Universität Rostock

Fachbereich Agrar- und Umweltwissenschaften

Professur für Aquakultur und Sea-Ranching

Die Immunantwort von Fischen und deren Bedeutung bei der Bekämpfung von Fischkrankheiten

HABILITATIONSSCHRIFT

zur

Erlangung des akademischen Grades

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Die meisten Probleme entstehen bei ihrer Lösung.

Leonardo da Vinci

Inhaltsverzeichnis

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Vorwort

Die vorliegende kumulative Habilitationsschrift befasst sich mit Fragestellungen zur zellulären Immunabwehr von Fischen. Als Zielorganismen wurden der Ginbuna-Karpfen (*Carassius auratus langsdorfii,* TEMMINCK & SCHLEGEL, 1846) und die Regenbogenforelle (*Onchorynchus mykiss,* WALBAUM, 1792) verwendet, wobei letztere Spezies ein weltweit bedeutsamer, in Aquakultur gehaltener Speisefisch ist. Die Arbeit beinhaltet sowohl grundlegende Untersuchungen zur zellvermittelten Immunität als auch anwendungsorientierte Arbeiten zum Einsatz von Impfstoffen in der Aquakultur.

Die entsprechenden Veröffentlichungen haben wesentlich zum Fortschritt auf dem Gebiet der zellvermittelten Zytotoxizität bei Fischen beigetragen. Einige grundlegende Phänomene der Immunologie, wie Homing und Transplantat-Gegen-Wirt-Reaktion wurden vom Autor dieser Arbeit erstmals bei Fischen beschrieben. Weiterhin wurden ein neues lymphatisches Organ in der Kieme entdeckt sowie Subpopulationen von Immunzellen und deren physiologische Leistungen bei Fischen erstmals charakterisiert.

In der hier vorgelegten Habilitationsschrift werden insgesamt 15 relevante Publikationen (**Teil I**) verarbeitet (12 davon im Peer-Review-Verfahren, 1 als Buch- und 2 als Konferenzbeiträge veröffentlicht). Der geschätzte Anteil an den Arbeiten ist unter dem Kapitel Übersicht über die verwendeten Arbeiten (Seite IX) jeweils prozentual in Klammern angegeben, aufgeschlüsselt nach Konzeption (**K**), Probennahme (**P**), Datenanalyse (**D**) und Texterstellung (**T**). Publikationen, bei denen der Autor dieser Habilitationsschrift Projektleiter war, sind zusätzlich mit **PL** gekennzeichnet. Alle **zur Habilitationsleistung gehörenden Arbeiten** sind im Textfluss bei ihrer ersten Nennung fortlaufend **römisch** nummeriert. Da die Inhalte der verarbeiteten Originalarbeiten in einigen Fällen themenübergreifend sind, kann es in den einzelnen Unterkapiteln der Habilitationsschrift zu Mehrfachnennungen kommen. Die Originalarbeiten von Drittautoren sowie weitere Veröffentlichungen des Verfassers dieser Habilitationsschrift werden, wie in wissenschaftlichen Arbeiten üblich, mit Nennung des Erstautors und des Erscheinungsjahres im Textfluss zitiert.

Die vorgelegte Habilitationsschrift enthält weiterhin bisher unveröffentlichte Daten. Diese wurden ausgewählt, um das Gesamtbild dieser Schrift abzurunden und um gleichwohl dazu überzuleiten, welche Arbeiten aktuell in

der Arbeitsgruppe des Autors durch- und weitergeführt werden. Darauf wird insbesondere im Kapitel **Schlussfolgerungen und Ausblick** eingegangen.

Die **Einleitung** dieser Arbeit führt unter Kapitel 1 in die Problematik der Fischkrankheiten und deren Bedeutung für die Aquakultur als wichtigen Wirtschaftsfaktor ein. Im Folgenden werden die Thesen der Habilitationsschrift erarbeitet.

Danach werden in Kapitel 2 zunächst vorwiegend **grundlagenorientierte Publikationen** des Autors zum Immunsystem von Fischen vorgestellt und diskutiert. Nach einem einleitenden Abschnitt zur Organisation des Immunsystems von Fischen wird das angeborene Immunsystem als Basis der Abwehr aller lebenden Organismen behandelt. Im nächsten Abschnitt geht es um das adaptive Immunsystem als Grundlage für eine erregerspezifische Abwehr und damit um die zentrale Aussage, dass Fische vakziniert werden können. Danach werden in Kapitel 3 die **translationalen und angewandten Forschungsarbeiten** des Autors zum Immunsystem von Fischen erklärt und diskutiert, wobei wiederum für die Vakzination von Fischen relevante Arbeiten im Vordergrund stehen. Da Fische poikilotherme Vertebraten sind, unterstreicht das Kapitel 4 zur **Temperatur und Immunantwort** die Bedeutung dieses Phänomens für die Physiologie von Fischen. Schließlich wird in den beiden letzten Kapiteln 5 bzw. 6 die **Bedeutung** der in der Habilitationsschrift verwendeten eigenen Arbeiten für die Aquakultur diskutiert, um im Anschluss dazu entsprechende **Schlussfolgerungen**, insbesondere für **geplante weitere Arbeiten**, zu ziehen. Referenzen sind unter Punkt 7 aufgeführt.

Im Teil II (Verwendete eigene Publikationen) sind die habilitationsrelevanten Originalarbeiten des Autors dieser Habilitationsschrift römisch durchnummeriert und entsprechend in der Reihenfolge ihrer ersten Nennung erfasst. Weitere unter der Autorenschaft des Verfassers erschienene Arbeiten, die zu einem besseren Verständnis dieser Schrift führen, sind gelistet. Diese sind jedoch entweder bereits abgegolten oder entstammen früheren, nicht ursächlich dieser Habilitation dienenden Forschungsthemen.

Zielsetzung

Der wissenschaftliche Anspruch dieser Arbeit besteht in der zusammenfassenden Darstellung der unter der Koautorenschaft des Verfassers dieser Habilitationsschrift gewonnenen grundlegenden Erkenntnisse zur zellulären Immunantwort bei Fischen und darin, diese Erkenntnisse anwendungsorientiert zu überbauen. Hinsichtlich translationaler und angewandter Forschungsergebnisse für die Aquakultur erhebt der Autor den Anspruch, wissenschaftliche Impulse für die gezielte Immunprophylaxe von Regenbogenforellen durch Vakzination gegeben zu haben. Auf diesem Fachgebiet sind zukünftig weitere entscheidende Fortschritte zu erwarten.

Im Rahmen dieser Habilitationsschrift werden die relevanten Publikationen zu den folgenden Schwerpunkthemen, welche die Immunantwort von Fischen beschreiben, verarbeitet:

GRUNDLAGENARBEITEN

- ORGANISATION DES IMMUNSYSTEMS
- ANGEBORENES IMMUNSYSTEM
- ADAPTIVES IMMUNSYSTEM

TRANSLATIONALE / ANWENDUNGSORIENTIERTE ARBEITEN

- ONTOGENESE UND IMMUNKOMPETENZ
- VAKZINATION
- TEMPERATUR UND IMMUNANTWORT

Urheberrechte

Teil I

Habilitationsschrift

Abkürzungsverzeichnis

APC	Antigen Presenting Cell
APP	Akute-Phase-Protein
BALT	Bronchus Associated Lymphoid Tissue
Batf	Basaler Leuzin-Zipper-Transkriptionsfaktor
Cas	CRISPR-Associated
CD	Cluster of Differentiation
CRISPR	Regularly Interspaced Short Palindromic Repeat
CTL	Zytotoxische T-Zelle (Cytotoxic T Lymphocyte)
DAMP	Danger-Associated Molecular Pattern
DC	Dendritic Cell
DNA	Desoxyribonukleinsäure (Deoxyribonucleic Acid)
GALT	Gut Associated Lymphoid Tissue
GVHD	Graft-Versus-Host Disease
GVHR	Graft-Versus-Host Reaction
Ig	Immunglobulin
ILT	Interbranchial Lymphoid Tissue
MHC	Haupthistokompatibilitätskomplex (Major Histocompatibility Complex)
IFN	Interferon
IHN	Infektiöse Hämatopoetische Nekrose
IHNV	Virus der Infektiösen Hämatopoetischen Nekrose
IPN	Infektiöse Pankreasnekrose
IRAK	Interleukin-Rezeptor-Assoziierte Kinase
ISAV	Infectious Salmon Anaemia Virus
ITAM	Immunoreceptor Tyrosine-based Activating Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
IRF	IFN-regulatorisches Protein
KHV	Koiherpesvirus
KHV-I	Koiherpesvirus-Infektion
KIR	Killer-cell Immunoglobulin-like Receptor
LILR	Leukocyte Immunoglobulin-Like Receptor
LPS	Lipopolysaccharid

MARCH	Membrane-Associated RING-CH
NET	Neutrophil Extracellular Trap
NILT	Novel Immunoglobulin-like Transcript
NITR	Novel Immune-Type Receptor
MAC	Membrane Attack Complex
PBL	Periphere Blutleukozyten
PRR	Pattern Recognition Receptor
RAG	Recombination Activating Gene
RNA	Ribonukleinsäure (Ribonucleic Acid)
SAA	Serum-Amyloid A
SPD	Salmon Pancreas Disease
SVC	Spring Viraemia of Carp
SVCV	Spring Viraemia of Carp Virus
TCR	T Cell Receptor
Th	T-Helfer
TLR	Toll-Like Receptor
TOLLIP	Toll-Interacting-Protein
VHS	Virale Hämorrhagische Septikämie
VHSV	Virus der Viralen Hämorrhagischen Septikämie
WTO	World Trade Organisation

Übersicht über die verwendeten Arbeiten

GRUNDLAGENORIENTIERTE ARBEITEN ZUM FISCHIMMUNSYSTEM

ARBEITEN ZUR ORGANISATION DES IMMUNSYSTEMS

I **FISCHER, U.**, KOPPANG, E.O., NAKANISHI, T. (2013): Teleost T and NK cell immunity. Fish Shellfish Immunol. 35:197-206. K: 70; T: 60; Review

II YAMAGUCHI, T., TAKIZAWA, F., **FISCHER, U.**, DIJKSTRA, J.M. (2015): Along the axis between type 1 and type 2 immunity; principles conserved in evolution from fish to mammals. Biology 4(4):814-859. K: 20; T: 20; Review

DIJKSTRA, J.M., **FISCHER, U.**, SAWAMOTO, Y.A., OTOTAKE, M., NAKANISHI, T. (2001): Exogenous antigens and the stimulation of MHC class I restricted cell-mediated cytotoxicity: possible strategies for fish vaccines. Fish & Shellfish Immunol. 11:437-458. K: 30; T: 30; Review

III SOMAMOTO, T., KOPPANG, E.O., **FISCHER, U.** (2014): Antiviral functions of CD8+ cytotoxic T cells in teleost fish. Dev. Comp. Immunol. 43(2):197-204. K: 70; T: 70; Review

IV TAFALLA, C., LEAL, E., YAMAGUCHI, T., **FISCHER, U.** (2016): T cell immunity in the teleost digestive tract. Dev. Comp. Immunol. 64:167-177. K: 40; T: 40; Review

V GRANJA, A.G., LEAL, E., PIGNATELLI, J., CASTRO, R., ABÓS, B., KATO, G., **FISCHER, U.**, TAFALLA, C. (2015): Identification of teleost skin CD8α+ dendritic-like cells, representing a potential common ancestor for mammalian cross-presenting dendritic cells. J. Immunol. 195(4):1825-1837. K: 20; P: 10; D: 10; T: 20

ARBEITEN ZUM ANGEBORENEN IMMUNSYSTEM

MUSTERERKENNUNG

REBL, A., SIEGL, E., KÖLLNER, B., **FISCHER, U.**, SEYFERT, H.-M. (2007): Characterization of twin toll-like receptors from rainbow trout (*Oncorhynchus mykiss*): Evolutionary relationship and induced expression by *Aeromonas salmonicida salmonicida*. Dev. Comp. Immunol. 31(5):499-510. K: 10; P: 20; D: 10; T: 20

REBL, A., HOYHEIM, B., **FISCHER, U.**, KÖLLNER, B., SIEGL, E., SEYFERT, H.-M. (2008): Tollip, a negative regulator of TLR-signalling, is encoded by twin genes in salmonid fish. Fish Shellfish Immunol. 25:153-162. K: 10; P: 20; D: 10; T: 20

REBL, A., GOLDAMMER, T., **FISCHER, U.**, KÖLLNER, B., SEYFERT, H.-M. (2009): Characterization of two key molecules of teleost innate immunity from rainbow trout (*Oncorhynchus mykiss*): MyD88 and SAA. Vet. Immunol. Immunopathol. 131(1-2):122-126. K: 10; P: 20; D: 10; T: 20

VI REBL, A., KÖBIS, J.M., **FISCHER, U.**, TAKIZAWA, F., VERLEIH, M., WIMMERS, K., GOLDAMMER, T. (2011): MARCH5 gene is duplicated in rainbow trout, but only fish-specific gene copy is up-regulated after VHSV infection. Fish Shellfish Immunol. 31(6):1041-1050. K: 10; P: 20; D: 10; T: 20

ZELLULÄRE ANGEBORENE ABWEHR

FISCHER, U., OTOTAKE, M., NAKANISHI, T. (1998a): *In vitro* cell-mediated cytotoxicity against allogeneic erythrocytes in ginbuna crucian carp and goldfish using a non-radioactive assay. Dev Comp Immunol. 22(2):195-206. K: 70; P: 100; D: 90; T: 80; PL

FISCHER, U., UTKE, K., OTOTAKE, M., DIJKSTRA, J.M., KÖLLNER, B. (2003): Adaptive cell-mediated cytotoxicity against allogeneic targets by CD8-positive lymphocytes of rainbow trout (*Oncorhynchus mykiss*). Dev Comp Immunol. 7:323-337. K: 60; P: 60; D: 60; T: 70; PL

UTKE, K., BERGMANN, S., LORENZEN, N., KÖLLNER, B., OTOTAKE, M., **FISCHER, U.** (2007): Cell-mediated cytotoxicity in rainbow trout, *Oncorhynchus mykiss*, infected with viral haemorrhagic septicaemia virus. Fish Shellfish Immunol. 22(3):182-196. K: 60; P: 40; D: 30; T: 50; PL

UTKE, K., KOCK, H., SCHÜTZE, H., BERGMANN, S.M., LORENZEN, N., EINER-JENSEN, K., KÖLLNER, B., DALMO, R.A., VESELY, T., OTOTAKE, M. & **FISCHER, U.** (2008): Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. Dev Comp Immunol. 32:239–252. K: 60; P: 40; D: 30; T: 50; PL

KOCK, H. & **FISCHER, U.** (2008): A novel immunoglobulin-like transcript from rainbow trout with two Ig-like domains and two isoforms. Mol Immunol. 45:1612–1622. K: 60; P: 30; D: 30; T: 40; PL

FISCHER, U., OTOTAKE, M. & NAKANISHI, T. (1999): Effect of environmental temperature on in vitro cell-mediated cytotoxicity (CMC) and graft-versus-host reaction (GVHR) in ginbuna crucian carp (*Carassius auratus langsdorfii*), Fish Shellfish Immunol. 9:233-236. K: 70; P: 100; D: 90; T: 80; PL

I **FISCHER, U.**, KOPPANG, E.O., NAKANISHI, T. (2013): Teleost T and NK cell immunity. Fish Shellfish Immunol. 35:197-206. K: 70; T: 60; Review

KÖLLNER, B., **FISCHER, U.**, ROMBOUT, J.H.W.M., TAVERNE-THIELE, J.J., HANSEN, J.D. (2004): Potential involvement of rainbow trout thrombocytes in immune functions: A study using a panel of monoclonal antibodies and RT-PCR. Dev Comp Immunol. 28:1049-1062. K: 30; P: 20; D: 20; T: 30

OHASHI, K., TAKIZAWA, F., TOKUMARU, N., NAKAYASU, C., TODA, H., **FISCHER, U.**, MORITOMO, T., HASHIMOTO, K., NAKANISHI, T., DIJKSTRA, J.M. (2010): A molecule in teleost fish, related with human MHC-encoded G6F, has a cytoplasmic tail with ITAM and marks the surface of

thrombocytes and in some fishes also of erythrocytes. Immunogenetics. 62(8):543-559. K: 10; P: 10; D: 10; T: 10

COMPLEMENT

LOVOLL, M., **FISCHER, U.**, MATHISEN, G.S., BOGWALD, J., OTOTAKE, M., DALMO, R.A. (2007): The C3 subtypes are differentially regulated after immunostimulation in rainbow trout, but head kidney macrophages do not contribute to C3 transcription. Vet Immunol Immunopathol. 117(3-4):284-295. K: 10; P: 20; D: 10; T: 20

ARBEITEN ZUM ADAPTIVEN IMMUNSYSTEM

ZELLULÄRE KOMPONENTEN DER ADAPTIVEN IMMUNANTWORT

KOPPANG, E.O., **FISCHER, U.**, MOORE, L., TRANULIS, M.A., DIJKSTRA, J.M., KÖLLNER, B., AUNE, L., JIRILLO, E. & HORDVIK, I. (2010): Salmonid T cells assemble in the thymus, spleen and in novel interbranchial lymphoid tissue. J. Anat. 217(6):728-39. K: 40; P: 30; D: 30; T: 30

VII TAKIZAWA, F., DIJKSTRA, J.M., KOTTERBA, P., KORYTÁŘ, T., KOCK, H., KÖLLNER, B., JAUREGUIBERRY, B., NAKANISHI, T., **FISCHER, U.** (2011): The expression of CD8α discriminates distinct T cell subsets in teleost fish. Dev Comp Immunol. 35(7):752-763. K: 40; P: 30; D: 30; T: 40; PL

V GRANJA, A.G., LEAL, E., PIGNATELLI, J., CASTRO, R., ABÓS, B., KATO, G., **FISCHER, U.**, TAFALLA, C. (2015): Identification of Teleost Skin CD8α+ Dendritic-like Cells, Representing a Potential Common Ancestor for Mammalian Cross-Presenting Dendritic Cells. J Immunol. 195(4):1825-1837. K: 20; P: 10; D: 10; T: 20

VIII KOPPANG, E.O., KVELLESTAD, A., **FISCHER, U.** (2015): Fish mucosal immunity: gill. In: Benjamin Beck and Eric Peatman (eds.): Mucosal Health in Aquaculture, Academic Press, Elsevier Inc. ISBN: 978-0-12-417186-2. K: 30; T: 30; Review

FISCHER, U., OTOTAKE, M., NAKANISHI, T. (1998b): Life span of circulating blood cells in ginbuna crucian carp (*Carassius auratus langsdorfii*), Fish Shellfish Immunol. 8:339-349. K: 70; P: 100; D: 90; T: 80; PL

IX YAMAGUCHI, T., **FISCHER, U.** (2016): Adoptive transfer of immunity against red mouth disease in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. 53:65. 2nd International Conference of Fish & Shellfish Immunology June 26th – 30th, 2016 Portland, Maine. Vortrag. K: 60; P: 50; D: 60; T: 60; PL

MHC - MAJOR HISTOCOMPATIBILITY COMPLEX

FISCHER, U., OTOTAKE, M., NAKANISHI, T. (1999): Effect of environmental temperature on in vitro cell-mediated cytotoxicity (CMC) and graft-versus-host reaction (GVHR) in ginbuna crucian carp (*Carassius auratus langsdorfii*), Fish Shellfish Immunol. 9:233-236. K: 70; P: 100; D: 90; T: 80; PL

FISCHER, U., OTOTAKE, M., NAKANISHI, T. (1998a): In vitro cell-mediated cytotoxicity against allogeneic erythrocytes in ginbuna crucian carp and goldfish using a non-radioactive assay. Dev Comp Immunol. 22(2):195-206. K: 70; P: 100; D: 90; T: 80; PL

FISCHER, U., UTKE, K., OTOTAKE, M., DIJKSTRA, J.M., KÖLLNER, B. (2003): Adaptive cell-mediated cytotoxicity against allogeneic targets by CD8-positive lymphocytes of rainbow trout (*Oncorhynchus mykiss*). Dev Comp Immunol. 7:323-337. K: 60; P: 60; D: 60; T: 70; PL

DIJKSTRA, J.M., KÖLLNER, B., AOYAGI, K., SAWAMOTO, Y., KURODA, A., OTOTAKE M., NAKANISHI, T., **FISCHER, U.** (2003): The rainbow trout classical MHC class I molecule Onmy-UBA*501 is expressed in similar cell types as mammalian classical MHC class I molecules. Fish Shellfish Immunol. 14:1-23. K: 50; P: 90; D: 90; T: 60

SARDER, R.I., **FISCHER, U.**, OKAMURA, K., KÖLLNER, B., DIJKSTRA, J.M., KIRYU, I., HASHIMOTO, K., NAKANISHI, T., OTOTAKE, M. (2003): The MHC class I linkage group is a major determinant in the in vivo rejection of allogeneic erythrocytes in rainbow trout (*Oncorhynchus mykiss*). Immunogenetics. 55:315-324. K: 20; P: 20; D: 20; T: 30

UTKE, K., BERGMANN, S., LORENZEN, N., KÖLLNER, B., OTOTAKE, M., **FISCHER U.** (2007): Cell-mediated cytotoxicity in rainbow trout, *Oncorhynchus mykiss*, infected with viral haemorrhagic septicaemia virus. Fish Shellfish Immunol. 22(3):182-196. K: 60; P: 40; D: 30; T: 50; PL

UTKE, K., KOCK, H., SCHÜTZE, H., BERGMANN, S.M., LORENZEN, N., EINER-JENSEN, K., KÖLLNER, B., DALMO, R.A., VESELY, T., OTOTAKE, M. **FISCHER, U.** (2008): Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. Dev Comp Immunol. 32:239–252. K: 60; P: 40; D: 30; T: 50; PL

V GRANJA, A.G., LEAL, E., PIGNATELLI, J., CASTRO, R., ABÓS, B., KATO, G., **FISCHER, U.**, TAFALLA, C. (2015): Identification of Teleost Skin CD8α+ Dendritic-like Cells, Representing a Potential Common Ancestor for Mammalian Cross-Presenting Dendritic Cells. J Immunol. 195(4):1825-37. K: 20; P: 10; D: 10; T: 20

TRANSLATIONALE UND ANGEWANDTE FORSCHUNGSARBEITEN ZUM FISCHIMMUNSYSTEM

ARBEITEN ZUR ONTOGENESE UND IMMUNKOMPETENZ

FISCHER, U., DIJKSTRA, J.M., KÖLLNER, B., KIRYU, I., KOPPANG, E.O., HORDVIK, I., SAWAMOTO, Y. & OTOTAKE, M. (2005): The ontogeny of MHC class I expression in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. 18:49-60. K: 60; P: 90; D: 90; T: 70

ARBEITEN ZUR VAKZINATION VON FISCHEN

DNA-IMMUNISIERUNG

UTKE, K., KOCK, H., SCHUETZE, H., BERGMANN, S.M., LORENZEN, N., EINER-JENSEN, K., KÖLLNER, B., DALMO, R.A., VESELY, T., OTOTAKE, M. & **FISCHER, U.** (2008): Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. Dev. Comp. Immunol. 32:239–252. K: 60; P: 40; D: 30; T: 50; PL

X CASTRO, R., TAKIZAWA, F., CHAARA, W., LUNAZZI, A., DANG, T.H., KOELLNER, B., QUILLET, E., SIX, A., **FISCHER, U.**, BOUDINOT, P. (2013): Contrasted TCRβ diversity of CD8+ and CD8- T cells in rainbow trout. PLoS One. 8(4): e60175. doi: 10.1371/journal.pone.0060175. Epub 2013 Apr 2. K: 40; P: 50; D: 30; T: 30

MUKOSALE IMMUNISIERUNG

VIII KOPPANG, E.O., KVELLESTAD, A., **FISCHER, U.** (2015): Fish mucosal immunity: gill. In: Benjamin Beck and Eric Peatman (eds.): Mucosal Health in Aquaculture, Academic Press, Elsevier Inc. ISBN: 978-0-12-417186-2. K: 30; T: 30; Review

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ARBEITEN ZU TEMPERATUR UND IMMUNANTWORT

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1 Einleitung

1.1 Fischkrankheiten

Fischkrankheiten finden nachweislich seit dem Altertum Beachtung. Ein ägyptisches Gemälde aus dem Jahre 1450 v.d.Z. zeigt einen Tilapia-Buntbarch mit aufgetriebenem Abdomen (MAWDESLEY-THOMAS, 1972) und gilt damit als ältestes Zeugnis der Darstellung von pathologischen Veränderungen bei Fischen. Die Beschreibung von übertragbaren Fischkrankheiten begann in der Neuzeit mit den parasitären, gefolgt von bakteriellen und viralen Erkrankungen (SNIEZKO, 1975). Als erste zusammenfassende Arbeit über Fischkrankheiten gilt ein von HOFER (1904) verfasstes Handbuch. Es folgten entsprechende Veröffentlichungen von PLEHN (1924) und SCHÄPERCLAUS (1954).

Unter den Bakteriosen war es die Furunkulose, hervorgerufen durch *Aeromonas salmonicida (A. salmonicida)*, die als erste Eingang in die bakteriologische Forschung bei Fischen gefunden hat. Die Erstbeschreibung der Erkrankung erfolgte bereits 1890 in einer Bachforellenzucht in Bayern durch EMMERICH & WEIBEL. Erst ein halbes Jahrhundert später wurde eine auch heute noch wirtschaftlich bedeutsame Bakteriose bei Regenbogenforellen beschrieben, die durch *Yersinia ruckeri (Y. ruckeri)* hervorgerufen wird, und Auslöser der Rotmaulseuche ist (RUCKER, 1966). WEISSENBERGER (1914) schlussfolgerte aus dem Vorhandensein von Einschlusskörpern erstmals die virale Ätiologie einer Fischkrankheit (Lymphozystis-Erkrankung), wobei der endgültige Nachweis entsprechender viraler Partikel mittels Elektronenmikroskopie (GRÜTZNER, 1956) und ihrer Infektiosität (WOLF et al., 1966) erst viel später erfolgte.

Unter den viralen Fischkrankheiten sind in Deutschland drei durch Rhabdoviren hervorgerufene Krankheiten von hoher ökonomischer Bedeutung, die Virale Hämorrhagische Septikämie der Salmoniden (VHS, Erstbeschreibung des Erregers in Dänemark durch JENSEN, 1965), die Infektiöse Hämatopoetische Nekrose der Salmoniden (IHN, Erstbeschreibung des Erregers in den USA durch WATSON et al., 1954) und die Frühjahrsvirämie der Karpfen (Spring Viraemia of Carp, SVC, Erstbeschreibung des Erregers durch FIJAN et al., 1971). Eine weitere Erkrankung der Salmoniden, die weltweit hohe Verluste verursacht, ist die Infektiöse Pankreasnekrose (IPN). Die IPN wird hervorgerufen durch ein Birnavirus, welches erstmals durch WOLF et al. (1960)

in den USA isoliert wurde. Neben VHS und IHN gehört in der Europäischen Union (EU) weiterhin die Koiherpesvirus-Infektion (KHV-I) zu den anzeigepflichtigen Fischseuchen. Diese führt seit ihrer Erstbeschreibung Ende der 1990-er Jahre (BRETZINGER et al., 1999) insbesondere bei Nutzkarpfen und Koi (*Cyprinus carpio*, LINNAEUS, 1758) zu hohen ökonomischen Verlusten. Durch retrospektive Untersuchung historischer Proben aus dem Vereinigten Königreich (HAENEN et al., 2004) konnte festgestellt werden, dass das KHV in Europa seit mindestens 1996 existiert.

Obwohl durch empirische Beobachtung bereits seit dem Altertum angewendet, gilt Vakzination spätestens seit Anfang des 19. Jahrhunderts als erfolgreiche Option zur Bekämpfung von Infektionskrankheiten. Der Begriff „Vaccination“ wurde geprägt durch Edward JENNER (1798), der durch Inokulation von lebenden Kuhpockenerregern an Menschen einen Schutz vor Pocken induzieren konnte. Die früheste Erwähnung einer Vakzination von Fischen erfolgte im Jahre 1938. SNIESZKO et al. (1938) hatten damals erfolgreich Karpfen gegen die Erythrodermatitis, ausgelöst durch das Bakterium *Aeromonas punctata*, immunisiert. 1942 folgte eine Arbeit von DUFF, in der er die Vakzination von Regenbogenforellen gegen Furunkulose nach oraler und parenteraler Inokulation beschrieb. 1965 gelang ROSS & KLONTZ die orale Immunisierung von Regenbogenforellen gegen die Rotmaulseuche.

Insbesondere seit Ausbreitung der kommerziellen Aquakultur spielt das Auftreten und die Bekämpfung von Fischkrankheiten eine zunehmende Rolle. Dabei bewegt sich das Auftreten einer Krankheit immer im Spannungsfeld zwischen den jeweiligen Umweltbedingungen, dem jeweiligen Krankheitserreger und dem Gesundheitsstatus des Erregerwirts. Laut Weltgesundheitsorganisation (WHO, 1948) ist Gesundheit „*ein Zustand des vollständigen körperlichen, geistigen und sozialen Wohlergehens und nicht nur das Fehlen von Krankheit oder Gebrechen*.“ Diese eigentlich für den Menschen gültige Definition lässt sich heute auch auf Tiere übertragen, wobei „körperliches, geistiges und soziales Wohlergehen“ bei Tieren „Tierwohl“ bzw. Tiergerechtheit (engl. „animal welfare“) zum Korrelat hat. Auf Grundlage des Brambell-Reports aus dem Jahre 1965 entwickelte das britische Farm Animal Welfare Council 1979 das Konzept der „Fünf Freiheiten“ als Grundlage für Mess- und Bewertungssysteme zur Tiergerechtheit:

1. Freiheit von Hunger und Durst,
2. Freiheit von haltungsbedingten Beschwerden,
3. Freiheit von Schmerz, Verletzungen und Krankheiten,
4. Freiheit von Angst und Stress,
5. Freiheit zum Ausleben normaler Verhaltensmuster.

Dabei erscheint die Freiheit von Stress unmöglich, da nach geltender Definition Stress die betroffenen Lebewesen durch äußere Reize (Stressoren) zur Bewältigung besonderer Anforderungen befähigt (nach Wikipedia). Der Begriff Stress wurde von H. SELYE (1936) erstmals verwendet, wobei er zwischen Eustress als physiologisch notwendige Aktivierung des Organismus und Distress als belastend und schädlich wirkend unterschied.

Der Gesundheitsstatus eines Individuums (Mensch oder Tier) wird in bedeutendem Maß durch seinen Immunstatus beeinflusst. Der Immunstatus beschreibt den Zustand des Immunsystems eines Individuums und seine Fähigkeit, Infektionen durch eine adäquate Immunantwort abzuwehren. Die Immunantwort erfolgt bei Vertebraten (also auch bei Fischen) nicht nur, wie bei Pflanzen und Nicht-Vertebraten, durch ein angeborenes (engl. innate) Immunsystem, sondern zusätzlich auch über ein adaptives Immunsystem. Beide Systeme werden jeweils durch zelluläre und humorale (von lat. Humor = Flüssigkeit, Feuchtigkeit) Komponenten repräsentiert, weswegen auch zwischen einem zellvermittelten Immunstatus und einem humoralen Immunstatus unterschieden wird. Die adaptive Komponente des Immunsystems beinhaltet ein immunologisches Gedächtnis, was die Grundlage für die Immunisierbarkeit von Vertebraten und damit den Einsatz von Impfstoffen zum gezielten Schutz vor bestimmten Infektionskrankheiten bildet.

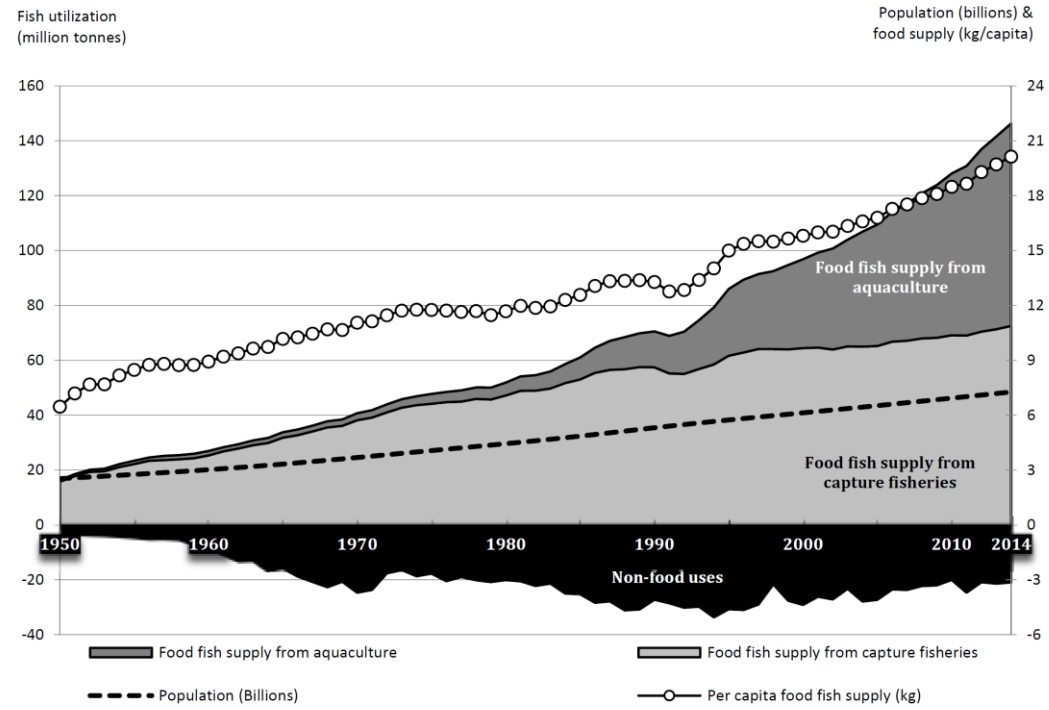
Infektionskrankheiten treten immer dann auf, wenn die Homöostase zwischen Umweltbedingungen, Erreger und Gesundheitsstatus des Wirtes gestört ist. Vakzinierte Fische erkranken bei Eintrag eines Erregers normalerweise nicht an der entsprechenden Krankheit, gegen die sie immunisiert wurden. Der Immunschutz kann dennoch durchbrochen werden, wenn die Erregerlast die Abwehrkapazität des Immunsystems übersteigt oder wenn schlechte Haltungsbedingungen eine Immunsuppression zur Folge haben. Insbesondere in der intensiven Aquakultur können hohe Besatzdichten

(engl. crowding) eine stressbedingte Immunsuppression (BLY et al., 1997; MAGNADÓTTIR, 2006) zur Folge haben. Hohe Besatzdichten können gleichermaßen zu hohen Erregerlasten für noch nicht infizierte Fische in der Hälterungseinheit führen, da zahlreiche bereits infizierte Fische unter Umständen enorme Mengen an Erregern ausscheiden können. Dies kann zu Erregerkonzentrationen im Hälterungswasser führen, wie sie in Wildgewässern kaum anzutreffen sind. Deshalb müssen in ein erfolgreiches Gesundheitsmanagement immer die drei Komponenten Umwelt, Erreger und Gesundheitsstatus des Fisches mit einbezogen werden.

1.2 Aquakultur – ein wichtiger Wirtschaftsfaktor

Aquakultur gilt als einer der am stärksten prosperierenden Bereiche der Nahrungsmittelproduktion. Während die Fangmengen aus der Fischerei (Fisch, Krebstiere, Muscheln und andere Invertebraten) seit einem Höchststand in 1996 weltweit stagnierten, war in der Aquakultur ein rapider Anstieg der Produktionsmengen um fast das Doppelte zu verzeichnen. Dieser Anstieg ist insbesondere der überdurchschnittlichen Entwicklung der Aquakultur in Asien und Afrika zu verdanken. Dennoch überstiegen in 2014 die weltweiten Fangmengen immer noch die Produktionsmengen in der Aquakultur (93.445,234 t versus 73.783,725 t, FAO 2014).

Figure 1
World fish utilization and supply/Utilisation et disponibilités mondiales de poisson/ Utilización y suministro mundiales de pescado



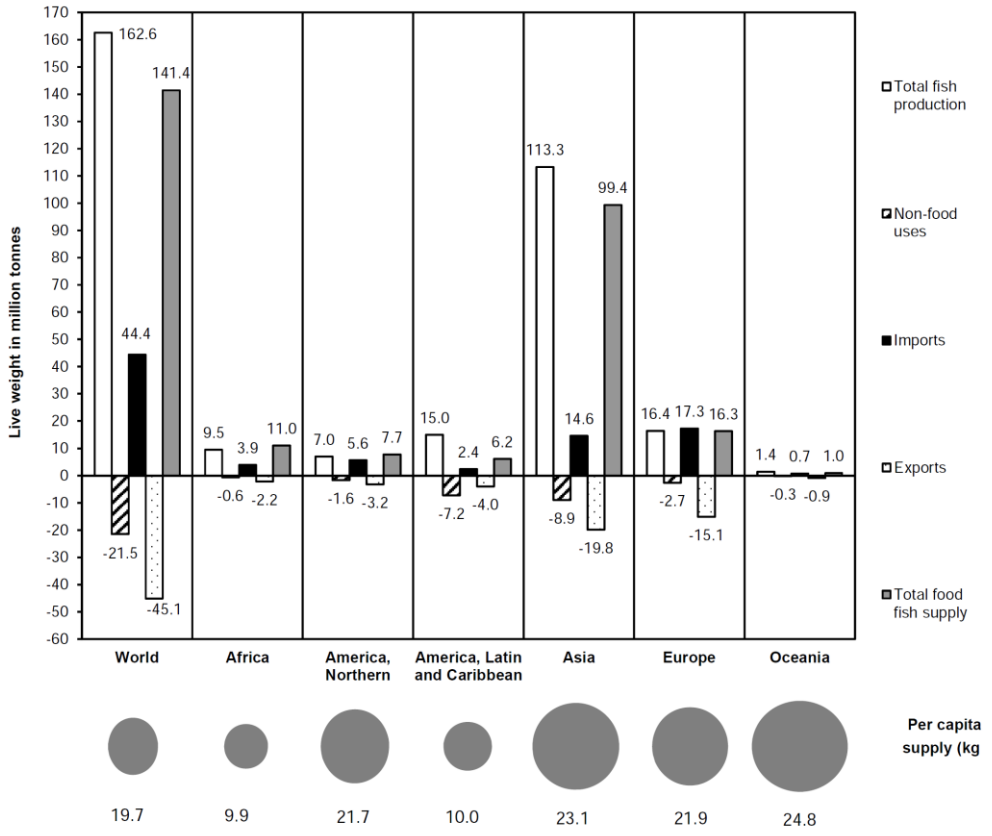
Quelle: FAO (2014): Fishery and Aquaculture Statistics, http://www.fao.org/fishery/static/Yearbook/YB2014_CD_Master/booklet/i5716t.pdf

Während China in 2014 weltweit ca. ein Drittel der Gesamtmenge an Fischen, Krebstieren, Muscheln und anderen Invertebraten in Aquakultur produzierte, fanden die in Deutschland erwirtschafteten Mengen in der FAO-Statistik des Jahres 2014 keine Erwähnung, was wegen der hierzulande erzeugten geringen Produktionsmengen nicht überrascht. Angesichts eines Niedergangs der erwerbsmäßigen Fangfischerei insbesondere in Deutschland, wo die Zahl der statistisch erfassten Fischer in den letzten 20 Jahren von 4.646 auf 2.469 einbrach (FAO, 2014), kann die Aquakultur in Deutschland auch für die Schaffung neuer und nachhaltiger Arbeitsplätze an Bedeutung gewinnen. Das trifft insbesondere für das Land Mecklenburg-Vorpommern zu, wo die in 2015 erfasste Anzahl von 20 Betrieben mit Erzeugung in Aquakultur (Deutschland insgesamt: 3.285 Betriebe, Statistisches Bundesamt, 2016) noch ausbaufähig erscheint. Die Produktion von Speisefischen aus Aquakultur wird in Deutschland erst seit 2011 statistisch erfasst, wobei im Vergleich zur weltweiten Entwicklung der Aquakultur die Wachstumsraten in Deutschland relativ moderat

ausfallen. So betrug laut Statistischem Bundesamt die Aquakulturproduktion in 2012 19.600 t und in 2015 26.900 t. Im Jahr 2015 war lediglich ein Wachstum von 2,5 % gegenüber 2014 zu verzeichnen. Gleichzeitig ging die Erzeugung von Fischen insgesamt um 9,5 Prozent zurück, bei gleichzeitigem starkem Anstieg bei der Erzeugung von Muscheln. Der Rückgang betraf insbesondere Baden-Württemberg und Bayern, wo zuletzt ein hoher Infektionsdruck durch VHS und IHN zu verzeichnen war (Statistik Nationales Referenzlabor für VHS und IHN am FLI, unveröffentlicht). Entsprechend nannten die betroffenen Betriebe in einer Befragung neben dem trockenen Sommer Fischseuchen als Grund für den deutlichen Rückgang in der Erzeugung von Fischen. Das zeigt sehr deutlich, dass die Bekämpfung von Fischseuchen prioritärer Bestandteil einer stabilen Aquakulturproduktion ist.

Figure 2

Food fish balance in live weight and per capita supply (2013)/Bilan alimentaire de poisson en équivalent poids vif et disponibilité par habitant (2013)/Balance de alimentos pesqueros y suministro per cápita, en peso vivo (2013)



Note: food fish supply equals production less non-food use plus imports less exports plus or less variation in stocks. Variation in stocks not shown.

Quelle: FAO (2014): Fishery and Aquaculture Statistics, http://www.fao.org/fishery/static/Yearbook/YB2014_CD_Master/booklet/i5716t.pdf

1.3 Besonderheiten der Aquakultur aus epidemiologischer Sicht

In der Aquakultur sind Fische Umweltbedingungen ausgesetzt, die ihre Artgenossen in natürlichen Ökosystemen nicht vorfinden. Dazu gehören insbesondere hohe Besatzdichten, die u.a. zu erhöhtem Stress (YARAHMADI et al., 2016), zu erhöhter Verletzungsgefahr (NORTH et al., 2006) und einer damit verbundenen erhöhten Anfälligkeit für Infektionen (PICKERING & POTTINGER, 1989) bei erhöhter gegenseitiger Ansteckung führen kann. Deshalb werden von der FAO für wirtschaftlich bedeutsame Zuchtfische maximale Besatzdichten empfohlen (z.B. für die Regenbogenforelle von 30-40 kg/m^3; FAO, 2016), um das Auftreten von Fischkrankheiten zu reduzieren.

Was den Domestikationsgrad von Fischen in Aquakultur betrifft, so ist dieser im Vergleich zu den meisten terrestrischen Nutztieren eher gering. Dieses liegt daran, dass im Gegensatz zu den terrestrischen Pflanzen und Tieren die Süß- und Meerwasserkulturarten erst seit wenigen Jahrzehnten kultiviert werden (DUARTE et al., 2007). Intensive selektive Züchtungsprogramme, beispielsweise für Regenbogenforellen (*Oncorhynchus mykiss*), den atlantischen Lachs (*Salmo salar,* LINNAEUS, 1758), den Katzenwels (*Ictalurus punctatus,* GILL, 1861) und Tilapien (*Oreochromis niloticus,* LINNAEUS, 1758), haben nach Ansicht von DUNHAM et al. (2001) im Vergleich zu domestizierten Säugern zwar zu sehr viel höheren Futterkonversionsraten aber auch zu einem erheblichen Verlust an genetischer Variabilität geführt. Ein Verlust an genetischer Variabilität birgt jedoch auch die Gefahr in sich, dass nicht nur einzelne Individuen, sondern die gesamte Population anfälliger gegenüber einer bestimmten Erregervariante wird. Das ist insbesondere dann der Fall, wenn Erreger und Wirt genetisch bedingte Phänotypen entwickelt haben, die den Erregereintritt, seine Vermehrung, seine krankmachenden Eigenschaften und seine Ausbreitung befördern. Bezieht sich die eingeschränkte genetische Variabilität jedoch nur auf ein bestimmtes Gen, von dem bekannt ist, dass es dem Wirt einen evolutionären Vorteil gegenüber dem Erreger in Form von Resistenz verschafft, ist dies durchaus eine gewollte Eigenschaft. Hierfür sind IPN-resistente atlantische Lachse ein gutes Beispiel wo die Selektionszüchtung auf Basis genetischer Marker (GHEYAS et al., 2010) erfolgreich eingesetzt wird.

Ein aus epidemiologischer Sicht ungünstiger Aspekt in der traditionellen Aquakultur ist die Verbindung der Hälterungsanlagen mit natürlichen Ökosystemen sowie deren offener Zugang für lebende Vektoren wie Schadnager, Raubtiere und wildlebende Wasservögel. Somit kann es sehr leicht zur Erregereinschleppung aus natürlichen Erregerreservoiren, insbesondere aus Wildfischpopulationen kommen. In naturnahen Teichwirtschaften mit empfänglichem Wildfischbesatz, deren komplette Räumung aus logistischer und naturschützerischer Sicht ausgeschlossen ist, kann die Tilgung eines Erregers nahezu unmöglich werden. Zudem ist die Einschleppung von weiteren, u.U. auch neuen Erregervarianten sehr wahrscheinlich.

Durch die Haltung von Fischen in geschlossenen Fischzuchtsystemen, insbesondere in Kreislaufanlagen, ist die Übertragung von Parasiten mit komplexen Lebenszyklen durch das Fehlen von Zwischenwirten zwar nahezu unmöglich, dafür sind die Bedingungen für die Übertragung von Viren und Bakterien sowie bestimmten Proto- und Metazoen mit direkten (monoxenen) Lebenszyklen günstig (MURRAY & PEELER, 2005). Dieses gilt insbesondere bei dem Besatz von Jungfischen aus anderen Aquakulturanlagen, wodurch sich diese Schadorganismen übertragen lassen. Einmal befallene Fischbestände in offenen aber auch in geschlossenen Aquakultursystemen lassen sich dann nur sehr schwer bekämpfen. Aufgrund hoher Besatzdichten, der damit verbundenen beschleunigten Erregerpassagen und Replikationszyklen sowie aufgrund der besonders für Viren typischen hohen Mutations- und Rekombinationsraten können Infektionserreger schneller eine höhere Virulenz erlangen als unter den Bedingungen extensiver Aquakultur oder als in Wildpopulationen (PULKKINEN et al., 2010; KURATH & WINTON, 2011).

Infektionserreger werden in geschlossene Kreisläufe vor allem durch den Handel mit infizierten, nicht zertifizierten Fischen sowie durch kontaminierte Gegenstände eingetragen. Kontrollmaßnahmen beinhalten deswegen die weitgehende Abschirmung solcher Aquakulturanlagen vor der Umwelt, restriktiven Zutritt, Besatz mit ausschließlich zertifizierten Fischen, risikobasiertes regelmäßiges Screening auf Erreger, regelmäßige Desinfektion und Vakzination. Diese Maßnahmen müssen risikobasiert, d.h. unter Berücksichtigung der epidemiologischen Situation erfolgen (PEELER & TAYLOR,

2011). Risikoanalysen haben ergeben, dass die Wahrscheinlichkeit der Einschleppung gefährlicher Fischseuchen zu großen Teilen über handelspolitische Restriktionen verringert werden kann, wobei globale, EU-weite und nationale Maßnahmen ineinandergreifen sollten. Die Welthandelsorganisation (WTO) hat mit einer „Vereinbarung über die Einhaltung Sanitärer und Phytosanitärer Maßnahmen“ [Agreement on the Application of Sanitary and Phytosanitary (SPS) Measures; WTO, 1995] die Erstellung von Internationalen Risikoanalysen (IRA) vorangetrieben, und das Internationale Tierseuchenamt (OIE) hat in 2004 entsprechende Richtlinien erarbeitet, die im „Aquatic Animal Health Code“ zusammengefasst sind. Am „Aquatic Animal Health Code“ und am “Manual of Diagnostic Tests for Aquatic Animals” in der jeweils aktualisierten Fassung orientieren sich wiederum europäische und nationale Gesetzgebungen zur Fischseuchenbekämpfung. Diese sind beispielsweise EU-weit in einem einheitlichen Diagnostikhandbuch für Fische [Durchführungsbeschluss (EU) 2015/1554 der Kommission vom 11. September 2015 mit Durchführungsbestimmungen zur Richtlinie 2006/88/EG hinsichtlich der Anforderungen an die Überwachung und der Diagnosemethoden] und national in der Fischseuchenverordnung (FischSeuchV) vom 24. November 2008 (BGBl. I S. 2315), zuletzt geändert am 3. Mai 2016 (BGBl. I S. 1057), berücksichtigt.

1.4 THESEN:

Erregerbedingte Fischkrankheiten gelten bis heute als ein großes Hemmnis bei der Ausweitung der Aquakultur. Obwohl durch moderne Diagnoseverfahren spezifische Erreger in der Regel schnell diagnostiziert werden können, ist eine entsprechende Therapie schwierig und insbesondere bei viralen Erregern nahezu unmöglich. Als Konsequenz bleibt laut FischSeuchV in der Regel die Bestandsräumung. Die Prophylaxe gefährlicher Fischseuchen durch Vakzination erscheint somit als logische Alternative. Laut § 11 der FischSeuchV besteht jedoch gegen anzeigepflichtige exotische Fischseuchen (solche, die in der EU nicht vorkommen) ein absolutes und gegen anzeigepflichtige nicht exotische Seuchen (solche die in der EU vorkommen) ein grundsätzliches Impfverbot. Anzeigepflichtig sind derzeit ausschließlich virale Fischkrankheiten, so dass dieses Impfverbot nicht für bakterielle Erkrankungen gilt, wo in den letzten Jahren der Verbrauch von Antibiotika durch den Einsatz von Vakzinen stark zurückgegangen ist (WHO, 2015). Angesichts dieser Ausgangssituation wird bei Bakteriosen weiterhin an solchen Impfstoffen gearbeitet, die gegen ökonomisch bedeutsame Fischseuchen eingesetzt werden können.

Gründe für ein Impfverbot gegen anzeigepflichtige virale Krankheiten liegen in handelspolitischen Restriktionen, da eine Unterscheidung zwischen dem der Vakzine zugrundeliegenden Impfantigen und einem viralen Felderreger bzw. der daraus resultierenden Antikörper derzeit nicht möglich ist. Des Weiteren neigen die meisten viralen Fischseuchenerreger zur Persistenz, d.h. einmal erkrankte aber auch vakzinierte und danach infizierte Fische bleiben lebenslang Träger von viralen Erregern, die sie an andere Fische weitergeben können. Bei viralen Impfstoffen stehen sogenannte DIVA (Discrimination between Infected and Vaccinated Animals)-Vakzinen im Vordergrund, die eine Unterscheidung zwischen infizierten und geimpften Fischen ermöglichen. Solche Vakzinen könnten in Zukunft zu einer Aufhebung des Impfverbotes gegen anzeigepflichtige nicht exotische virale Fischseuchen führen.

Die zielgerichtete Entwicklung bakterieller und viraler Impfstoffe setzt in jedem Fall tiefgreifende Kenntnisse der spezifischen Immunmechanismen bei Fischen sowie fundiertes Wissen über die sich daraus ergebende Epidemiologie und

Pathogenese der entsprechenden Krankheit voraus. Aufbauend auf diesen Überlegungen werden folgende Thesen formuliert:

Forschungsansatz

i. Kenntnisse zur Immunantwort von Fischen dienen dem Verständnis der Pathogenese von Infektionskrankheiten und der Entwicklung von Präventions- und Bekämpfungsmaßnahmen.

Grundlagenorientierte Forschung

ii. Fische verfügen über die phylogenetisch sehr alte Fähigkeit, „Eigen“ von „Fremd“ zu unterscheiden. Sie sind in die Lage, Transplantate abzustoßen und eine Transplantat-Gegen-Wirt-Krankheit (Graft-Versus-Host-Disease, GVHD) auszulösen.

iii. Fische besitzen ein angeborenes Immunsystem. Das angeborene Immunsystem verfügt über breitenspezifische Mustererkennungsrezeptoren in Form von Toll-Like-Rezeptoren (TLR), Novel Immunoglobulin-like Transcripts (NILT), über Komplement, Thrombozyten und über funktionelle natürliche Killer (NK)-Zellen. NK-Zellen und zytotoxische T-Zellen (CTL, cytotoxic T lymphocytes) von Fischen können allogene (körperfremde, selbe Spezies) Zellen und virusinfizierte körpereigene Zellen eliminieren.

iv. Das Adaptive Immunsystem bildet die Grundlage für eine gezielte Immunisierbarkeit. Fische besitzen ein antigenspezifisches adaptives Immunsystem, was durch die Expression entsprechender Gene und Proteine, sowie durch funktionelle Studien bewiesen ist. Fische exprimieren u.a. Haupthistokompatibilitätsmoleküle (MHC) der Klassen I und II, CD8, CD4 sowie Antikörper der Klassen IgM und IgT. Einige der genannten Moleküle werden bei Regenbogenforellen bereits während der Embryogenese exprimiert, was einen frühen Immunisierungserfolg wahrscheinlich macht. Auf zellulärer Ebene interagieren entsprechend infizierte antigenpräsentierende Targetzellen mit zytotoxischen T-Zellen und B-Zellen, was auf funktioneller Seite Schutz vor Infektion basierend auf neutralisierenden Antikörpern und zellvermittelter Zytotoxizität zur Folge hat. Spezifische zellvermittelte Zytotoxizität gegen allogene und gegen virusinfizierte körpereigene Zellen ist bei Fischen MHC Klasse I-restringiert. Immunzellen von vakzinierten Donorfischen zeigen nach

adoptivem Transfer auf homolog und heterolog vakzinierte syngene Rezipienten ein Virusantigen-spezifisches Homing.

v. Aufgrund des Vorhandenseins der Masterregulatorgenhomologe der T-Helferzellrespons EOMES (Eomesodermin) und GATA-3 sowie von Interleukin 4/13A bei Forellen, kann davon ausgegangen werden, dass Fische ähnlich wie Säuger über T-Helfer (Th)1- und Th2-Zellen verfügen, mit denen sie die zytotoxische bzw. antikörpervermittelte Immunantwort steuern.

Translationale und angewandte Forschung

vi. Fische können oral verabreichtes Antigen über den Darm aufnehmen, was einen wirksamen Immunschutz zur Folge hat. Die Darmschleimhaut von Fischen beherbergt Zellen, die Eigenschaften von M-Zellen der Säuger aufweisen.

vii. Die Kieme ist ein wichtiges Organ der angeborenen und adaptiven Immunität. Fische verfügen über ein Interbranchiales Lymphatisches Gewebe (Interbranchial Lymphoid Tissue, ILT). Das Kiemenepithel von Fischen ist in der Lage, bakterielles Impfstoffantigen über verschiedene Zellen aufzunehmen, die Eigenschaften von M-Zellen, Makrophagen und dendritischen Zellen (DC) aufweisen. Solche Zellen befinden sich auch im Epithel des ILT. Viren können gleichfalls über die Kiemen aufgenommen werden, wo sie die Expression von Immunglobulinen in B-Zellen und Effektormolekülen zytotoxischer T-Zellen induzieren. Die Rekrutierung von Immunzellen in die Kieme wird durch Chemokine begünstigt.

viii. Kreuzpräsentierende DCs sind wichtig, um extrazelluläre Antigene, die normalerweise über MHC Klasse II-Präsentation eine gute Antikörperantwort induzieren, auch über MHC Klasse I zu präsentieren. MHC Klasse I triggert zellvermittelte Zytotoxizität, was insbesondere für die virale Abwehr essentiell ist. Kreuzpräsentation ist somit wichtig für die Wirksamkeit inaktivierter Virusvakzinen, deren antigene Bestandteile den Immunzellen extrazellulär angeboten werden. Fische verfügen über einen Schleimhaut-assoziierten Phänotyp von DCs, der MHC-Klasse-II- sowie CD8-positiv ist.

ix. Mukosale Immunisierung (Bad) gegen VHSV induziert bei Forellen eine Verschiebung des T-Zellrezeptor (TCR-)-Repertoires als Ausdruck eines fokussierten Spektrums von antigenspezifischen T-Zellen. Mit Hilfe der

Spektratypisierung der variablen TCR-Domänen lassen sich Rückschlüsse auf den Vakzinationserfolg ziehen.

x. DNA-Immunisierung ist eine empfehlenswerte Methode, um in Forellen Immunreaktionen gegen diskrete virale Proteine zu vergleichen. Ähnlich wie bei einer Virusreplikation werden bei der DNA-Immunisierung virale Proteine intrazelluär translatiert. Antivirale zellvermittelte Zytotoxizität gegen das Glykoprotein G des VHSV ist stärker ausgeprägt als gegen das Nukleokapsidprotein N. Die antivirale zellvermittelte Zytotoxizität gegen das Glykoprotein G des IHNV erfolgt durch antigenspezifische zytotoxische T-Zellen.

xi. Intramuskuläre DNA-Immunisierung induziert an der Injektionsstelle eine lokale inflammatorische Reaktion als Folge der myozytären Expression DNA-kodierter viraler Proteine. Diese Reaktion ist gekennzeichnet durch einen chemokininduzierten Influx von Entzündungszellen, insbesondere IgM- und IgT-exprimierenden B-Zellen. Durch intramuskuläre Injektion ölbasierter Hilfsstoffe (Adjuvanzien) werden hingegen vor allem Granulozyten rekrutiert.

xii. Immunität gegen die Rotmaulseuche lässt sich bei Forellen passiv durch Zellen immunisierter Donoren übertragen, wobei die Träger einer wirksamen Abwehr wahrscheinlich Memory-B-Zellen sind.

xiii. Gedächtnis- (Memory-) Zellen bilden die Grundvoraussetzung für eine aktive Immunisierung (Vakzination). Lymphozyten von Fischen, insbesondere B-Zellen, zeigen eine hohe Überlebensdauer (mindestens 200 Tage), was auf das Vorhandensein von Gedächtnis-Zellen hinweist. Ein Teil der langlebigen Zellen zeigt noch nach Monaten proliferative Eigenschaften.

xiv. Die Immunantwort und insbesondere die zellvermittelte Zytotoxizität unterliegen bei Fischen einer jahreszeitlichen und Temperaturabhängigkeit. Das hat Bedeutung für Vakzinationsstrategien.

xv. Viele Impfstoffe sind zur Steigerung ihrer Wirksamkeit mit Adjuvanzien versetzt. Gewebszerfallsprodukte triggern das Immunsystem von Fischen in Form von sogenannten Gefahrenassoziierten Molekularen Mustern (Danger Associated Molecular Pattern, DAMP). Solche Zerfallsprodukte können bei Fischen als Adjuvanzien eingesetzt werden.

Ausblick

xvi. Die parenterale Verabreichung von Impfstoffen an Fische per Nadel ist kostenintensiv, weshalb mukosale Impfstoffe im Fokus der Entwicklung stehen. Orale und Badvakzinen sind nur dann wirksam, wenn eine effektive Aufnahme der entsprechenden Antigene über die Schleimhäute gewährleistet ist, ohne dabei Toleranz zu induzieren. Das setzt tiefe Kenntnisse der damit im Zusammenhang stehenden grundlegenden Mechanismen voraus.

xvii. Adjuvanzien können die Wirksamkeit von Impfstoffen erhöhen. Adjuvanzien stimulieren das adaptive Immunsystem indirekt über angeborene Immunmechanismen. Deshalb ist eine genaue Kenntnis des Zusammenspiels dieser beiden Komponenten des Immunsystems wichtig.

xviii. Zytokine sind wichtiger Bestandteil der Steuerung des Immunsystems. Bestimmte Interleukine wie das Interleukin-15 sind verantwortlich für die Induktion zytotoxischer Zellen, die wiederum essentieller Bestandteil der antiviralen Abwehr sind. Weiterhin trägt Interleukin-15 zur Rekrutierung von Memory-T-Zellen bei, was Interleukin-15 als mögliches molekulares Adjuvans ausweist.

xix. Zukünftige Forschungen bei Fischen sollten sich deshalb auf Untersuchungen zur Schleimhautimmunität und zu Adjuvanzien konzentrieren.

2 Grundlagenorientierte Forschung zum Fischimmunsystem

2.1 Die Organisation des Immunsystems von Fischen

Die Hauptaufgabe des Immunsystems besteht in der Aufrechterhaltung der Homöostase eines Organismus und damit im Schutz vor Noxen (Substanzen oder Umstände, die einem Organismus Schäden zufügen). Zu den belebten Noxen gehören Viren, Bakterien, Pilze und Parasiten, aber auch Transplantate. Einmal eingedrungen obliegt es dem Immunsystem, die gestörte Homöostase durch Eliminierung dieser Noxen wiederherzustellen. Bevor eine Eliminierung erfolgen kann, muss der Organismus zwischen Eigen und Fremd unterscheiden können, um auszuschließen, dass das Immunsystem gegen körpereigene Strukturen reagiert (Autoimmunreaktion). Eine klassische Situation der Unterscheidung zwischen Eigen und Fremd ist die Transplantatabstoßung, die bereits bei frühen Nichtvertebraten wie Nesseltieren (Cnidaria) entwickelt ist (DIONNE, 2013). Sie dient im weiteren Sinne dazu, die genotypische und phänotypische Integrität des Individuums aufrecht zu erhalten. Transplantatabstoßung ist beim Menschen seit geraumer Zeit bekannt, jedoch waren deren Ursachen lange unklar. Immer wieder wurde versucht, fehlendes oder krankes durch gesundes Gewebe zu ersetzen, was in den meisten Fällen nicht zum Erfolg führte. Trotzdem gelang angeblich im 4. Jahrhundert die Transplantation eines Beines (DA VARAGINE, 1952) und im Mittelalter wurden autologe Hautlappen erfolgreich zur Rekonstruktion verletzter Nasen eingesetzt (TAGLIACOZZI, 1597). Ein Durchbruch gelang in den 1970-er Jahren mit der Entdeckung der Haupthistokompatibilitätsantigene (DAUSSET, 1981), wobei sich Histokompatibilität mit Gewebsverträglichkeit übersetzen lässt. Aufbauend auf den Arbeiten von DAUSSET zum MHC (Major Histocompatibility Complex, MHC) erkannte BENACERRAF (1978) die eigentliche physiologische Bedeutung der MHC-Moleküle bei der adaptiven Immunantwort, wofür beide Wissenschaftler 1980 den Nobelpreis für Physiologie erhielten. Der Bedeutung des MHC bei Fischen ist in dieser Arbeit unter Punkt 2.3.3 ein eigenes Unterkapitel gewidmet.

Das Immunsystem von Vertebraten und somit auch das von Fischen besteht nach klassischer Einteilung aus einer angeborenen (spontanen) und einer adaptiven (lernenden, erinnerungsfähigen) Komponente, die ihrerseits wiederum aus humoralen (flüssigen, löslichen) und zellulären Kompartimenten

bestehen (**I**). Angeborene humorale Kompartimente des Immunsystems sind beispielsweise antimikrobielle Peptide, Lysozym und Komplement, während die entsprechenden zellulären Komponenten durch natürliche Killerzellen, Makrophagen und Granulozyten vertreten sind. Adaptive humorale Komponenten des Immunsystems werden durch die Immunglobuline (Antikörper) repräsentiert, deren zelluläre Gegenstücke durch B-Lymphozyten und T-Lymphozyten verkörpert werden. T-Lymphozyten werden grob in zytotoxische T-Zellen (engl. Cytotoxic T Lymphocytes - CTLs) und T-Helferzellen (Th) eingeteilt. Letztere nehmen innerhalb des Immunsystems eine regulierende Funktion ein und gliedern sich im Wesentlichen in Th1-, Th2- und Th17-Zellen. Th1-Zellen stimulieren vor allem zellvermittelte Zytotoxizität durch Sekretion von Gamma-Interferon (IFN-γ) und exprimieren den Masterregulator T-bet. Th2-Zellen triggern die B-Zellantwort mittels Interleukin (IL)-4 und exprimieren den Masterregulator GATA-3 (**II**). Th17 aktiviert vor allem Granulozyten und hat proinflammatorische Funktionen (**II**). Die Hauptaufgabe von Antikörpern besteht im Zusammenspiel mit weiteren humoralen Faktoren in der Neutralisation von Erregern, während NK-Zellen und CTLs veränderte und fremde (beispielsweise tumoröse und infizierte bzw. transplantierte) Zellen eliminieren.

Die Übersichtsartikel DIJKSTRA, **FISCHER** et al. (2001), **I**, **II**, **III** und **IV** geben ein Überblick zum aktuellen Wissensstand über NK- und T-Zellen bei Knochenfischen. In der Arbeit **I** wird auf diejenigen Moleküle eingegangen, die bei Fischen im Zusammenhang mit zytotoxischen Immunreaktionen bekannt sind sowie auf deren Organisation und Verteilung in Fischen. Schließlich werden die bislang bekannten funktionellen Leistungen zytotoxischer Zellen bei Fischen beschrieben, wobei auch der Einfluss von T-Helferzellreaktionen beleuchtet wird. Ein besonderes Augenmerk wird auf die Regulierung von Immunzellinteraktionen durch Zytokine gelegt, d.h. von Botenstoffen, die das Zellwachstum und die Zelldifferenzierung regulieren. Das Netzwerk der T-Helferzellen im Zusammenspiel mit Zellen der angeborenen Abwehr ist Gegenstand der Arbeit **II**, wobei hier insbesondere vergleichende und evolutionäre Aspekte der Immunabwehr von Fischen und Säugern aufgegriffen werden. In der Arbeit **III** wird der Kenntnisstand zu den Hauptakteuren der adaptiven zellvermittelten Zytotoxizität, den CTL, zusammengefasst. Besondere

Berücksichtigung findet in dieser Arbeit die antivirale Abwehr, deren MHC-Klasse-I-Restriktion und der Bezug zur Vakzination von Fischen mit herkömmlichen und mukosalen Impfstoffen, die über die Schleimhäute aufgenommen werden. Möglichen Strategien zur Stimulation des MHC Klasse I und damit der adaptiven zellvermittelten Immunreaktion durch exogene Antigene, wie sie bei klassischen inaktivierten Impfstoffen bei Fischen zum Einsatz kommen könnten, werden bereits in einer Übersichtsarbeit aus dem Jahre 2001 (DIJKSTRA, **FISCHER** et. al.) aufgezeigt. Dieser Gedanke wurde später wieder aufgegriffen und mündete in einer Arbeit über Kreuzpräsentation mukosaler dendritischer Zellen (DC) bei Forellen (**V**), deren Inhalt in den Unterkapiteln 2.3.2 und 2.3.3 erläutert wird.

Der aktuelle Kenntnisstand zur Schleimhautimmunität in Bezug auf den Intestinaltrakt von Fischen wird in der Übersichtsarbeit **IV** vertieft. Hier wird insbesondere auf die orale Toleranz eingegangen. Orale Toleranz ist ein wichtiger Bestandteil der Immunregulation um zu verhindern, dass das Immunsystem keine sinnlosen Abwehrmechanismen gegenüber Nahrungsmitteln aufbaut, was im schlimmsten Falle zu Nahrungsmittelallergien führen kann. Gleichzeitig stellt orale Toleranz jedoch auch ein großes Hemmnis beim Einsatz von oralen Impfstoffen dar, wobei das Immunsystem Impfstoffantigene toleriert, die über die Schleimhäute aufgenommen werden, anstatt einen protektiven Schutz gegen den entsprechenden Erreger aufzubauen.

2.2 Das angeborene Immunsystem als Grundlage der Abwehr

2.2.1 Einführung

Fische und Rundmäuler sind entwicklungsgeschichtlich die ersten Organismen, die nicht nur über ein angeborenes, sondern auch über ein adaptives Immunsystem verfügen (Übersicht bei BOEHM et al., 2012). Letzteres erscheint jedoch in seiner Struktur gegenüber dem Immunsystem von Säugern primitiv, da Fischen im Gegensatz zu Säugern beispielsweise bestimmte Immunglobulinklassen sowie Lymphknoten und ein Lymphgefäßsystem fehlen. Da sich Fische über einen erdgeschichtlich sehr langen Zeitraum evolviert haben, inzwischen die artenreichste Wirbeltierklasse stellen und sich somit sowohl an sich verändernde Ökosysteme als auch an neue Erreger und

Erregertypen anpassen konnten, ist eine Voraussetzung dafür ihre immunologische Kompetenz. Dabei liegt die Vermutung sehr nahe, dass angeborene Komponenten des Immunsystems die Primitivität der adaptiven Komponenten kompensieren konnten. Weit über 95 % aller Tiere sowie alle Pflanze verfügen ausschließlich über ein angeborenes Immunsystem. Dennoch können sie erfolgreich gegenüber sich ebenfalls kontinuierlich weiterentwickelnden Krankheitserregern bestehen, weshalb der Untersuchung des angeborenen Teils des Immunsystems bei Fischen eine große Bedeutung beigemessen werden sollte.

2.2.2 Mustererkennung

Die angeborene Komponente des Immunsystems wurde früher als unspezifisch bezeichnet, da eine Immunreaktion durch diese Komponente spontan erfolgt und ein Schutz auch gegenüber verwandten Erregern erzielt werden kann. Solche verwandten Erreger weisen antigene Muster (PAMPs - Pathogen Associated Molecular Patterns) auf, die durch Mustererkennungs-Rezeptoren (PRRs – Pattern Recognition Receptors) detektiert werden können (Übersicht bei HANSEN et al., 2011). Diese Muster weisen zwar eine eingeschränkte Spezifität auf, bestimmte PRRs können jedoch nicht alle PAMPs sondern nur bestimmte, verwandte antigene Determinanten erkennen. Zudem setzt bereits die Unterscheidung des Immunsystems zwischen „Eigen“ und „Fremd“ eine gewisse Spezifität voraus. Typische PRRs sind beispielsweise die TLRs.

TLRs sind evolutionär verwandt mit den Toll-Molekülen von Fruchtfliegen, die während der Ontogenese und der antimikrobiellen Abwehr eine Rolle spielen (HASHIMOTO et al., 1988). Später wurden Homologien zu bestimmten PRRs bei Säugern festgestellt, weswegen diese als Toll-ähnliche (-like) Rezeptoren bezeichnet wurden (MEDZHITOV et al., 1997; ROCK et al., 1998). TLRs können verschiedene PAMPs binden, wozu bakterielle Komponenten und virale Nukleinsäuren gehören. Dabei gibt es sowohl genetische als auch funktionelle Homologien zwischen TLRs von Säugern und Fischen, weswegen einige piscine TLRs derselben Nomenklatur folgen wie der von Säugern (Übersicht bei PIETRETTI & WIEGERTJES, 2014). Einer der ersten bei Säugern beschriebenen TLRs war TLR4, welcher Lipopolysaccharid (LPS), eine wichtige Komponente der Zellmembran von Gram-negativen Bakterien, bindet (CHOW et

al., 1999). Obwohl es bei Fischen Sequenzhomologien zum TLR4 von Säugern gibt (OSHIUMI et al., 2003), bindet LPS nicht an piscine TLR4-Moleküle (SULLIVAN et al., 2009). Damit sind piscine TLR4-Gene paralog aber nicht ortholog zu denen von Säugern (PALTI, 2011). Einige piscine TLR-Gene weisen bemerkenswert große Unterschiede zu denen von Säugern auf, so dass diese eine heterologe Bezeichnung erhielten. In der Arbeit von REBL, **FISCHER** et al. (2007) wurde ein bereits beim Atlantischen Lachs beschriebener neuer TLR (AM233509) in der Forelle kloniert und charakterisiert. Da es für diesen TLR kein entsprechendes orthologes Korrelat zu Säuger-TLRs gab, wurde ihm eine neue fortlaufende Nummer, TLR22, zugewiesen. Im Unterschied zum Atlantischen Lachs liegt das TLR22-Gen bei der Forelle jeweils als Zwillingsgen, d.h. in zwei Kopien vor. Entfernte Homologien konnten zu intrazellulären Säuger-TLR-Genen (TLR3, 7, 8, 9, 13) festgestellt werden, die für die Erkennung von Erreger-Nukleinsäuren zuständig sind. TLR22 wird am stärksten in der Milz exprimiert und lässt sich durch inaktivierte *A. salmonicida*-Bakterien stimulieren, wie sie auch in Vakzinen zum Einsatz kommen.

In Forellen wird ein weiteres Zwillingsgen exprimiert, das für ein Homologon des Toll-Interacting-Proteins (TOLLIP) kodiert (REBL, **FISCHER** et al., 2008). TOLLIP fungiert als Inhibitor und hemmt die Interaktion zwischen dem Myeloiden Differenzierungs-Antigen 88 (MyD88) und der Interleukin-Rezeptor-Assoziierten Kinasen 1 und 4 (IRAK-1, IRAK-4). Letztere sind in der TLR-Signaltransduktionskaskade von Säugern dem TLR2 und dem TLR4 nachgeschaltet (O'NEILL, 2000). Damit ist TOLLIP in der Lage, die Wirkung von PAMPs wie die von bakteriellem Lipopeptid bzw. Lipopolysaccharid (LPS) zu regulieren. Eine weitere Funktion von TOLLIP besteht in der Inhibition von IL-1β (einem pro-inflammatorischen Zytokin), dessen Rezeptor ebenfalls die Moleküle MyD88 und IRAK nachgeschaltet sind (Übersicht CAPELLUTO, 2012). Somit spielt TOLLIP eine wichtige Rolle bei der Regulation der Entzündung. Interessanterweise wird TOLLIP bei VHSV-infizierten Forellen aufreguliert, was möglicherweise einer virusassoziierten Entzündungsreaktion entgegenwirkt (REBL, **FISCHER** et al., 2008). Die Arbeit von REBL, **FISCHER** et al. (2009) befasst sich mit dem bereits erwähnten Molekül der TLR-Kaskade MyD88 und einem weiteren Molekül, dem Serum-Amyloid A (SAA). SAA gehört zu den Akute-Phase-Proteinen (APP). Die akute Phase einer Entzündung wird als

systemische Reaktion des Organismus verstanden, wobei APPe bei Säugern als Folge der Bindung exogener infektiöser oder exogener inflammatorischer Noxen an TLRen gebildet werden. SAA wiederum kann an TLR2 von Monozyten binden, was die weitere Expression von proinflammatorischen Zytokinen und Chemokinen zur Folge hat (CHENG et al., 2008; DE BUCK et al., 2016). Sowohl MyD88- als auch SAA-Gene von Forellen zeigen hohe strukturelle Konserviertheiten im Vergleich zu orthologen Genen von Säugern, die auf entsprechende orthologe Funktionen hinweisen. Beide Gene konnten in Forellen nach Infektion mit VHSV transkriptionell stimuliert werden, was auf deren Bedeutung bei der antiviralen TLR-vermittelten Infektabwehr schließen lässt.

Weitere in die angeborene Immunantwort von Säugern involvierte Moleküle sind die MARCH- (Membrane-Associated RING-CH)-Ubiquitin-E3-Ligasen, von denen es beim Menschen 11 unterschiedliche gibt und die eine große Variabilität hinsichtlich ihrer Zellmembranproteinliganden aufweisen (FUJITA et al., 2013). March5 ist bei Säugern in den mitochondrialen Metabolismus involviert (PARK et al., 2014). Orthologe duplizierte March5-Gene wurden bei Forellen in der Arbeit **VI** charakterisiert. Auch MARCH5 wurde in Forellen transkriptionell durch Infektion mit VHSV signifikant aufreguliert, wobei hiervon nur eines der beiden vorhandenen Genkopien betroffen war.

2.2.3 Zelluläre angeborene Abwehr

Zu den zellulären Komponenten des angeborenen Immunsystems zählen die NK–Zellen, deren Existenz und Bedeutung bei Fischen aus der Fähigkeit zur Transplantatabstoßung (**FISCHER** et al., 1998a) und spontaner Zytotoxizität gegenüber allogenen (**FISCHER** et al., 2003) und virusinfizierten Zellen (UTKE, **FISCHER** et al., 2007 und 2008) geschlussfolgert werden kann.

Transplantatabstoßung ist bei Säugern ein komplexer Prozess, bei dem zuallererst Monozyten, Makrophagen, Neutrophile Granulozyten, Thrombozyten und NK-Zellen involviert sind (SPAHN et al., 2014). NK-Zellen erkennen transplantierte Zellen als fremd, wenn deren Rezeptoren keine körpereigenen MHC Klasse I-Moleküle auf der Oberfläche somatischer Zellen finden (sog. Missing-Self-Reaktion). Zu diesen Rezeptoren gehören beispielsweise die Killer-cell Immunoglobulin-like Receptors (KIRs), die Leukocyte

Immunoglobulin-Like Receptors (LILRs), Ly49 und CD94-NKG2. Bei Bindung an körpereigene MHC Klasse I-Moleküle werden in der diesen Rezeptoren nachgeschalteteten Signaltransduktionskaskaden sogenannte ITIMs (Immunoreceptor Tyrosine-based Inhibitory Motifs) aktiviert, was zur Anergie (Reaktionslosigkeit) von NK-Zellen führt und eine Zytolyse gesunder körpereigener Zellen verhindert. Wiederum andere KIRs mit nachgeschalteten ITAMs (Immunoreceptor Tyrosine-based Activating Motifs) aber auch TLRs verschieben das Gleichgewicht zwischen ITIMs und ITAMs hin zu einer Aktivierung von NK-Zellen (Übersicht LANIER, 2008; DELLA CHIESA et al., 2014; SPAHN et al., 2014). Putative Mustererkennungsrezeptoren (PRRs) mit ITAMs und ITIMs wurden auch bei Fischen beschrieben und aufgrund der großen Unterschiede zu Säuger-PRRs als NITRs (Novel Immune-Type Receptors) und als NILTs (Novel Immunoglobulin-Like Transcripts) bezeichnet (YODER et al., 2010). Das weltweit erste NILT von Regenbogenforellen wurde in einer Arbeit von KOCK & **FISCHER** (2008) beschrieben und als Onmy-NILT2D bezeichnet. Es kann alternativ in eine lange membrangebundene und in eine kurze, voraussichtlich sekretierte Variante gespleißt werden. Beide Varianten verfügen über zwei Immunoglobulin-ähnliche Domänen. Da die meisten NILTs und NITRs lediglich als exprimierte Gene beschrieben sind, ist deren Funktion bei Fischen weitgehend unbekannt. Bemerkenswerterweise wird an der zytoplasmatischen Region der langen Variante von Onmy-NILT2D sowohl ein ITAM als auch ein ITIM exprimiert, was als Hinweis darauf verstanden werden kann, dass dieser PRR gleichzeitig über aktivierende und inhibierende Eigenschaften verfügt.

Ein Phänomen der Transplantatabstoßung ist die Transplantat-gegen-Wirt-Krankheit (GVHD, Graft-Versus-Host Disease) oder Transplantat-Gegen-Wirt-Reaktion (Graft-Versus-Host Reaction, GVHR). GVHD ist eine ernstzunehmende Komplikation bei der Knochenmarkstransplantation, wenn immunkompetente Spenderzellen auf einen immunsupprimierten Empfänger übertragen werden. Der Empfänger wird vor der Knochenmarksübertragung einer immunsuppressiven Therapie (Bestrahlung und/oder Chemotherapie) unterzogen, die einerseits seine krankhaften Immunzellen eliminieren und die andererseits verhindern soll, dass die übertragenen Spenderzellen vom Empfänger abgestoßen werden. Die übertragenen Knochenmarkszellen bzw.

deren Tochterzellen sind jedoch voll immunkompetent und können den für sie fremden Empfängerorganismus bzw. dessen Zellen attackieren, was vor allem zu Epithelschäden mit fataler Prognose führen kann (Übersicht VRIESENDORP & HEIDT, 2016). Bei Fischen wurde die GVHD weltweit erstmals von **FISCHER** et al. (1999) beschrieben. Hierbei wurde ein experimentelles System aus triploiden Ginbunas und tetraploiden Ginbuna-Goldfisch (*Carassius auratus,* LINNAEUS 1758)-Hybriden verwendet. Die Ginbuna-Goldfisch-Hybride unterschieden sich von den triploiden Ginbunas genetisch lediglich im Goldfischallel. Daraus folgte, dass ausschließlich die Leukozyten von Ginbunas Körperzellen der Ginbuna-Goldfisch-Hybriden als fremd erkennen können, jedoch nicht umgekehrt. Wurden nun Leukozyten von triploiden Ginbunas auf die tetraploiden Hybride übertragen, begannen die Spenderzellen, die Rezipientenzellen zu attackieren, was zu Epithelschäden in der Haut und im Gastrointestinaltrakt der Rezipienten mit letalem Ausgang führte. Diese Experimente haben eindrucksvoll gezeigt, dass Fische über ihr angeborenes Immunsystem zu ähnlich komplexen Immunreaktionen wie bei höheren Säugetieren befähigt sind.

Natürliche Killer (NK)-Zellen sind wichtige Effektoren der antiviralen Immunantwort. Viele humanpathogene Viren regulieren während der Replikation die Expression von MHC Klasse I und damit die Präsentation viraler Peptide herunter, um auf diese Weise der Erkennung durch CD8-positive zytotoxische T-Zellen (CTL) zu entgehen. Durch fehlende MHC Klasse I-Moleküle wird jedoch ein Missing-Self-Status erreicht, in dessen Folge wiederum bislang anerge (sich in Ruhe befindende) NK-Zellen aktiviert werden. Eine weitere Aktivierung wird erreicht, indem virusinfizierte Zellen auf ihrer Oberfläche Liganden exprimieren, die von Rezeptoren auf NK-Zellen erkannt werden können. Zu diesen gehören u.a. NKG2D, DNAM-1, CD94-NKG2C, NKp46, NKp30 und NKp44, die ihrerseits wiederum aktivierende ITAM-Motive besitzen (Übersicht BELTRÁN & LÓPEZ-VERGÈS, 2014). Auch bei Fischen wird eine NK-Zell-vermittelte Zytotoxizität gegen allogene und virusinfizierte Zellen beschrieben (**I**). So werden VHSV-infizierte Zellen in einem MHC Klasse I-unabhängigen Kontext lysiert. Zur entsprechenden Zytolyse von allogenen (MHC Klasse I-inkompatiblen) virusinfizierten Zellen waren Forellenleukozyten befähigt, die entweder durch sublethale Infektion mit VHSV generiert (UTKE, **FISCHER** et al., 2008) oder durch DNA-Immunisierung gegen das Glykoprotein G

des VHSV (UTKE, **FISCHER** et al., 2007) aktiviert wurden. Solche zytotoxischen Zellen lysierten die infizierten Targetzellen unabhängig davon, ob deren MHC Klasse I mit dem der immunisierten Forellen übereinstimmte oder nicht, weswegen davon ausgegangen werden kann, dass es sich um eine NK-Zell-vermittelte Zytolyse handelte. Letztere Arbeit gilt als Erstbeschreibung einer zellvermittelten Zytotoxizität nach DNA-Immunisierung bei Fischen.

Weitere wichtige Zellen der angeborenen Abwehr insbesondere gegenüber Bakterien sind Makrophagen (KÖLLNER, **FISCHER** et al., 2001) und Granulozyten (Übersicht RIEGER & BARREDA, 2011). Granulozyten verfügen über ein äußerst flexibles Repertoire an Mechanismen der Erregerabwehr. Dazu gehören die Phagozytose („Fressen“ von invasiven Erregern), die Exkretion reaktiver Sauerstoffspezies, die zu einer Oxidation und damit Abtötung von Erregern führt, sowie NETs (Neutrophil Extracellular Traps; z.B. BROGDEN et al., 2012) und zellvermittelte Zytotoxizität (**FISCHER** et al., 1998a) führt.

Wie bei Säugern spielen Thrombozyten von Fischen eine wichtige Rolle bei der Blutgerinnung. Eine Besonderheit der Thrombozyten von Vögeln und Fischen im Vergleich zu Säugern besteht jedoch in deren Kernhaltigkeit. Weiterhin wurde die Fähigkeit von Thrombozyten zur Phagozytose nachgewiesen (NAGASAWA et al., 2014). Thrombozyten von Fischen lassen sich *ex vivo* morphologisch oft schwer von Lymphozyten unterscheiden, weshalb die Etablierung monoklonaler Antikörper zu deren sicheren Erkennung wichtig war (KÖLLNER, **FISCHER** et al., 2004). Forellenthrombozyten verfügen über eine canaliculäres System, welches sich durch deren Zytoplasma zieht und möglicherweise durch Oberflächenvergrößerung die Aufnahme von Partikeln im Nanometerbereich befördert. Die Fähigkeit zur Phagozytose würde auch erklären, warum Thrombozyten von Fischen MHC Klasse II exprimieren (KÖLLNER, **FISCHER** et al., 2004). Beides sind Merkmale von professionellen Antigen-präsentierenden Zellen (Antigen Presenting Cells, APC), die essentiell für die Präsentation bakterieller Antigene und die nachfolgende Etablierung einer antikörpervermittelten Immunantwort sind.

Thrombozyten von Fischen spielen, wie bei Säugern, auch eine Rolle bei der Blutgerinnung. Einer der aktivierenden Faktoren ist Kollagen, das Thrombozyten von Zebrafischen über den Rezeptor G6fL aktiviert (HUGHES et

al., 2012). Bei Goldfischen und Ginbunas verfügt G6fl über ein ITAM (OHASHI, **FISCHER** et al., 2010), was dessen aktivierenden Eigenschaften unterstreicht. Interessanterweise wird G6fL in Goldfischen und Ginbunas auch von Erythrozyten exprimiert, was auf Funktionen schließen lässt, die sowohl Thrombozyten als auch Erythrozyten eigen sind und auf eine gemeinsame hämatopoetische Vorläuferzelle hinweist.

2.2.4 Komplement

Das angeborene Immunsystem wird bei Fischen neben zellulären auch durch humorale (lösliche) Komponenten repräsentiert, wozu beispielsweise antimikrobielle Peptide, Lysozym und Komplement gehören. Diese sind sowohl im Blut als auch im Schleim vorhanden und stellen somit eine erste Barriere gegenüber eindringenden Erregern dar (Übersicht NAKAO et al., 2011, BRINCHMANN, 2016). Immunmodulatorische Substanzen wie β-Glukane können zu einer Aktivierung dieser Barrieren führen und werden zur Stärkung des Immunsystems als Beimischung zum Fischfutter im großen Maßstab eingesetzt (Übersicht SONG et. al, 2014). Neben anderen PAMPs (wie z.B. bakteriellem LPS) wirken auch β-Glukane über die Aktivierung des angeborenen Immunsystems, wobei es zu einer erhöhten Aktivität von Makrophagen, Granulozyten und Lysozym sowie zur Aktivierung des Komplementsystems kommt. Zentrale Schaltstelle aller drei Aktivierungswege des Komplementsystems ist der Komplementfaktor C3. Als Folge der Aktivierung des klassischen, alternativen oder Lektinweges durch Bakterien werden die dem C3 nachgeschalteten Elemente der Komplementsignaltransduktions-kaskade aktiviert, bis schließlich der sogenannte Membranangriffskomplex (Membrane Attack Complex - MAC) gebildet wird, was schlussendlich zur Lyse von Bakterienzellen führt (Übersicht ZHANG & CUI, 2014). C3 wird vor allem in der Leber gebildet (ALPER et al., 1969), wobei Makrophagen von Säugern ebenfalls C3 exprimieren (MOGILENKO et al., 2012). In einer Studie mit Salmoniden (LOVOLL, **FISCHER** et al., 2007) wurde gezeigt, dass naive Forellen- und Lachsmakrophagen *in vitro* keine C3-Gene transkribieren. Obwohl die Stimulation solcher Makrophagen mit LPS und β-Glukan eine erhöhte Expression von proinflammatorischen Zytokinen zur Folge hatte, blieb die

Transkription von C3 in den Makrophagen weiterhin aus. Dennoch war C3-mRNA nach *in vivo*-Stimulation von Forellen mit LPS und β-Glukan transkriptionell aufreguliert, was vergleichbare Daten bei anderen Fischspezies, wie z.B. beim gefleckten Seewolf (*Anarhichas minor,* OLAFSEN 1772), zu bestätigen scheint, wonach C3 auch unter der Abwesenheit von Makrophagen induziert werden kann (NORUM et al., 2005).

2.3 Das adaptive Immunsystem als Grundlage der Vakzination

2.3.1 Einführung

Die entwicklungsgeschichtlich aus einem gemeinsamen Vorfahren hervorgegangenen Kieferlosen (Agnatha) und Kiefermäuler (Gnathostomata) sind aus phylogenetischer Sicht die frühesten Organismen, die über ein adaptives Immunsystem verfügen (Übersicht bei BOEHM et al., 2012). Während die Kieferlosen mit den Rundmäulern (Cyclostomata) in eine entwicklungsgeschichtliche Sackgasse geraten sind, haben sich die ersten primitiven Kiefermäuler zunächst zu Knorpel-, im weiteren Verlauf zu den Knochenfischen und letztendlich zu Säugetieren entwickelt. Zur frühen Entwicklung der Wirbeltiere haben diverse Ereignisse von Genomduplikationen wesentlich beigetragen, was wahrscheinlich zu einer erweiterten Diversität und Variabilität von Komponenten des Immunsystems geführt hat (Übersicht bei WARD & ROSENTHAL, 2014). Das adaptive Immunsystem von Kiefermäulern ist bereits durch ein erweitertes Repertoire an somatisch hypermutierten B-Zell- und T-Zell-Antigenrezeptoren gekennzeichnet, die hochspezifisch für das entsprechende Antigen sind und somit über die eingeschränkte Spezifität der für das angeborene Immunsystem typischen Mustererkennung hinausgehen. Gleichwohl als Basis für eine Vakzination verfügt das adaptive Immunsystem über ein immunologisches Gedächtnis, das zu einer schnelleren und effizienteren sekundären Immunantwort gegen homologes Antigen befähigt.

Der humorale Teil des adaptiven Immunsystems wird bei Fischen durch drei Immunglobulin-Klassen IgM, IgD und IgT (IgZ Homologon bei Zebrafischen, *Danio rerio,* HAMILTON 1822) repräsentiert. Gleichzeitig fungieren die Immunglobuline als B-Zellrezeptoren, wenn sie auf B-Zellen verankert sind. Ebenso wie bei Säugern unterliegen Immunglobuline der Fische einer somatischen Rekombination ihrer variablen Domänen, was deren hohe

Variabilität in der spezifischen Erkennung antigener Determinanten zur Folge hat (CANNON et al., 2004). Bei Fischen erfolgt jedoch keine gezielte Rekombination der schweren Immunglobulinketten und damit kein Immunglobulinklassenswitch (WAKAE et al., 2006), was sich bei Säugern in einem Wechsel von IgM oder IgD zu den anderen bekannten Ig-Klassen IgG, IgE und IgA äußert (Übersicht, STAVNEZER & SCHRADER, 2014). Da bei Fischen zudem die entsprechenden Fcε-, Fcα- bzw. Fcγ-Rezeptormoleküle fehlen, an die Ig-Isotypen von Säugern mit ihrem Fc-Teil der schweren Kette binden könnten, erfolgt bei Fischen auch keine entsprechende Spezialisierung hin zu antiparasitären (IgE), schleimhaut- (IgA) bzw. serumspezifischen (IgG) Effektorfunktionen und damit keine weitere gewebs- bzw. erregerspezifische Fokussierung (AKULA et al., 2014). Eine Ausnahme hiervon bildet das IgT, dem eine spezifische Funktion in der Schleimhautimmunität von Fischen nachgesagt wird (PARRA et al., 2016).

Ein zusätzlicher Hinweis für die im Gegensatz zu höheren Vertebraten eingeschränkte Spezialisierung des Immunsystems von Fischen ist die Tatsache, dass ihre lymphatischen Organe einfacher strukturiert sind als die höherer Vertebraten. Das betrifft beispielweise das Fehlen von Lymphknoten und Keimzentren, in denen bei Säugern wichtige Interaktionen zwischen B- und T-Zellen, incl. Immunglobulinklassenswitch (Übersicht, DE SILVA & KLEIN, 2015), stattfinden. Weiterhin fehlt ein organisiertes darmassoziiertes lymphatisches Gewebe (Gut Associated Lymphoid Tissue, GALT). Fische besitzen jedoch, genau wie Säuger, einen Thymus und eine Milz.

Die zellulären Komponenten des adaptiven Immunsystems werden durch T- und B-Zellen repräsentiert. Die Herkunft der B-Zellen ist bei Fischen umstritten, wobei experimentelle Studien zur Expression von RAG (Recombination Activating Gene) sich dahingehend verdichten, dass die Kopfniere als wahrscheinlichster Bildungsort diskutiert wird (HUTTENHUIS et al., 2005; WANG et al., 2014b).

2.3.2 Zelluläre Komponenten der adaptiven Immunantwort

Bei Vertebraten und somit auch bei Fischen rekrutieren sich die T-Zellen aus dem Thymus (ZAPATA et al., 2006). So sind dort CD3 (Marker für T-Zellen)- (KOPPANG, **FISCHER** et al., 2010) sowie CD8 (Hauptmarker für zytotoxische T-

Zellen - CTL)- und CD4 (Hauptmarker für Th-Zellen)-positive Zellen (**VII**) zu finden. Eine wichtige Aufgabe des Thymus besteht bei Säugern in der Selektion von T-Zellen, wobei anerge (keine Reaktion zeigende) und hypererge (zu stark reagierende) T-Vorläuferzellen in den programmierten Zelltod (Apoptose) geschickt werden (Übersicht KURD & ROBEY, 2016). Während dieses Selektionsprozesses sind die Thymozyten „doppelt positiv", d.h. sie tragen im Gegensatz zu extrathymischen T-Zellen der meisten Säuger gleichzeitig CD8- und CD4-Moleküle. Solche doppelt positiven Thymozyten konnten auch bei Forellen in einer Studie mit durchflusszytometrisch sortierten CD8-positiven Thymozyten eindeutig belegt werden (**VII**), während im peripheren Blut und den sekundären lymphatischen Organen und Geweben nur Zellen nachweisbar waren, die entweder CD8 oder CD4 exprimieren. Eine Ausnahme von dieser Regel kann beispielsweise bei Schweinen beobachtet werden, bei denen auch extrathymische doppelt positive T-Zellen vorkommen (Übersicht GERNER et al., 2009). Überraschend für Fische (hier Regenbogenforellen) ist der äußerst geringe Anteil von CD8-positiven Zellen im Blut (<1 % der Lymphozyten) sowie in der Milz und der Kopfniere (<5 % der Lymphozyten), während der Prozentsatz dieser Zellen in den Schleimhäuten etwa zwischen 10-25 % (Kieme) und 50 % (Darm) liegt. Ähnliche Ergebnisse erbrachten Untersuchungen zur Verteilung von CD8-positiven Zellen in Ginbuna-Karpfen (TODA et al., 2011). Dies unterstreicht die Bedeutung mukosaler CTL bei der Infektabwehr.

Vor der Erstbeschreibung eines monoklonalen Antikörpers gegen Forellen-CD8 (**VII**) war es nicht möglich, CTL oder Subpopulationen von DC (**V**) bei Forellen darzustellen. Deshalb ist die Etablierung solcher Antikörper ein Meilenstein bei der Untersuchung der zellulären Immunabwehr bei Fischen. Die Arbeit **VII** beinhaltet nicht nur die Verteilung von CD8-positiven Zellen in den lymphatischen Organen sondern auch deren Charakterisierung. So wurden durchflusszytometrisch sortierte CD8-positive und CD8-negative Lymphozyten auf die Expression von immunologisch relevanten Genen untersucht. Während extrathymische CD8-positive Lymphozyten mRNA kodierend für CD8, aber keine kodierend für IgM (B-Zellmarker) und CD4 (T-Helferzellmarker) exprimierten, konnte umgekehrt in CD8-negativen Lymphozyten Genexpression von CD4 und IgM aber nicht von CD8 mittels RT-PCR festgestellt werden. Nach

Stimulation exprimierten CD8-positive Zellen die für sie typischen Effektorgene *Perforin* und *Granulysin* (vermitteln Zytolyse von Targetzellen) sowie *IFN-γ* (wichtiges aktivierendes Zytokin für CTL und von Th1-Zellen, die ihrerseits wiederum die CTL-Respons triggern). Folgerichtig war in CD8-negativen Lymphozyten (beinhalten Th1- und Th2-Zellen) neben dem Th2-Zytokingen *IL-4/13A* (triggern die antikörpervermittelte Abwehr) auch *IFN-γ*-Expression feststellbar. Obwohl diese Expressionsstudien grundlegende Charakteristika des Immunsystems von Säugern widerspiegeln, wurden auch Unterschiede zu Säugern, wie der geringe Gehalt an CD8-positiven Zellen im Blut und deren Abundanz im respiratorischen Gewebe (Forellen-Kieme versus Säuger-Lunge), gezeigt. Die Kieme kann durch den direkten Kontakt mit Wasser höheren Erregerlasten ausgesetzt werden als die Lunge durch die Luft, weshalb eine hohe Abundanz von Immunzellen im piscinen respiratorischen Gewebe sinnvoll erscheint.

Diese Annahme wird durch das Vorhandensein eines bis vor Kurzem unbekannten lymphatischen Gewebes in der Kieme von Salmoniden unterstrichen, das 2010 in einer Arbeit von KOPPANG, **FISCHER** et al. (siehe auch **VIII**) erstmalig beschrieben wurde und den Namen Interbranchiales Lymphatisches Gewebe (Interbranchial Lymphoid Tissue, ILT) erhielt. Dieses nichtvaskularisierte Gewebe ist im Gegensatz zu dem bei Säugern und Vögeln vorhandenen subepithelialen BALT (Bronchus Associated Lymphoid Tissue) eine intraepitheliale Struktur, da deren lymphatische Zellen (vornehmlich CD3-positive/CD8-positive Zellen) oberhalb der epithelialen Basalmembran lokalisiert sind, und das ILT von Zytokeratin durchzogen ist. Obwohl die Vermutung nahelag, dass es sich beim ILT durch seine unmittelbare Nachbarschaft zum Thymus um ein hämatopoetisches Gewebe handelt, konnten keine morphologischen Korrelate zu einer für den Thymus typischen Medulla (Mark) oder zu einer Cortex (Rinde), keine Rekombination (fehlende Expression von RAG) soweit keine apoptotischen Ereignisse festgestellt werden, die bei der Reifung und Selektion von Thymozyten normalerweise gehäuft anzutreffen sind.

Eine grundlegende Eigenschaft des adaptiven Immunsystems ist sein immunologisches Gedächtnis. Im weitesten Sinne besitzen bereits Prokaryoten eine adaptive Abwehr mit Gedächtnisfunktion, die mit Hilfe des Clustered

Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas (CRISPR-assoziierten)-Systems bewerkstelligt wird (Übersicht BOEHM & SWANN, 2014). Dieses Gedächtnis wird jedoch auf Einzelzellebene realisiert und ist nicht das Produkt einer klassischen systemischen Reaktion, wie sie bei Vertebraten abläuft und in deren Verlauf ein ganzes Netzwerk von Immunzellen und Molekülen involviert ist. Die Erlangung eines Impfschutzes ist deshalb ausschließlich den Vertebraten vorbehalten, da sie über ein klassisches adaptives Immunsystem und damit über ein klassisches immunologisches Gedächtnis (engl. memory) verfügen. Dies bedeutet, dass nach wiederholtem Kontakt mit einem homologen infektiösen Agens schneller auf eine Infektion reagiert werden kann als es ein naives Immunsystem vermag. Bereits PANUM konnte 1846 belegen, dass der Schutz vor einer Maserninfektion für mindestens 65 Jahre anhält. Träger des immunologischen Gedächtnisses sind langlebige Memoryzellen, die nach Abklingen einer Immunantwort noch Monate bzw. Jahre im Körper zirkulieren können (Übersicht SLIFKA & AMANNA, 2014). Auch bei Fischen existieren offenbar solche langlebigen Immunzellen (**FISCHER** et al., 1998b). In einer entsprechenden Studie mit Ginbunas, welchen fluorochromierte autologe Leukozyten übertragen wurden, konnten Lymphozyten durchflusszytometrisch über einen Zeitraum von mehr als 145 Tagen *in vivo* nachgewiesen werden. Der Subtyp der Lymphozyten (B-Zellen oder T-Zellen) konnte zu diesem Zeitpunkt in Ermangelung entsprechender, Marker-spezifischer Antikörper noch nicht ermittelt werden. In einer einzigartigen, unlängst abgeschlossenen Langzeitstudie (JAAFAR, YAMAGUCHI & **FISCHER**, unveröffentlicht) mit fluorochromierten Forellenleukozyten aus der Milz, die an isogene Forellen desselben Klons übertragen wurden, waren es vor allem die IgM-positiven B-Zellen und weniger die CD8-positiven Lymphozyten, die über einen Zeitraum von mehr als 200 Tagen *in vivo* nachweisbar waren. Eine spezifische Präferenz dieser Zellen (Homing) für die Milz war nicht zu erkennen. Interessanterweise kam es bei einem Teil der langlebigen B-Zellen nach Übertragung mehrfach zur Zellteilung. Diese Zellteilung konnte durchflusszytometrisch ermittelt werden, da markierte Mutterzellen immer jeweils die Hälfte des Farbstoffes an ihre Tochterzellen weitergaben, was anhand einer verringerten Fluoreszenzintensität festgestellt werden konnte

(Abb. 1). Ein Teil der übertragenen langlebigen B-Zellen teilte sich nicht und deutet somit auf den ruhenden Pool von Memory-B-Zellen hin.

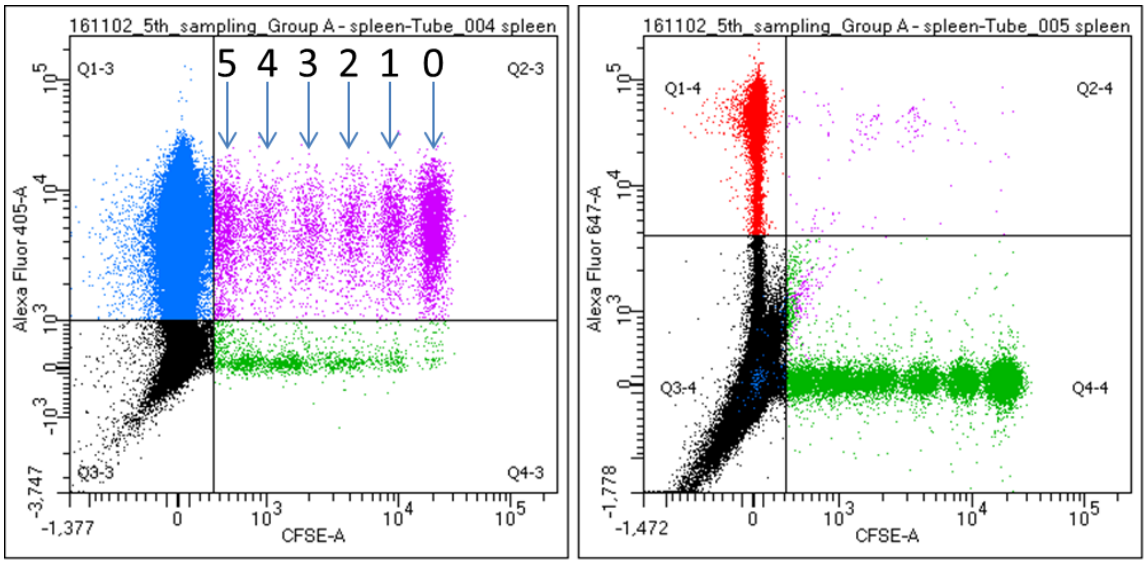
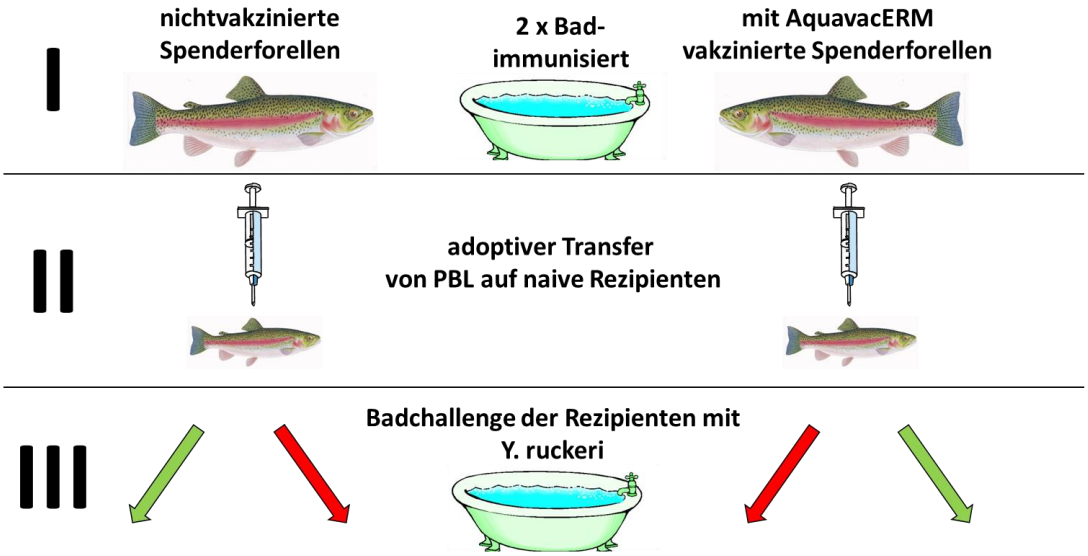


Abb. 1: Darstellung CFSE-gefärbter Donoren-Forellenmilzzellen (CFSE-positive Zellen befinden sich jeweils in den rechten Quadranten Q2 und Q4) 200 Tage nach Übertragung an naive Rezipientenforellen. Nach der Entnahme aus den Rezipienten wurden die Gesamtmilzzellen mit Antikörpern gegen IgM und AlexaFluor 405 (linker Plot) sowie mit Antikörpern gegen CD8 und AlexaFluor 647 (rechter Plot) gefärbt (IgM- bzw. CD8-positive Zellen befinden sich jeweils in den oberen Quadranten Q1 bzw. Q2). Während noch ein beträchtlicher Teil von übertragenen (CFSE-positiven) Zellen IgM positiv war (siehe Q2-3), wurden kaum noch transferierte CD8-positive Zellen (siehe Q2-4) gefunden. Während einige übertragene Zellen sich nicht geteilt hatten (Pfeil 0), ist deutlich zu sehen, dass ein Teil dieser Zellen bis zu 5 Teilungszyklen (Pfeile 1-5) erfahren haben, was anhand der nach links sequenziell abnehmenden Fluoreszenzintensität ablesbar war (JAAFAR, YAMAGUCHI & **FISCHER**, unveröffentlicht).

Parallel übertragene isogene Leukozyten aus dem Darm hatten eine sehr kurze Überlebensdauer, weshalb davon auszugehen ist, dass im Darm von Forellen kaum Memoryzellen generiert werden.

Dass B-Zellen wichtige Träger einer protektiven Immunität bei bakteriellen Infektionen sind, konnte weltweit erstmalig bei Fischen in der Arbeit **IX** (Vortrag, Publikation in Vorbereitung) belegt werden. Hierzu wurden klonale, genetisch identische Spender-Forellen bei 15 °C im Abstand von 10 Wochen mit einer Vakzine (AquavacERM, MSD) gegen die Rotmaulsäuche immunisiert und nach

weiteren 2 Wochen deren periphere Blutleukozyten (PBL) isoliert (Abb. 2, I). Die PBL wurden an naive Rezipienten-Forellen des gleichen Klons transferiert (Abb. 2, II), wonach diese mit *Y. ruckeri* infiziert wurden (Abb. 2, III). Während Kontrollforellen, die PBL von nichtvakzinierten Spendern übertragen bekamen, eine kumulative Mortalität von 63,6 % (14 von 22 Empfängern moribunt) aufwiesen, überlebten alle Forellen, die PBL von immunisierten Spendern übertragen bekamen (Abb. 2).



Scheininfektion	Y. ruckeri		Y. ruckeri	Scheininfektion
Mortalitäten (verendete Rezipienten/Gesamtzahl infiziert)				
0/22	**14/22**		**0/22**	**0/22**

Abb. 2: Schutz vor Infektion nach adoptivem Transfer von immunkompetenten PBL auf syngene naive Rezipienten. Dazu wurden Spenderforellen gegen *Y. ruckeri* badimmunisiert (I, rechte Hälfte), deren PBL isoliert und auf Rezipienten übertragen (II). Die Rezipienten wurden danach mit *Y. ruckeri* infiziert (III, rote Pfeile) und deren Mortalitäten ausgelesen. Die Übertragung von Immunzellen schützte die Empfänger vor Infektion (rechter roter Pfeil).

Weiterhin wurden von den Spendern und Empfängern Blutseren gewonnen und auf das Vorhandensein von Antikörpern gegen *Y. ruckeri* untersucht. Die Spenderforellen zeigten nach Vakzination moderate IgM-Extinktionen. Nachdem deren Blutleukozyten auf naive Rezipienten übertragen

worden waren, kam es nach einem wiederholten Antigenkontakt (hier Challenge) zur Induktion sehr hoher IgM-Extinktionen, während die alleinige Übertragung solcher Blutleukozyten in den Rezipienten keine Antikörper induzierte (Abb. 3). Dadurch konnte gezeigt werden, dass es wahrscheinlich die übertragenen Memory-B-Zellen waren, die nach Challenge eine rasche IgM-Produktion induzierten. Dies wiederum unterstützte die These, dass B-Zellen und in Folge die Produktion von *Y. ruckeri*-spezifischem IgM für den Schutz vor Infektion verantwortlich waren. Die Vermutung, dass IgM-Antikörper einen Schutz gegen die Rotmaulseuche vermitteln, äußerten auch RAIDA et al. (2011), die eine neutralisierende Wirkung von Blutplasmen immunisierter Forellen gegen homologe *Y. ruckeri*-Bakterien feststellen konnten, während EVENHUIS et al. (2014) durch Übertragung von Seren *Y. ruckeri*-immunisierter Forellen auf naive Forellenrezipienten bei Letzteren einen belastbaren Schutz vor *Y. ruckeri*-Infektion etablierte. EVENHUIS et al. (2014) haben zusätzlich, ähnlich wie in der Arbeit **IX** beschrieben, PBL auf syngene Forellen übertragen, konnten hiermit aber keinen Schutz vermitteln. Das mag daran gelegen haben, dass EVENHUIS et al. (2014) nicht konsequent Badimmunisierung und Badinfektion miteinander kombiniert haben, was wiederum dem natürlichen Infektionsweg bei der Rotmaulseuche entsprochen hätte, sondern Impfstoff und Vakzine wurden teilweise mittels Injektion appliziert.

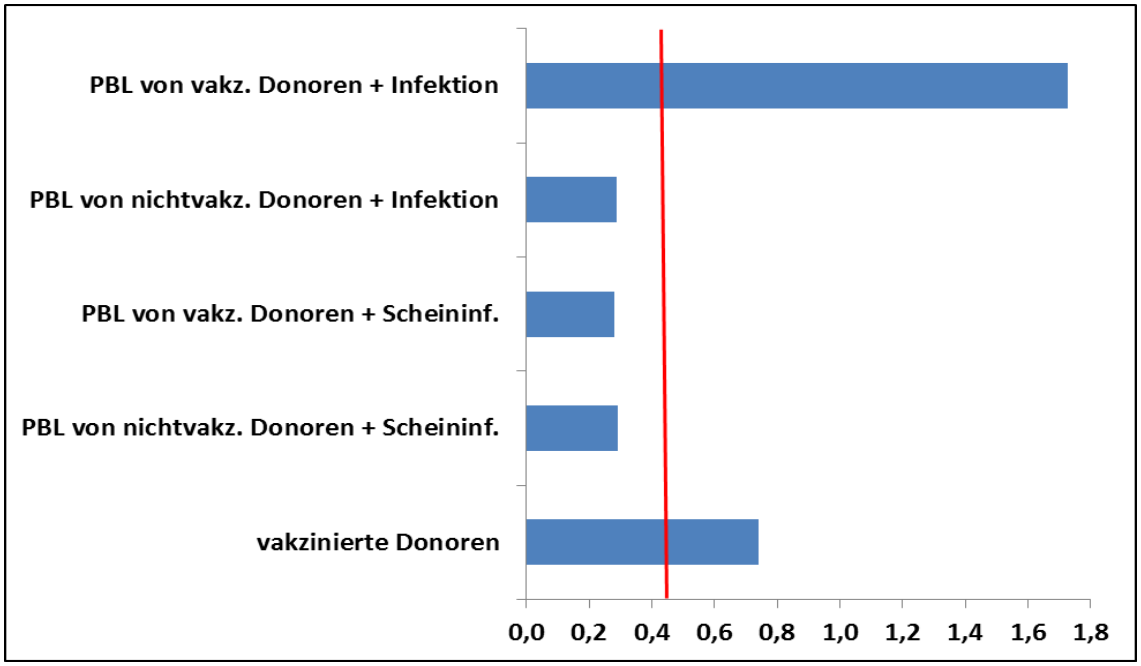


Abb. 3: Induktion von *Y. ruckeri*-spezifischem IgM in Forellenseren nach Vakzination gegen *Y. ruckeri* (untere Säule) sowie in Forellen, die Blutleukozyten von

vakzinierten Spendern übertragen bekamen und danach mit *Y. ruckeri* infiziert worden waren (obere Säule). Übertragung von solchen Leukozyten auf Rezipienten-Forellen allein oder Infektion von Forellen, denen naive Leukozyten übertragen wurden, induzierte keine Antikörper (mittlere drei Säulen). Die rote Linie markiert den sogenannten Cut-Off unterhalb bzw. oberhalb dessen eine Unterscheidung zwischen IgM-negativen und IgM-positiven Zellen getroffen wurde.

2.3.3 MHC - Major Histocompatibility Complex

In der intensiven Aquakultur besteht die Gefahr, mit Fischlinien zu arbeiten, die eine eingeschränkte genetische Diversität aufweisen. Dies kann dazu führen, dass bestimmte Linien gegenüber manchen Erregern bzw. Erregervarianten empfänglicher sind, als es für die gesamte genetische Bandbreite einer Population der Fall wäre. Solche für die Immunabwehr bedeutsamen genetischen Unterschiede sind insbesondere in den Haupthistokompatibilitäts-Antigenen zu suchen, deren Bedeutung für die Resistenz gegenüber Krankheiten (z.B. GRIMHOLT et al., 2003) aber auch für das Verhalten (AZUMA, **FISCHER** et al., 2005) bei Fischen nachgewiesen wurde. Die MHC-Antigene kommen prinzipiell in zwei Varianten vor, dem MHC Klasse I und dem MHC Klasse II. Diese beiden Varianten unterscheiden sich in Struktur und Aufgabe. Der MHC Klasse I-Proteinkomplex besteht aus einer membranverankerten Domäne, einer aus drei Domänen (α1, α2 und α3) bestehenden schweren α-Kette (Heavy Chain, HC) sowie einer löslichen Untereinheit, dem β2-Mikroglobulin (β2m), die wiederum der schweren Kette Stabilität verleiht (BERKO et al., 2005). Die Aufgabe von MHC Klasse I besteht darin, antigene Peptide intrazellulären Ursprungs (beispielsweise die von viralen Proteinen) dem TCR von CD8-positiven zytotoxischen T-Zellen (CTL) zu präsentieren. Dabei wird das entsprechende Peptid in einer Grube gebunden, die durch die Domänen α1 und α2 gebildet wird. Demgegenüber besteht der MHC Klasse II-Proteinkomplex aus zwei membranverankerten Untereinheiten, den α- und β-Ketten, die wiederum jeweils zwei extrazelluläre Domänen bilden (α1 und α2 bzw. β1 und β2). Beim MHC Klasse II werden Peptide von Antigenen extrazellulären Ursprungs (z.B. von durch Phagozytose aufgenommenen Bakterien) in einer Grube präsentiert, die von den α1- und β1-Domänen gebildet wird. Hier erfolgt die Präsentation durch Bindung an den TCR von Th-

Zellen, wobei als akzessorisches Molekül anstelle von CD8 hier das CD4-Molekül fungiert. Während der MHC Klasse I von allen kernhaltigen Zellen exprimiert wird, verfügen nur sogenannte professionelle APC (Makrophagen, DC und B-Zellen) über einen MHC Klasse II. Antigenpräsentation durch MHC Klasse I triggert die CTL-vermittelte Zytotoxizität, während durch MHC Klasse II präsentierte Peptide die Th-Zellen ansprechen, was im Falle von Th1-Zellen zu einer Verschiebung der Immunbalance in Richtung zellvermittelte Zytotoxizität und im Falle von Th2-Zellen die B-Zellantwort und damit die Bildung von Antikörpern in den Vordergrund rückt (Übersicht z.B. bei JANEWAY et al., 2001; VAN STIPDONK et al., 2001).

Die wichtigste Aufgabe der MHC-Moleküle besteht nicht, wie unmittelbar nach deren Entdeckung zunächst angenommen, in der Abstoßung von Transplantaten, sondern in der Präsentation von antigenen Erregerstrukturen gegenüber den Zellen des adaptiven Immunsystems. Dennoch hält die Transplantationsimmunologie auch für den Fischimmunologen wichtige Modelle zur Untersuchung grundlegender Immunmechanismen bereit. Das betrifft beispielsweise die Transplantat-gegen-Wirt-Krankheit (GVHD, Graft-Versus-Host-Disease) (**FISCHER** et al., 1999, siehe Unterkapitel 2.2.3) sowie die zellvermittelte Zytotoxizität gegen allogene (fremdes Individuum derselben Spezies) Zellen (**FISCHER** et al., 1998a). Aus der Überlegung heraus, dass piscine Erythrozyten kernhaltige Zellen sind und somit, im Gegensatz zu Säugern, MHC Klasse I exprimieren, haben **FISCHER** et al. (1998a) weltweit erstmals kernhaltige allogene Erythrozyten als Targetzellen zytotoxischer Zellen eingesetzt. Um alloreaktive zytotoxische Zellen zu generieren, wurden Schuppen von klonalen tetraploiden Ginbuna-Goldfisch-Hybriden auf klonale triploide Ginbunas transplantiert. Danach wurden Leukozyten von den so sensibilisierten triploiden Rezipienten gewonnen und *in vitro* gegen Erythrozyten der tetraploiden Spender zur Reaktion gebracht, wobei eine klare zytotoxische Reaktion anhand der Freisetzung von Hämoglobin aus den lysierten Erythrozytentargets gemessen werden konnte. Dadurch hatte sich der bis dahin übliche Einsatz von radioaktivem Chrom-51 zur Messung der Targetzelllyse erübrigt. Vergleichbare allospezifische zytotoxische Reaktionen gegen allogene Targetzellen (hier permanente Zellkulturen) konnten auch

HASEGAWA et al. (1998) unter Verwendung eines auf Radioaktivität basierenden Chrom-51-Assays feststellen.

Die Untersuchungen des Verfassers dieser Arbeit an Ginbuna-Karpfen wurden nachfolgend an klonalen Regenbogenforellen verfeinert (**FISCHER** et al., 2003). Dazu wurden Leukozyten von Fischen isoliert, die zuvor mit Allotransplantaten (Haut) sensibilisiert wurden. Nun wurden die Leukozyten nicht als Gesamtpool eingesetzt, sondern durch magnetische Zellsortierung mittels Antikörpern gegen IgM, Granulozyten, Monozyten und Thrombozyten zusätzlich in entsprechende Subpopulationen separiert. Nur die IgM-negativen Lymphozyten vermochten allogene Targeterythrozyten zu lysieren. Anhand von RT-PCR-Untersuchungen wurde ferner gezeigt, dass nur die IgM-negativen Lymphozyten CD8 exprimieren, und dass deren CD8-Expression durch wiederholten Kontakt mit dem homologen Alloantigen noch gesteigert werden konnte. Dies ließ darauf schließen, dass CTLs die Hauptakteure der allospezifischen zellvermittelten Zytotoxizität waren und dass, wahrscheinlich wie bei der Allotransplantatabstoßung in Säugern (WANG et al., 1998), MHC-assoziierte Peptide im Fokus der allospezifischen CTL stehen.

Später konnten DIJKSTRA, **FISCHER** et al. (2003) unter Verwendung eines erstmals bei Fischen etablierten Antikörpers gegen MHC Klasse I die theoretische Überlegung zur Expression von MHC Klasse I-Molekülen auf kernhaltigen Forellenerythrozyten durch einen Direktnachweis dieser Moleküle untermauern. In derselben Arbeit wurde festgestellt, dass MHC Klasse I in weiteren kernhaltigen Zellen von Forellen vorkommt, insbesondere in Lymphozyten, Makrophagen und Granulozyten, was deren Bedeutung bei der Präsentation über MHC Klasse I-Moleküle und als professionelle APC unterstreicht. Auf Organebene konnten die höchsten Proteinexpressionen von MHC Klasse I in den lymphatischen Organen und den mukosalen Oberflächen (Darm, Kieme) sowie in verschiedenen Endothelien festgestellt werden (DIJKSTRA, **FISCHER** et al., (2003). Gleichzeitig haben Sarder, **FISCHER** et al. (2003) gezeigt, dass MHC Klasse I einer der Hauptdeterminanten für die Abstoßungsreaktion gegenüber allogenen piscinen Erythrozyten ist. Um letztere Schlussfolgerung machen zu können, wurden Forellenerythrozyten mit unterschiedlichem MHC Klasse I-Hintergrund jeweils mit verschiedenen Fluorochromen markiert und auf Rezipienten übertragen, die aus derselben

Geschwistergruppe stammten wie die Donoren. Dabei stellte sich heraus, dass eine Abstoßung immer im Zusammenhang mit individuellen Unterschieden im MHC Klasse I stand.

Einige piscine Viren wie das ISAV (Infectious Salmon Anaemia Virus) replizieren in Erythrozyten (WORKENHE et al., 2008), was Erythrozyten auch in den Focus der antiviralen MHC Klasse I-restringierten CTL-vermittelten Abwehr rückt. Da piscine Viren wie alle Viren ausschließlich intrazellulär replizieren, ist das MHC Klasse I-Molekül auch bei Fischen von zentraler Bedeutung bei der CTL-vermittelten viralen Abwehr (UTKE, **FISCHER** et al., 2007 und 2008). In beiden Arbeiten wurde gezeigt, dass Leukozyten von virusinfizierten Fischen bzw. von Fischen, die gegen eine virale Erkrankung DNA-immunisiert wurden, MHC Klasse I-syngene virusinfizierte Targetzellen besser lysieren als solche mit einem heterologen MHC Klasse I. Daraus konnte geschlussfolgert werden, dass MHC Klasse I-restringierte CTLs verantwortlich für die Zytolyse von virusinfizierten Zellen waren. Diese Experimente zeigten auch, dass andere zytotoxische Zellen als CTL (vermutlich NK-Zellen) infizierte Zellen lysieren können. Diese grundlegenden Arbeiten gelten weltweit als Erstnachweis einer zellvermittelten Zytotoxizität nach DNA-Immunisierung bei Fischen bzw. nach einer VHSV-Infektion bei Forellen.

Grundsätzlich präsentieren MHC Klasse II-Moleküle antigene Peptide extrazellulären Ursprungs, wie beispielsweise die von über Phagozytose aufgenommenen Bakterien. Werden extrazelluläre Antigene über MHC Klasse I präsentiert, spricht man von Kreuzpräsentation. Einige kreuzpräsentierende dendritische Zellen (DC) sind bei Säugern durch eine ansonsten den CTL vorbehaltene Expression von CD8-Molekülen charakterisiert (Übersicht bei GUTIÉRREZ-MARTÍNEZ et al., 2015). In der Arbeit **V** wurde erstmalig ein entsprechender DC-Phänotyp bei Forellen beschrieben. Dies ist umso bemerkenswerter, als dass die Existenz von DCs in Fischen bislang umstritten war. In der entsprechenden Publikation (**V**) wird eine hoch komplexe Subpopulation von Leukozyten beschrieben, die gleichzeitig MHC Klasse II und CD8 exprimiert. Diese Subpopulation von DCs, die gut 1 % der in der Haut von Forellen erfassten Leukozyten ausmachte, zeigte phänotypische und funktionelle Charakteristika unreifer Säuger-DCs, die angesichts des Fehlens von Lymphknoten bei Fischen an der Regulation dezentraler bzw. lokaler

mukosaler Immunreaktionen einschließlich mukosaler Toleranzinduktion beteiligt sein könnte. Diese Forellen-DCs exprimierten ansonsten keine weiteren T-Zellmarker (CD3, TCRα, TCRβ, CD8β), jedoch konnten hohe Expressionslevel der DC-Marker DC-SIGN und LAMP3 gezeigt werden. Weitere DC-typische Eigenschaften äußerten sich in deren Fähigkeit zur Phagozytose von apoptotischen Zellen und zur Stimulierbarkeit durch PAMPs. Zusätzliche Charakteristika dieser DC-Subpopulation waren die Expression von CD141 (BDCA-3/Thrombomodulin) und CD103 (IntegrinαE) sowie weiterer für kreuzpräsentierende Zellen charakteristischer Moleküle wie des PRRs TLR3, des Basalen Leuzin-Zipper-Transkriptionsfaktors ATF-ähnlich (Batf)3, und des IFN-regulatorischen Proteins (IRF)8.

3 Translationale und angewandte Forschungsarbeiten zum Fischimmunsystem

3.1 Ontogenese und Immunkompetenz

Bereits in der frühen Ontogenese, d.h. während der Embryogenese und im larvalen Stadium, sind Fische oft einem hohen Infektionsdruck ausgesetzt. Trotz des während dieser Phase noch nicht vollständig entwickelten Immunsystems, können Erreger durch die bei einigen Spezies im Dottersack enthaltenen erregerspezifischen maternalen Antikörper neutralisiert werden, wenn die Laichner bereits Kontakt mit dem selben Erreger hatten (ZHANG et al., 2013). Fische können aus rein technologischer Sicht während des larvalen Dottersackstadiums lediglich durch Badexposition immunisiert werden. Eine Applikation von Impfstoffen über die Nadel ist wegen der geringen Größe der Fische nicht möglich und die orale Immunisierung, insbesondere bei Salmoniden (z.B. Lachse, Forellen, Saiblinge) durch die spät einsetzende Futteraufnahme, ausgeschlossen. Zu beachten ist ferner, dass eventuell im Dottersack vorhandene maternale Antikörper das Impfantigen neutralisieren und damit für das Immunsystem nicht mehr zugänglich machen könnten. Weiterhin neigen juvenile Organismen dazu, die in der Frühphase der Ontogenese aufgenommenen Antigene später zu tolerieren (Übersicht LØKKA & KOPPANG, 2016). Dies kann dazu führen, dass solche Fische keine belastbare Immunität ausprägen oder bei Erregerkontakt eine chronische Erkrankung entwickeln, die klinisch inapparent verläuft. Aufgrund des Carrier-Status solcher

klinisch unauffälligen Fische stellen diese als permanente Ausscheider von pathogenen Erregern eine Gefahr für andere Fische dar. Eine Immunisierung von Fischen macht im larvalen Stadium aus diesen Gründen oft keinen Sinn.

Für die Entwicklung von Impfstrategien ist es deshalb von herausragender Bedeutung zu wissen, ab welchem Zeitpunkt während der Ontogenese Immunkompetenz vorliegt. Informationen darüber, ab wann bestimmte Komponenten des Immunsystems verfügbar sind, geben wichtige Hinweise darauf, ob diese Komponenten bereits potentiell durch Vakzination angesprochen werden können. Anhand von Expressionsstudien zu immunologisch relevanten Molekülen der Antigenpräsentation (MHC Klasse I) sowie der adaptiven zellvermittelten Zytotoxizität (CD8, TCR) konnten (**FISCHER** et al., 2005) nachweisen, dass diese immunrelevanten Gene bereits sehr früh während der Embryogenese exprimiert werden. In derselben Arbeit kam auch erstmals ein Antikörper gegen MHC Klasse I zum Einsatz, mit dessen Hilfe das entsprechende Molekül während der Ontogenese erstmalig bei Fischen nicht nur auf mRNA- sondern auch auf Proteinebene dargestellt werden konnte. Neben weitgehender Bestätigung der Ergebnisse der RT-PCR war ein überraschend früher Nachweis von MHC Klasse I in den Neuronen der Forelle möglich. Letzterer Befund deutet darauf hin, dass MHC Klasse I auch bei Fischen eine Bedeutung bei der Ontogenese von neuronalem Gewebe hat (HUH et al., 2000; ELMER & MCALLISTER, 2012). In der späteren Ontogenese ging die MHC Klasse I-Expression im Forellengehirn stark zurück, wie es auch für Neuronen bei adulten Säugern unter normalphysiologischen Bedingungen beschrieben ist (NARDO et al., 2016). Weiterhin konnten **FISCHER** et al. (2005) ab dem Beginn der Fütterung der juvenilen Fische sehr hohe Expressionsraten von MHC Klasse I in den Oberflächenepithelien, insbesondere des Darmes, feststellen, nachdem die Larven den Dottersack aufgebraucht hatten und mit der Fütterung begonnen worden war. Zu diesem Zeitpunkt waren die Epithelien über das Wasser und die orale Aufnahme erstmalig in größerem Maßstab nutritiven Antigenen ausgesetzt, was die höheren Expressionsraten von MHC Klasse I erklären würde.

In einer späteren Arbeit mit Regenbogenforellen (HEINECKE et al., 2014) wurden die Expressionsergebnisse von **FISCHER** et al. (2005) weitgehend bestätigt und erweitert. Während Gene von Molekülen der angeborenen

Abwehr (wie Komplementkomponenten und SAA) aber auch einige entsprechenden Elemente der adaptiven Abwehr, wie CD8, MHC Klasse II und IgM, relativ früh exprimiert wurden, konnte mRNA kodierend für CD4, TCR und IgT erst später detektiert werden. Ob die Expression dieser Gene auch einen Vakzinationserfolg und damit Schutz vor Infektion garantiert, kann letzten Endes nur der Tierversuch zeigen.

Bei der Seezunge (*Solea solea,* LINNAEUS, 1758) kommt zu den beiden Schlüsselereignissen der Fischontogenese Schlupf und Fütterungsbeginn noch die Metamorphose der Augenverlagerung hinzu. Überraschenderweise scheinen bei dieser Spezies Gene des adaptiven Immunsystems wie MHC Klasse I und TCR früher exprimiert zu werden als bestimmte Gene der angeborenen Immunität, deren Expression erst nach der Metamorphose nachweisbar war (FERRARESSO et al., 2016).

3.2 Vakzination von Fischen

3.2.1 DNA-Immunisierung

In Aquakultur gehaltene Fische haben ein weitaus geringeres individuelles Körpergewicht als die meisten landwirtschaftlichen Nutztiere. Um vermarktbare Mengen an Muskelfleisch in der Aquakultur zu produzieren, müssen sehr große Tierzahlen gehalten werden, deren individuelle Immunisierung per Nadel einen hohen Personaleinsatz erfordert. Das derzeit wirksamste, jedoch auch gleichzeitig teuerste, Verfahren ist die intraperitoneale (i.p.) Applikation von Impfstoffen per Nadel, wobei inzwischen auch computergesteuerte Impfautomaten zum Einsatz kommen. Hierbei können optimale Antigenmengen zusammen mit Adjuvanzien (Hilfsstoffe, die die Wirkung von Arzneistoffen verstärken) verabreicht werden. Bei diesen Hilfsstoffen handelt es sich oft um Mineralöl-basierte Adjuvanzien, welche durch ihre proinflammatorischen Eigenschaften die Immunantwort auf den Impfstoff triggern (Übersicht POWELL et al., 2015). Intramuskulär (i.m.) lassen sich solchermaßen adjuvantierte Impfstoffe aufgrund der mit der Adjuvanswirkung verbundenen erheblichen lokalen Nebenwirkungen nur eingeschränkt einsetzen. Demgegenüber können hocheffiziente DNA-Vakzinen auch ohne den Einsatz von Adjuvanzien intramuskulär injiziert werden (UTKE, **FISCHER** et al., 2008; Überblick EVENSEN &

LEONG, 2013), was wiederum den Einsatz von Impfautomaten unterstützen würde.

Bedenken zum Einsatz von DNA-Vakzinen bei Fischen stehen im Zusammenhang mit ihrer Stabilität, der Persistenz der Plasmide im Fisch, ungewollten Immunreaktionen und der möglichen chromosomalen Integration von Plasmid-DNA (GILLUND et al., 2008). Obwohl viele dieser Bedenken unbegründet sind, ist die öffentliche Akzeptanz von DNA-Vakzinen insbesondere im europäischen Raum nach wie vor niedrig (Überblick HØLVOLD et al., 2014). Erste Vorstöße auf EU-Ebene nähren jedoch die Hoffnung, dass DNA-Vakzinen ihr Stigma verlieren. So hat die European Medicines Agency (EMA) 2016 einen von der Firma Elanco entwickelten DNA-Impfstoff gegen das Salmon Pancreas Disease (SPD) zur Zulassung empfohlen.

Nach Vakzination sowie nach Infektion von Säugern wird appliziertes bzw. eingedrungenes Antigen durch das Lymphgefäßsystem in die dränagierenden Lymphknoten transportiert, wo komplexe Immunreaktionen in Gang gesetzt werden. Zu diesen Reaktionen gehört die Keimzentrumsreaktion, wo es nach Antigenprozessierung und Präsentation zur Proliferation von B-Zellen und zur Entwicklung von antikörperproduzierenden Plasmazellen kommt. Das Vorhandensein eines Lymphgefäßsystems, welches solche Antigeneintrittspforten dränagieren könnte, wird bei Fischen kontrovers diskutiert (RUMMER et al., 2014). Fische besitzen keinen Lymphknoten und in der Milz und der Kopfniere werden keine Keimzentren gebildet. Deshalb wird bei Fischen, neben zentralen Immunreaktionen (Milz und Kopfniere), den lokalen Immunreaktionen eine große Bedeutung beigemessen. In der Arbeit **XV** wurden komplexe lokale Immunreaktionen an der i.m. Injektionsstelle nach DNA-Vakzination gegen die Virale Hämorrhagische Septikämie (VHS) untersucht. Hierbei konnte festgestellt werden, dass es nach lokaler Expression von viralem Glykoprotein in den Muskelzellen zu einer lokalen Entzündungsreaktion kommt, die durch einen Influx bzw. die Proliferation von mononukleären Leukozyten und insbesondere B Zellen gekennzeichnet war. Überraschenderweise waren unter den infiltrierenden B-Zellen nicht nur, wie zu erwarten, IgM-positive Zellen, sondern auch eine ganze Reihe von IgT-positiven Zellen, obwohl letzteren gemeinhin eine exklusive Bedeutung in der Schleimhautimmunität zugesprochen wird (ZHANG et al., 2010; PARRA et al.,

2016). Der Influx von B-Zellen war begleitet von einer deutlichen Expression von Chemokin- und Chemokinrezeptorgenen.

Der Influx von Immunzellen in die Inkjektionsstelle von DNA-Vakzinen folgt bei Fischen einer antigenen Spezifität, was durch Homing-Experimente mittels adaptivem Transfer von Immunzellen gezeigt werden konnte (UTKE, **FISCHER** et al. (2008). Als Homing bezeichnet man die Rezirkulation von immunkompetenten Lymphozyten an den Ort ihrer Herkunft, was bei Säugern in der Regel deren Rückkehr in die Lymphknoten und die Milz bedeutet (GALLATIN et al., 1986). Um das Phänomen Homing bei Fischen überhaupt erstmals zu zeigen, haben UTKE, **FISCHER** et al. (2008) klonale Donor-Forellen mit einer DNA-Vakzine gegen das Glykoprotein des Rhabdovirus VHSV i.m. immunisiert. Aus der Applikationsstelle der DNA-Vakzine wurden später die Leukozyten isoliert sowie fluorochromiert und danach auf syngene Forellen-Rezipienten übertragen. Diese Rezipienten waren zuvor mit homologen und heterologen DNA-Vakzinen kodierend für die Glykoproteine der Rhabdoviren VHSV bzw. dem Tollwutvirus und dem SVCV (einem zypriniden Rhabdovirus) immunisiert worden. Nach adoptivem Transfer der Donorleukozyten zeigten diese in den Rezipienten eine präferentielle Affinität zur Applikationsstelle von homologen (aber nicht heterologen) DNA-Vakzinen. Diese Ergebnisse deuten darauf hin, dass Immunzellen von Forellen antigenspezifisch rezirkulieren.

3.2.2 Mukosale Immunisierung

Wegen des relativ hohen Arbeitsaufwandes bei der Injektion von Impfstoffen an Fische werden Immunisierungsverfahren angestrebt, die sich automatisieren bzw. bei denen sich Impfstoffe über die Schleimhäute (Haut/Kiemen: Bad- und Sprayimmunisierung; Magen-Darmtrakt: orale Immunisierung) verabreichen lassen (**VIII**). Bei der Aufnahme von oral applizierten Impfantigenen aus dem Darmlumen spielen bei Säugern die sogenannten M-Zellen (engl. multifold cells) eine Rolle, deren Homologe durch FUGLEM, **FISCHER** et al. (2010) bei Fischen (hier Forelle und Atlantischer Lachs) erstmals beschrieben wurden. Dadurch konnte ein wichtiger Beitrag zur Charakterisierung spezifischer Phänotypen von Antigen-resorbierenden Schleimhautzellen geleistet werden, was dem tieferen Verständnis der Wirksamkeit von mukosalen Vakzinen bei Fischen dient. M-Zellen von Säugern zeichnen sich durch eine apikal zum

Darmlumen hin gefaltete Oberfläche aus, die nur wenige Mikrovilli aufweist und durch die Antigene aus dem Darmlumen aufgenommen werden. Die basale Seite dieser Zellen verfügt über taschenartige Einbuchtungen, in denen Lymphozyten und APCs in engen Kontakt mit den M-Zellen treten können. Hier erfolgt eine Weitergabe von antigenen Komponenten (Übersicht WANG et al., 2014a). Die bei Salmoniden beschriebenen M-ähnlichen Zellen zeigten morphologische Parallelen zu denen von Säugern (kaum Mikrovilli auf der apikalen Seite) und waren in der Lage partikuläre Antigene aufzunehmen. Dabei exprimierten sie charakteristische Zuckerreste, die mit UEA1, einem Lektin aus dem Stechginster (*Ulex europaeus*) und charakteristischem M-Zellmarker, angefärbt werden konnten. Weiterhin waren an den basalen Seiten dieser M-ähnlichen Zellen Lymphozyten zu finden. M-ähnliche Zellen könnten bei Fischen für solche Infektionen von Bedeutung sein, bei denen die Eintrittspforte der Darm ist bzw. bei denen Impfantigene über die Darmschleimhaut aufgenommen werden (z.B. *Y. ruckeri*) (KHIMMAKTHONG et al., 2013; KORBUT et al., 2016).

Trotz gewisser Fortschritte im Verständnis der mukosalen Immunität bei Fischen (Übersicht SALINAS, 2015; **IV; IV**) gibt es noch erhebliche Wissenslücken. So erweist sich die eingeschränkte Wirksamkeit von oral bzw. durch Bad applizierten Impfstoffen über den Magen-Darm-Trakt, die Kiemen bzw. die Haut bei Fischen nach wie vor als ein großes Problem und ist deshalb Gegenstand intensiver Forschung (Übersicht MUNANG'ANDU et al., 2015). Insbesondere die Kieme scheint durch Ihre filigranes und großflächiges Epithel als ideale Eintrittspforte für die Aufnahme von Impfstoffantigenen prädestiniert zu sein, wobei die Organisation und die Abundanz lymphoider Strukturen in der Kieme (siehe hierzu Kapitel 2.3.2) dieses Postulat noch untermauern (KOPPANG, **FISCHER** et al., 2010; **VII**).

Verschiedene Arbeitsgruppen (z.B. COBO et al., 2014) konnten die Kieme von Forellen als Eintrittspforte von inaktivierten *A. salmonicida*-Bakterien nachweisen, jedoch waren diese Autoren nicht in der Lage, den Zelltyp zu bestimmen, der für die Aufnahme der Bakterien zuständig ist. Kürzlich konnte festgestellt werden, dass die zur Aufnahme von bakteriellen Antigen befähigten Zellen, sogenannte Gill Antigen Sampling (GAS)-Zellen, der Kiemenschleimhaut von Forellen ein charakteristisches Lektinbindungsmuster

aufweisen (**XI**), wie es auch für M-Zellen des Darmes bei Ratten beschrieben wurde (ZHAO et al., 2014). Hierzu wurden Forellen mit formalininaktivierten Bakterien (*A. salmonicida salmonicida* – *A.s.s.*) badvakziniert, deren Kiemen entnommen und mit Antikörpern gegen *A.s.s.* sowie mit dem fluorochromierten Lektin UEA1 als putativem M-Zellmarker doppelgefärbt. Ein Teil der Epithelzellen war intrazytoplasmatisch sowohl UEA1- als auch *A.s.s.*-positiv, was darauf hindeutet, dass M-ähnliche Zellen bakterielles Antigen aufgenommen hatten. Solche Zellen fanden sich vermehrt in der Nähe des ILT (Abb. 4). Neben den Lektin-positiven GAS-Zellen, waren auch Lektin-negative Zellen, wahrscheinlich Makrophagen und/oder DCs, zur Antigenaufnahme befähigt.

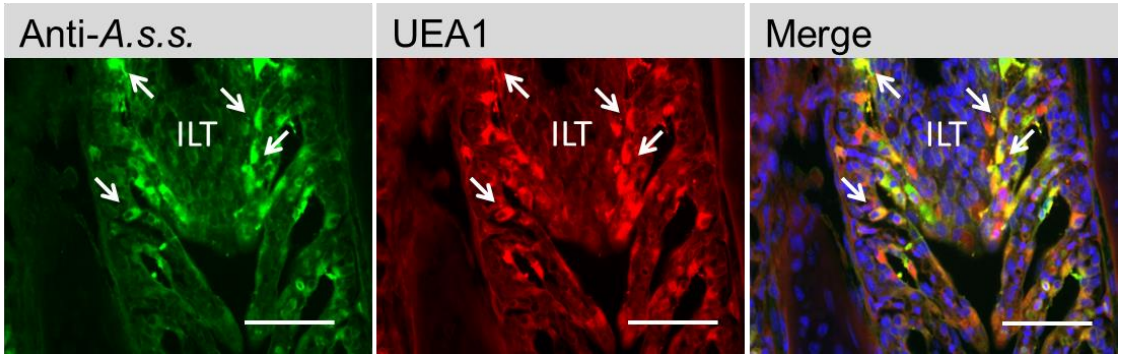


Abb. 4: Kryostatschnitte der Forellenkieme nach Badimmunisierung mit inaktivierten A.s.s.-Bakterien und anschließender Färbung mit Antikörpern gegen *A.s.s.* (grün) und UEA1 (rot). Nach Überlagerung der beiden Bilder erscheinen manche Zellen doppelgefärbt/orange (Pfeile). Einige Zellen sind entweder nur grün (putative Makrophagen) oder rot (andere als M-Zellen, z.B. Becherzellen, die ebenfalls lektinbindende Zucker enthalten). Blau = Zellkernfärbung; Weiße Balken = 50 µm.

Aufgrund ihres Lektinbindungsmusters sowie ihrer Eigenschaft, fluoreszenzmarkierte Bakterien (*A.s.s.*) über die Kieme aufzunehmen, wurden solche GAS-Zellen weiterhin durchflusszytometrisch in UEA1 / *A.s.s.*-doppelt-positive sowie *A.s.s.*-einfach-positive Zellen sortiert (Abb. 5). Von den sortierten Zellen wurden anschließend die Transkriptome mittels Next Generation Sequencing (NGS) bestimmt. Während Lektin-positive GAS-Zellen das für M-Zellen typische Annexin sowie IL-20 (charakteristisch für Epithelzellen) exprimierten, zeigten Lektin-negative GAS-Zellen ein Expressionsmuster, wie

es für Makrophagen und DCs typisch ist (Expression von CD83, IL-12 und MHC Klasse II).

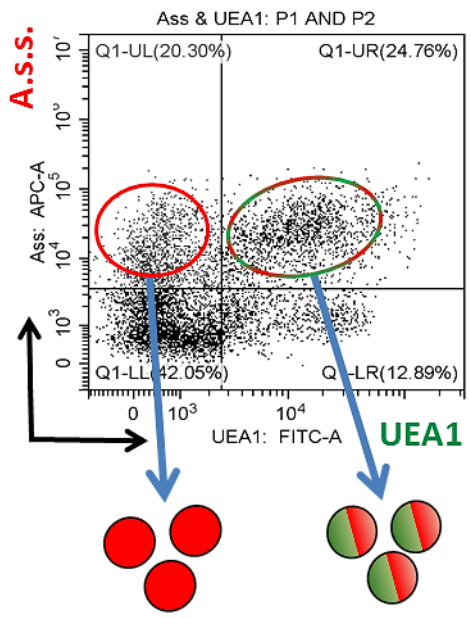


Abb. 5: Dotplot von Kiemenzellen nach Badimmunisierung von Forellen mit gefärbten *A.s.s.*-Bakterien (rot) und anschließender Färbung mit Antikörpern UEA1 (grün). Es wurden jeweils die UEA-negativen (oberer linker Quadrant, 20,30 %) und die UEA1-positiven (oberer rechter Quadrant, 24,76 %) GAS-Zellen durchflusszytometrisch sortiert.

Bei ähnlichen Experimenten zur Aufnahme von inaktivierten *Y. ruckeri*-Bakterien konnten die Ergebnisse mit *A. salmonicida* nicht nachvollzogen werden, was auf andere Eintrittspforten als die Kieme hindeutet (**XI**). So haben KHIMMAKTHONG et al. (2013) nach Badimmunisierung von Forellen sowie KORBUT et al. (2016) nach Badimmunisierung von Zebrafischlarven mit inaktivierten *Y. ruckeri*-Bakterien eine Aufnahme des Antigens über die Haut und den Darm festgestellt, weniger jedoch über die Kieme.

Nicht nur Bakterien, sondern auch Viren, wie z.B. VHSV, können über die Kiemen aufgenommen werden. Bei entsprechenden Untersuchungen (**XII**) kam es kurz nach Badimmunisierung mit attenuiertem VHSV zur Expression von Immunglobulingenen (*IgM* und *IgT*) in B-Zellen und zur Expression des Effektorgens zytotoxischer T-Zellen *Perforin* in CD8-positiven Zellen, was als Ausdruck einer adaptiven humoralen bzw. zellulären Immunreaktion zu werten ist. Die chemotaktische Rekrutierung dieser Zellen wurde unterstützt durch die Expression von Chemokinen. Solche und Untersuchungen anderer Autoren (Übersicht SALINAS, 2015) weisen die Kieme unzweifelhaft als ein zentrales Organ der angeborenen und adaptiven Abwehr aus.

Die artspezifische Kenntnis der Physiologie des Gastrointestinaltraktes hat große Bedeutung bei der Entwicklung von Impfstoffen für in Aquakultur gehaltene Fische. So können oral verabreichte Antigene durch Magensäure, insbesondere bei karnivoren Fischen wie Salmoniden, inaktiviert werden. In der Vergangenheit sind deshalb verschiedene Strategien entwickelt worden, um eine solche Inaktivierung zu verhindern (Übersicht MUTOLOKI et al., 2015), beispielsweise durch Neutralisation der Magensäure (ADELMANN, **FISCHER** et al., 2008). In letzterer Arbeit wurde eine attenuierte orale Lebendvirusvakzine aus abgeschwächtem VHSV mit einer Substanz versetzt, die in der Lage ist, während der Magenpassage das dortige saure Milieu zu neutralisieren und damit eine Inaktivierung der Lebendvirusvakzine zu verhindern. Nach Verfütterung des in Pelletform formulierten Impfstoffes an Forellen gelangte replikationsfähiges Virus bis in die hinteren Darmabschnitte, wo es im Epithel mittels Immunfluoreszenz nachweisbar war. Die so immunisierten Fische zeigten einen belastbaren Immunschutz gegenüber Challenge mit hochvirulentem VHSV. Leider haben solche Lebendvirusvakzine nur marginale Zulassungschancen, da die Gefahr besteht, dass die abgeschwächten, jedoch weiterhin replikationsfähigen, Erreger zu hochpathogenen Viren rückmutieren, bei Übertragung auf hochempfängliche Brütlinge Klinik bzw. Mortalität auslösen, und dass bei Austritt in Wildgewässer replikationsfähiges Virus unkontrolliert in der Umwelt zirkuliert.

Schleimhautimmunität wird ebenso wie andere Komponenten des Immunsystems durch T-Helfer (Th)-Zellen reguliert, wobei Th1-Zellen durch Aktivierung von zytotoxischen Zellen vor allem für pro-inflammatorische Immunreaktionen stehen, während eine Th2-dominierte Immunantwort anti-inflammatorisch wirkt und auf die Produktion von Immunglobulinen ausgerichtet ist (Übersicht **IV**). Th1 und Th2 wirken gegenseitig antagonistisch und werden durch ein komplexes Netzwerk von Masterregulatoren, Rezeptoren-Ligandeninteraktionen und Zytokinen (Übersicht in **II**) gesteuert. Die Charakterisierung dieser Netzwerkkomponenten gestattet Rückschlüsse auf die Qualität der Th-Antwort und kann somit ein wichtiges Instrument bei der Etablierung und Evaluierung von Vakzinen sein. Unter Kenntnis der Qualität einer Th-Antwort auf bestimmte Pathogene können Vakzinen derart konzipiert werden, dass sie präferenziell eine Th1- oder eine Th2-Antwort induzieren,

beispielsweise bei der Wahl von Adjuvanzien, in deren Ergebnis Th1- oder Th2-relevante Stimulation angezeigt erscheint.

In der Arbeit **XIII** werden zwei homologe Gene für Eomesodermin (*EOMES1* und *2*), einem Masterregulator der Th1-Antwort für Forellen und Ginbunas beschrieben. Neben einer hohen Expression von *EOMES* im Gehirn und den Ovarien, was u.a. eine Beteiligung an der Neurogenese bzw. Oogenese nahelegt, deutete die Expression in lymphatischen Organen auf eine Beteiligung bei der Immunantwort hin. In CD8-positiven/IgM-negativen Zellen (putative CTL) und CD8-negativen/IgM-negativen Zellen (putative Th1-Zellen und NK-Zellen) war die Expression von *EOMES* am höchsten, was weiterhin auf dessen Bedeutung bei der zellvermittelten Immunantwort durch CTL hinweist.

In einer weiteren Arbeit (**XIV**) des Verfassers dieser Schrift wurde festgestellt, dass die Schleimhäute von gesunden Forellen in erster Linie Th2-typische Gene exprimieren (insbesondere *interleukin-4/13A* und *GATA-3*, während in den lymphatischen Organen eher ein Th1-typisches Milieu (Expression von *EOMES* und *IFN-γ*) etabliert ist. Das macht aus physiologischer Sicht durchaus Sinn, da die Schleimhäute einem ständigen Antigenkontakt (z.B. ubiquitäre und kommensal-intestinale Keime, nutritive Antigene) ausgesetzt sind. Diese grundsätzlich ungefährlichen Noxen werden normalerweise toleriert, um widersinnige und gleichzeitig energieträchtige Immunreaktionen zu vermeiden. Solche physiologischen Gegebenheiten gilt es bei der Entwicklung von mukosalen Vakzinen zu berücksichtigen.

Das antiinflammatorische Th2-Milieu der Schleimhäute gesunder Fische wird bei Infektion rasch in den Status einer aus immunologischer Sicht adaptiven inflammatorischen Abwehrreaktion versetzt. So werden in der Kieme von Forellen nach Infektion mit VHSV eine ganze Reihe von Zellen rekrutiert, die sowohl zellvermittelte als auch humorale Immunmechanismen repräsentieren, wobei die Expression von Chemokinrezeptor- bzw. Effektormolekül-Genen auf die Rekrutierung und den funktionellen Phänotyp dieser Zellen schließen lässt (**XII**).

3.2.3 Adjuvanzien

Trotz vielversprechender Ansätze zur mukosalen Applikation von Impfstoffen an Fische werden parenteral (i.p. und i.m.) verabreichte Vakzinen aufgrund ihrer besseren Wirksamkeit und geringeren Herstellungskosten auch in Zukunft eine Rolle spielen. Zur Steigerung der Wirksamkeit parenteraler Fischimpfstoffe werden im großen Maßstab Adjuvanzien eingesetzt (siehe auch 3.2.1 und 3.2.2). Diese Hilfsstoffe triggern einerseits die Immunantwort auf den Impfstoff, andererseits können sie jedoch auch erhebliche Nebenwirkungen in Form von Peritonitis (Bauchfallentzündung) zur Folge haben. Diese tritt in Form von Verklebungen der inneren Organe zutage, was vom Standpunkt des Tierschutzes und der Vermarktbarkeit des Lebensmittels Fisch inakzeptabel ist (Übersicht EVENSEN et al., 2005; TAFALLA et al., 2013). Bei der durch Adjuvanzien hervorgerufenen Abwehrreaktion kommt es auch zum Gewebszerfall, wobei die Zellzerfallsprodukte durch bestimmte auf Immunzellen exprimierte PRRs erkannt werden. Solche Zerfallsprodukte werden auch als Damage- oder Danger-Associated Molecular Patterns (DAMPs) bezeichnet (SEONG & MATZINGER, 2004). DAMP ist ein Oberbegriff, hinter dem sich eine ganze Reihe von Zellzerfallsprodukten verbergen. Unter anderem können DAMPs durch autolytischen Zerfall von Proteinen und DNA entstehen (SILVA, 2010). Zerfallsprodukte von zellulären Proteinen und zellulärer DNA können auch *ex vivo* hergestellt werden. So konnten **FISCHER** & KOTTERBA (unveröffentlicht) solche artifiziellen DAMPs erfolgreich zur Steigerung der Wirksamkeit von Vakzinen gegen die Furunkulose der Forellen, hervorgerufen durch *A.s.s.*, als Adjuvans einsetzen.

Hierzu wurden Forellen mit einer traditionellen Vakzine aus formalininaktiviertem *A.s.s.*-Antigen oder mit derselben Vakzine unter Zusatz von DAMP-Adjuvans per i.p.-Injektion immunisiert und danach mit virulenten homologen Bakterien belastet. Dabei vermittelte die adjuvantierte Vakzine einen besseren Schutz vor Infektion als das *A.s.s.*-Antigen allein (Abb. 6).

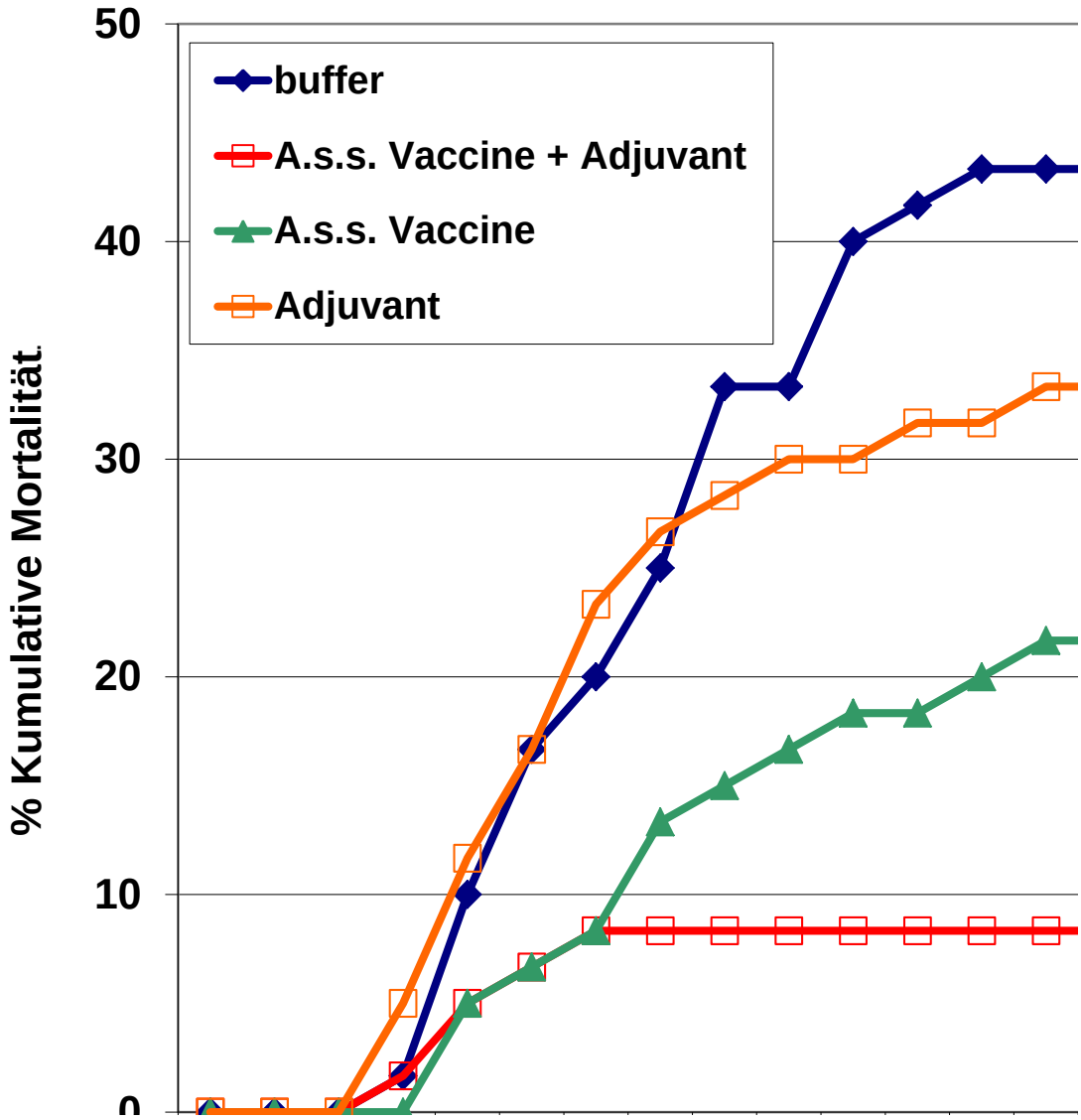


Abb. 6: Kumulative Mortalität nach Vakzination gegen Furunkulose mit und ohne Adjuvans sowie nachfolgende Belastungsinfektion mit *A.s.s.* Während Kontrollforellen, die entweder Puffer statt Vakzine oder nur DAMP-Adjuvans gespritzt bekamen, erhöhte Mortalitäten zeigten, waren Forellen mit einer Kombination aus Vakzine und Adjuvans am besten geschützt. Das Experiment wurde einmal wiederholt, wobei sich nach Challenge ein ähnlicher Immunschutz abzeichnete wie im ersten Experiment.

Nachfolgende *in vitro*-Experimente (YAMAGUCHI, **FISCHER**, unveröffentlicht) haben gezeigt, dass das verwendete DAMP-Adjuvans für Leukozyten der Forelle nicht zytotoxisch ist und die Genexpression der proinflammatorischen Zytokine IL-1β2 and IL-6 induziert (Abb. 7).

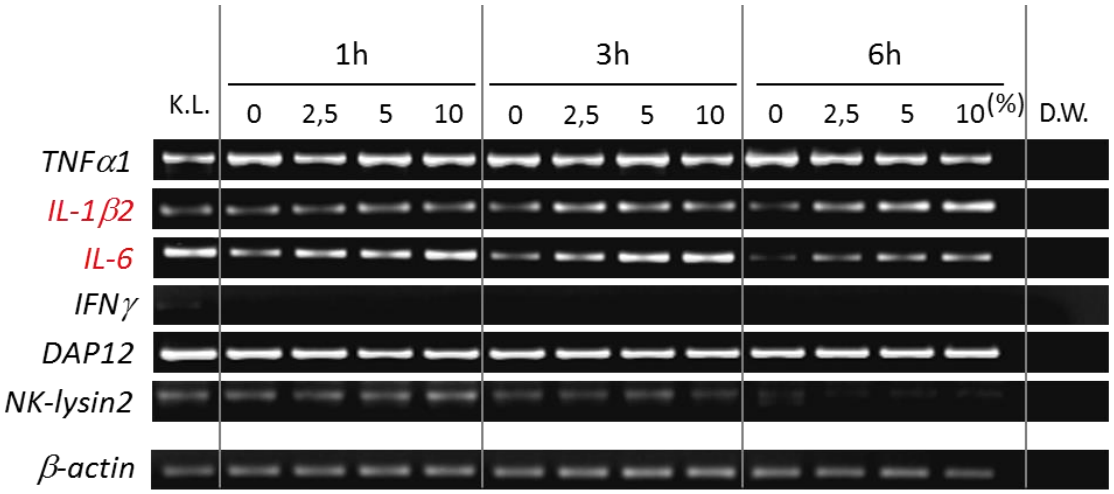


Abb. 7: Expression von immunrelevanten Genen nach 1-, 2-, und 6-stündiger Exposition von Pronephrozyten der Forelle mit DAMP-Adjuvans verschiedener Konzentrationen (2,5-10 %) im Zellkulturmedium.

Nach alleiniger i.p. Applikation von DAMP-Adjuvans an Forellen zeigten die Fische eine erhöhte NK-zellvermittelte zytotoxische Reaktivität (SOTO-LAMPE, **FISCHER**, unveröffentlicht). Dazu wurden aus den adjuvansbehandelten Forellen drei Tage nach Injektion die Milzleukozyten isoliert und mit fluorochromierten Maus-Mastozytomzellen (Zelllinie P815) zur Reaktion gebracht. Diese Zellen exprimieren keinen MHC Klasse I und machen sie zu Standardtargetzellen für NK-Effektorzellen, die diese im Sinne einer Missing-Self-Reaktion (siehe Unterkapitel 2.2.3) lysieren. Die Lyse der Targetzellen wurde durchflusszytometrisch gemessen (Abb. 8), wobei ungefärbte Milzleukozyten von gefärbten Maus-Mastozytomzellen durch ihre fehlende bzw. vorhandene Fluorochromierung unterschieden werden konnten. Lebende und tote Targetzellen wurden durch ein weiteres Fluorochrom diskriminiert, das ausschließlich Kerne toter (lysierter) Targetzellen mit permeabler Zellmembran anfärbt. Dabei wurde festgestellt, dass Milzleukozyten von DAMP-behandelten Fischen eine höhere NK-zellvermittelte Zytotoxizität entwickelten als solche von unbehandelten Fischen. Ein weiteres kommerzielles Adjuvans der Firma Seppic (Montanide) induzierte eine entsprechend höhere NK-Zellaktivität.

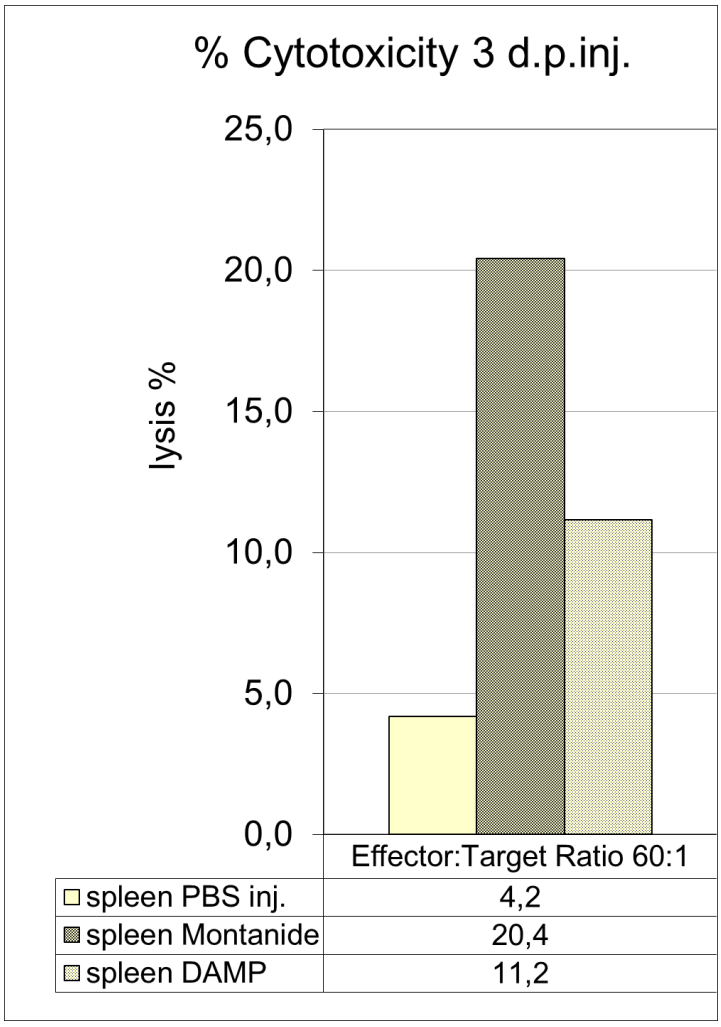


Abb. 8: NK-zellvermittelte Zytolyse von Maus-Mastozytomtargetzellen drei Tage nach i.p. Injektion zweier Adjuvanzien (Montanide, DAMP) an Forellen. Milzleukozyten der Forelle dienten hierbei als putative NK-Effektorzellen. Die Zytolyse wurde durchflusszytometrisch bei einem Effektor-/Targetzell-Ratio von 60 zu 1 gemessen (SOTO-LAMPE, **FISCHER**, unveröffentlicht).

Molekulare Adjuvanzien sind innovative Ansätze zur Steigerung der Wirkung eines Impfstoffes. Insbesondere DNA-Impfstoffe lassen sich durch weitere Vektoren komplementieren, die z.B. für Chemokine oder Zytokine kodieren. Dadurch können bestimmte Immunzellen an den Ort der Impfstoffapplikation rekrutiert und dort gezielt stimuliert werden.

In der Arbeit **XV** konnte durch i.m. Applikation sowohl rekombinanter Chemokine als auch Chemokin-kodierender Vektoren an der Injektionsstelle eine Rekrutierung von B-Zellen induziert werden. Diese Ergebnisse verdeutlichen, dass Chemokine potentielle Kandidaten für molekulare

Adjuvanzien sind, die den Influx von ausgewählten Immunzellen (hier B-Zellen) zur Applikationsstelle von Vakzinen befördern. Demgegenüber führte die i.m. Applikation eines traditionellen Öladjuvans zum Influx von myeloischen Zellen (Granulozyten und Makrophagen). Diese Experimente zeigten ferner, dass sich durch Wahl des Adjuvans lokale Immunreaktionen gezielt steuern lassen, was wiederum Einfluss auf den Vakzinationserfolg haben kann.

3.2.4 Induktion zellvermittelter Zytotoxizität durch Vakzination

Erste Anhaltpunkte zur Wirksamkeit von Vakzinen am geimpften Tier geben Antikörpertiter. Jedoch sind hohe Antikörpertiter nicht immer ein Garant für einen effektiven Immunschutz vor einer Belastungsinfektion. Insbesondere bei viralen Infektionen kommt neben den adaptiven humoralen, durch Antikörper vermittelten Immunreaktionen, auch den zellulären Abwehrmechanismen eine Bedeutung zu (GALLIMORE et al., 1998). Da Viren bei ihrer Replikation auf lebende Zellen angewiesen sind, geht es bei der zellvermittelten Zytotoxizität letzten Endes darum, infizierte Zellen als Replikationsorte von Viren durch zytotoxische T-Zellen (CTL) zu eliminieren. Naive CTL-Vorläufer, die im primären lymphatischen Organ Thymus rekrutiert werden, rezirkulieren bei Säugern zwischen den sekundären lymphatischen Organen (Milz und Lymphknoten), wo sie in Kontakt mit APCs (Makrophagen und DCs) kommen (COLES et al., 2002). Nach Kontakt mit APCs kommt es zur Aktivierung und klonalen Proliferation der CTLs, was die Eliminierung weiterer infizierter antigenpräsentierender Zellen und damit die Eindämmung der Erregerausbreitung zur Folge hat (Übersicht MACKAY, 1991; BUTCHER & PICKER, 1996). Eine Rezirkulation kann bei Fischen aufgrund des Fehlens von Lymphknoten nur in die Milz stattfinden, oder aber die Kaskade der Antigenpräsentation, Rekrutierung und Proliferation von Immunzellen findet in Teilen oder vollständig am Ort der Infektion statt, von wo diese in die Zirkulation über die Blutgefäße und damit in andere Organe gelangen.

UTKE, **FISCHER** et al. (2007 und 2008) konnten antivirale zellvermittelte Zytotoxizität sowohl nach Virusinfektion als auch nach DNA-Immunisierung (Erstbeschreibung bei Fischen) zeigen. Hierzu wurde ein System von MHC Klasse I-typisierten klonalen Forellen als CTL-Donoren und von infizierbaren im MHC Klasse I isogenen Zielzellen aufgebaut. Solche Systeme sind sehr

aufwändig und deshalb weltweit nur in sehr wenigen Laboren etabliert (**III**). Neben ihrer Effizienz als Impfstoff ist die DNA-Immunisierung eine elegante Methode, um die Immunantwort gegen einzelne virale Proteine separat zu untersuchen. So war bereits bekannt, dass Immunisierung mit rhabdoviralen DNA-Vakzinen, kodierend für das Glykoprotein G von IHNV (ANDERSON et al., 1996) und VHSV (BOUDINOT et al., 1998), den besten Immunschutz gegen eine letale Infektion mit homologem Vollvirus hervorruft, wobei ein antikörpervermittelter Schutz diskutiert wurde (LORENZEN et al., 2000). Dieser Immunschutz basiert jedoch auch auf zellvermittelter Zytotoxizität gegen virusinfizierte Zellen, die gleichermaßen dann am deutlichsten ausgeprägt war, wenn mit einer DNA-Vakzine immunisiert wurde, die für das G-Protein des VHSV kodiert (UTKE, **FISCHER** et al., 2007). Die zellvermittelte Zytolyse war hierbei sowohl gegen infizierte syngene (im MHC Klasse I kompatible) Zellen als auch gegen infizierte heterogene (im MHC Klasse I inkompatible) Zellen gerichtet, was den Schluss zulässt, dass sowohl CTL als auch NK-Zellen zur Zytolyse virusinfizierter Zellen befähigt sind.

Ob eine adaptive zelluläre Immunantwort, getragen durch CTL oder T-Helfer-Zellen (Th), mittels Vakzination bzw. Infektion induzierbar ist, kann ebenso durch Veränderungen im T-Zellrezeptor (TCR)-Repertoire bestimmt werden. Da für Forellen mittlerweile Antikörper gegen CD8 (Marker für CTL) (**VII**) und gegen CD4 (Marker für Th; TAKIZAWA et al., 2016) verfügbar sind, können somit in dieser Spezies die TCR- Repertoires beider Subpopulationen separat analysiert werden. Dieses erfolgte nach Sortierung der entsprechenden Antikörper-positiven Lymphozyten mittels sogenannter Spektratypisierung des Längenpolymorphismus im variablen Teil des TCR. Das bei immunologisch naiven Tieren normalverteilte Längenspektrum wird nach Antigenkontakt verschoben, wobei bestimmte antigenspezifische TCR-Spezies im Zuge der Immunantwort präferenziell exprimiert werden. Dieses Phänomen ist in der Arbeit **X** für T-Zellsubpopulationen infizierter (hier attenuiertes VHSV) Fische weltweit erstmals beschrieben worden. Während das TCR-Repertoire, gemessen an den Längenprofilen der Komplementarität bestimmenden Region (complementarity determining region, CDR) 3 des TCR von CD8-positven Lymphozyten in naiven Forellen eine hohe Diversität und Polyklonalität aufwies, war das von CD8-negativen Lymphozyten irregulär verteilt. Nach i.p.-

Immunisierung mit lebendem, attenuiertem VHSV waren das TCR-Repertoire von CD8-positven Lymphozyten nunmehr eingeschränkt, was darauf schließen lässt, dass es durch die virale Replikation zu einer klonalen Expansion von CTL mit bestimmten TCR-Eigenschaften gekommen war. Dieses betraf nach Vakzination auch die CD8-negativen Lymphozyten, deren CDR3-Längenprofile sich nun noch eingeschränkter darstellten als die von naiven Zellen.

Der wichtigste CTL-Marker ist CD8, dem die physiologische Bedeutung eines TCR-Korezeptors für die Bindung an MHC Klasse I/β2m/Peptid-Komplexe auf antigenpräsentierenden Zellen zukommt. Mittels Antikörper gegen CD8 lassen sich jedoch lediglich CTL darstellen, wobei deren antigene Spezifität unklar bleibt. Nach Erstkontakt von CTL mit antigenpräsentierenden Zellen gelangen insbesondere CTL mit hoher Avidität gegenüber dem MHC Klasse I/β2m/Peptid-Komplex zur klonalen Proliferation, wobei Populationen antigenspezifischer CTL entstehen, die eine große Zahl homolog virusinfizierter Zellen eliminieren können, während dieselben CTL Targetzellen, die mit einem heterologen Virus infiziert sind, nicht lysieren können (SNYDER et al., 2003). Solche antigenspezifischen CTL lassen sich *in vitro* durch Markierung mit rekombinanten MHC Klasse I/β2m/Peptid-Komplexen diagnostizieren. Voraussetzung hierfür ist, dass der gesamte Komplex eine hohe Avidität und Spezifität zum TCR aufweist. Deshalb kommen nur solche MHC Klasse I- und β2m-Moleküle in Frage, die aus demselben oder isogenen Organismus stammen, aus dem die CTL sich ableiten. Zunächst besteht die Schwierigkeit darin, geeignete Peptide zu finden, die im MHC Klasse I präsentiert werden. Hierfür gibt es verschiedene Methoden (ZHAO et al., 2003), deren Beschreibung den Rahmen dieser Schrift sprengen würde.

SOTO-LAMPE, TAKIZAWA & **FISCHER** (unveröffentlicht) haben sich für ein datenbankgestütztes System (NetMHCpan 3.0 der Danish Technical University) entschieden, wobei das entsprechende Computerprogramm bereits bekannte Daten zur erfolgreichen Bildung von MHC Klasse I/β2m/Peptid-Komplexen nutzt, um Peptide eines bestimmten viralen Proteins im Kontext mit bestimmten MHC Klasse I-Allelen vorauszusagen. Die Überprüfung der Korrektheit dieser Voraussage erfolgte durch SOTO-LAMPE, TAKIZAWA & **FISCHER** (unveröffentlicht), indem jeweils rekombinante MHC Klasse I- und β2m-Proteine mit den

vorausgesagten viralen Peptiden *in vitro* zur Reaktion gebracht wurden. Ein stabiler Komplex aus MHC Klasse I und β2m kommt nur dann zustande, wenn das Peptid korrekt in die durch die α1- und α2-Domänen des MHC Klasse I gebildete Grube passt (GRANDEA et al., 1997). Das vorausgesagte Peptid mit der größten Stabilisierungspotenz (Abb. 10) wurde danach zur Herstellung von rekombinanten MHC Klasse I/β2m/Peptid-Komplexen verwendet, wonach diese tetramerisiert, fluorochromiert und schließlich mit IHNV-spezifischen CTL von Forellen zur Reaktion gebracht wurden, die vorher gegen IHNV immunisiert worden waren.

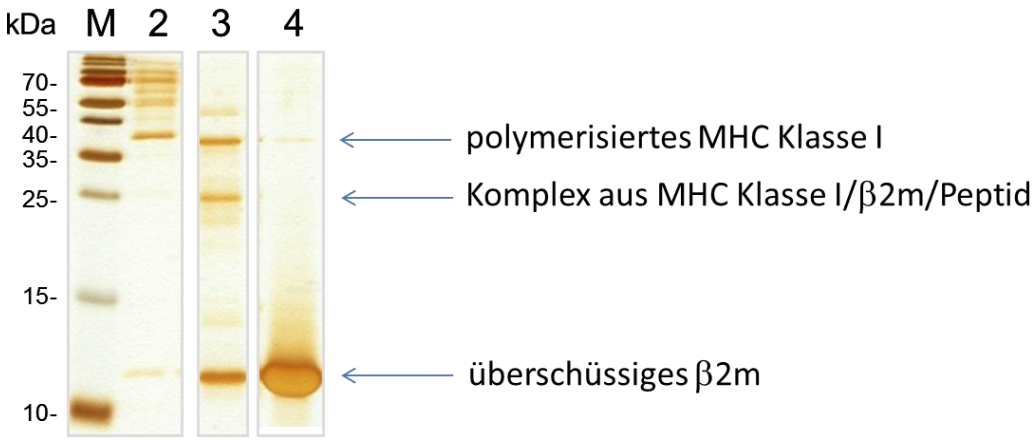


Abb. 10: Elektrophoretisch aufgetrennte, durch HPLC (High Performance Liquid Chromatography) gewonnene, Fraktionen eines Kompexierungsansatzes aus einem vorausgesagtem synthetischem Peptid des IHNV-Glykoproteins (nicht sichtbar da weit kleiner als 10 kDA), mit rekombinantem MHC Klasse I und β2m. Ein stabilisierter MHC Klasse I/β2m/Peptid-Komplex war in Fraktion 3 zu finden (SOTO-LAMPE, TAKIZAWA & **FISCHER**, unveröffentlicht).

Antigenspezifische CTL konnten durchflusszytometrisch als CD8/Tetramer-doppelt-positiv detektiert werden (Abb. 11).

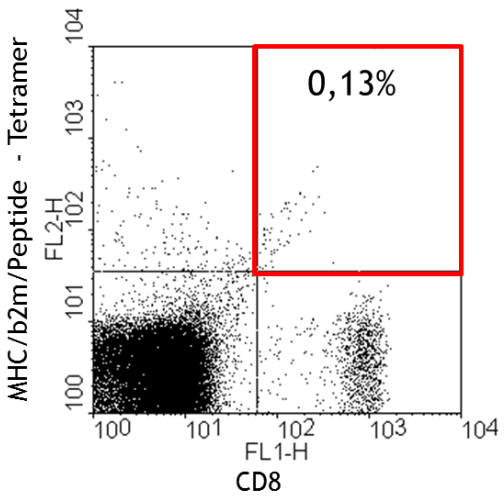


Abb. 11: Durchflusszytometrie von PBL nach Färbung mit MHC Klasse I/b2m/Peptid-Tetrameren und Antikörpern gegen CD8. Die PBL stammen aus Forellen, die zuvor mit einem vorausgesagten und nach Komlexierungsansatz als geeignet deklarierten IHNV-Glykoprotein-Peptid immunisiert und danach mit einer subletalen Dosis von IHNV belastet wurden. Im oberen rechten Quadranten sind die Tetramer-/CD8-doppelt-positiven antigenspezifischen CTL dargestellt (SOTO-LAMPE, TAKIZAWA & **FISCHER**, unveröffentlicht).

Der geringe Prozentsatz der antigenspezifischen CTL, wie hier und auch in anderen Arbeiten beschrieben (z.B. CHEN et al., 2010, SCHULTE et al., 2011), sollte nicht darüber hinwegtäuschen, dass CTL effiziente Killerzellen gegenüber virusinfizierten Zellen sind. Parallel durchgeführte Untersuchungen mit peripheren Blutleukozyten (PBL) aus Forellen, die zuvor mit demselben IHNV-Glykoprotein-Peptid immunisiert und danach mit einer subletalen Dosis von IHNV belastet wurden, haben gezeigt, dass solche PBL IHNV-infizierte, im MHC Klasse I syngene Targetzellen besser lysieren als IHNV-infizierte heterologe Targetzellen. (Abb. 12). Dies lässt den Schluss zu, dass virusantigenspezifische CTL gebildet wurden. Neben CHEN et al. (2010), die ähnliche Untersuchungen an virusinfizierten Graskarpfen durchführten, sind die Arbeiten an Regenbogenforellen von SOTO-LAMPE, TAKIZAWA & **FISCHER** (unveröffentlicht) weltweit die einzigen Nachweise von antigenspezifischen zytotoxischen Zellen bei Fischen.

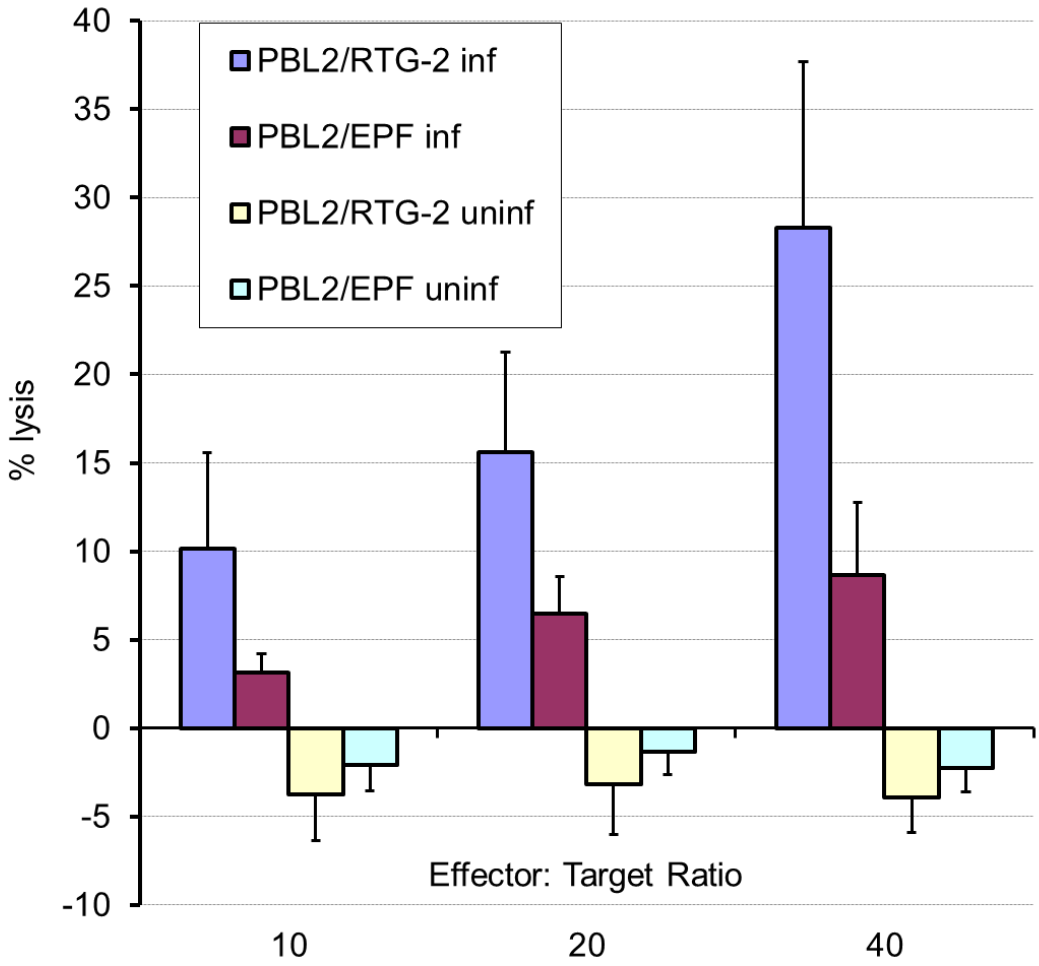


Abb. 12: Zellvermittelte Zytotoxizität von peripheren Blutleukozyten (PBL), angegeben in Prozent (Y-Achse) gegen IHNV-infizierte (inf) und nicht-infizierte (uninf) im MHC Klasse I syngene (RTG-2) und heterologe (EPF) Targetzellen. Die PBL wurden aus Forellen isoliert, die zuvor mit einem Peptid aus dem IHNV-Glykoprotein immunisiert und danach mit einer subletalen Dosis von IHNV belastet wurden (SOTO-LAMPE, TAKIZAWA & **FISCHER**, unveröffentlicht).

4 Temperatur und Immunantwort

Eine Besonderheit von niederen Vertebraten ist deren Poikilothermie. Fische sind nicht in der Lage, ihre Körpertemperatur aktiv zu beeinflussen. Sämtliche physiologischen Prozesse, einschließlich der Immunreaktionen, sind demnach von der Umgebungstemperatur abhängig. Das Immunsystem stellt sich in gewissen physiologischen Grenzen auf die unterschiedlichen Temperaturbedingungen ein. Bei niedrigeren Temperaturen kommen die phylogenetisch älteren Komponenten des angeborenen Immunsystems zum

Tragen, während bei den für die jeweilige Spezies höheren, permissiven, Temperaturen die Komponenten des adaptiven Immunsystems hervortreten (BOWDEN et al., 2007). Letzteres geht zwar mit einer höheren Effektivität der Erregerabwehr einher, demgegenüber vermehren sich viele Erreger bei erhöhten Temperaturen auch besser. So werden Fische in der Aquakultur oft unter Temperaturbedingungen gehalten, die optimal für eine Gewichtszunahme sind, gleichzeitig aber bestimmten Erregern optimale Replikationschancen ermöglichen. Temperatur ist zudem ein ernstzunehmender Stressfaktor bei schnellen Temperaturwechseln. Aufgrund dieser Tatsachen kommt es in offenen Aquakultursystemen insbesondere im Frühjahr gehäuft zu Seuchenausbrüchen, wenn die Fische nach der Winterruhe einen Temperaturbereich passieren, bei dem virale Erreger sich bereits gut vermehren können, bei dem das Immunsystem jedoch noch nicht optimal auf pathogene Erreger reagieren kann. Zwei typische Beispiele hierfür sind die Frühjahrsvirämie der Karpfen (Spring Viraemia of Carp, SVC) (AHNE et al., 2002) und die VHS der Salmoniden (JØRGENSEN et al., 1982).

Die Wassertemperatur hat eine direkte praktische Relevanz beim Einsatz von Impfstoffen in der Aquakultur. Eine Immunantwort erfolgt innerhalb des physiologischen Temperaturspektrums einer Spezies bei höheren Temperaturen rascher als bei niedrigen (Übersicht MAKRINOS & BOWDEN, 2016). Dieser Sachverhalt fließt in die Berechnung der sogenannten Tagesgrade ein, die sich aus dem Produkt der Umgebungstemperatur in °C und Tagen ergibt. Deshalb wird beispielsweise der Zeitrahmen von der Applikation eines Impfstoffes bis zum Eintritt einer protektiven Immunantwort in Tagesgraden angegeben.

Überraschenderweise haben Fische trotz ihrer Poikilothermie Strategien entwickelt, ihre Körpertemperatur zu regulieren. So wurde gezeigt, dass immunisierte bzw. kranke Fische, um die Effektivität ihres Immunsystems zu erhöhen, wärmere Regionen eines Habitats aufsuchen, was als Verhaltensfieber (behavioural fever) bezeichnet wird (z.B. GRÄNS et al., 2012).

Während die meisten Arbeiten zur Temperaturabhängigkeit der Immunantwort sich mit der Antikörperbildung oder mit Genexpressionsstudien beschäftigen, haben **FISCHER** et al. (1999) den Einfluss der Umgebungstemperatur auf die zellvermittelte Zytotoxizität bei Fischen (hier

Ginbuna) untersucht. Sowohl die *in vitro* gemessene allospezifische zellvermittelte Zytotoxizität als auch die GVHD (siehe Unterkapitel 2.2.3) als *in vivo*-Korrelat einer zellvermittelten Zytotoxizität waren bei niedrigen Temperaturen verzögert. Für die *in vitro*-Studie zur Temperaturabhängigkeit der alloreaktiven zellvermittelten Zytotoxizität wurden zunächst klonale triploide Ginbunas mit Erythrozyten- und Schuppen-Transplantaten von tetraploiden Goldfisch/Ginbuna-Hybriden sensibilisiert. Die triploiden Ginbunas wurden bei einer Wassertemperatur von 25 °C gehalten. Mittels Hämoglobin-Freisetzungstest, bei dem die alloreaktiven zytotoxischen Leukozyten aus den sensibilisierten triploiden Ginbunas *in vitro* mit Erythrozyten aus gesunden tetraploiden Hybriden zur Reaktion gebracht wurden, erfolgte die Messung der Temperaturabhängigkeit einer Erythrolyse bei 15, 20 und 25 °C. Für die *in vivo*-Studie zur Messung der Temperaturabhängigkeit der alloreaktiven zellvermittelten Zytotoxizität wurden die tetraploiden Empfänger (Goldfisch/Ginbuna-Hybriden) von alloreaktiven zytotoxischen Zellen unter dem gleichen Temperaturregime gehalten wie die Target- und Effektorzellen beim eingangs beschriebenen *in vitro* Versuch. *In vitro* waren die gemessenen Zytotoxizitäten bei höheren Temperaturen bis auf das 3-fache erhöht. Ein ähnlicher Trend zeigte sich *in vivo*, wobei die Mortalität durch GVHD bei den tetraploiden Hybriden bei einer Temperatur von 25 °C ca. 2 Wochen früher einsetzte als bei 20 °C, während bei 15 °C eine deutlich geringere Mortalität zu verzeichnen war als bei höheren Temperaturen.

Das Immunsystem von Fischen unterliegt nicht nur einem durch Temperaturwechsel und Licht geprägten jahreszeitlichem Rhythmus (Übersicht BOWDEN et al., 2007). Vielmehr scheinen Fische auch eine innere Uhr zu besitzen, die temperatur- und lichtunabhängig agiert. So fanden UTKE, **FISCHER** et al., 2008, dass es Unterschiede zwischen den Sommer- und Wintermonaten bei der *in vitro* gemessenen zellvermittelten Zytotoxizität gegen virusinfizierte Zellen gibt, obwohl die DNA-immunisierten Donorfische antiviraler zytotoxischer Zellen ganzjährig bei konstanter Temperatur und konstantem Lichtregime gehalten wurden. Die saisonale Abhängigkeit betraf vor allem die MHC Klasse I-restringierte zellvermittelte Zytotoxizität, die durch DNA-Immunisierung gegen das Nukleokapsidprotein induziert wurde und weniger die gegen das Glykoprotein induzierte.

5 Bedeutung der betrachteten Arbeiten für die Aquakultur

Der Forschungsansatz der vorliegenden Arbeit bestand in der Annahme, dass die Erweiterung von Kenntnissen zur Immunantwort bei Fischen dem Verständnis der Pathogenese von Infektionskrankheiten und der Entwicklung von entsprechenden Präventions- und Bekämpfungsmaßnahmen für die Aquakultur dient. Eine wichtige Präventions- und Bekämpfungsmaßnahme von Krankheiten ist die Vakzination. Vakzination zieht eine Immunantwort nach sich, um deren Aspekte es in der vorliegenden Arbeit ging. Die Untersuchungen umfassten grundlagenorientierte, translationale und angewandte Forschung mit Fokus auf zellvermittelte Immunmechanismen.

Innerhalb der Vertebraten erlangten Fische neben den Rundmäulern als erste die Fähigkeit „Eigen“ von „Fremd“ zu unterscheiden, was z.B. Ginbuna-Karpfen in die Lage versetzt, Transplantate abzustoßen (**FISCHER** et al., 1998a) und eine Transplantat-Gegen-Wirt-Reaktion (Graft-Versus-Host-Reaktion, GVHR) (**FISCHER** et al., 1999) auszulösen.

Wie auch Nichtvertebraten und Pflanzen besitzen Fische ein angeborenes Immunsystem. So exprimieren Forellen breitenspezifische Mustererkennungsrezeptoren in Form von Toll-Like-Rezeptoren (TLR) (REBL, **FISCHER** et al., 2007, 2008, 2009 und **VI**) und Novel Immunoglobulin-like Transcripts (NILTs) (KOCK & **FISCHER**, 2008). Sie verfügen über Komplement (LOVOLL, **FISCHER** et al., 2007), Thrombozyten (KÖLLNER, **FISCHER** et al., 2004; OHASHI, **FISCHER** et al., 2007) und über funktionelle natürliche Killer (NK)-Zellen (**FISCHER** et al., 1998a; UTKE, **FISCHER** et al., 2007 und 2008). NK-Zellen von Ginbuna-Karpfen und Forellen können allogene Zellen und virusinfizierte Zellen eliminieren.

Auch zytotoxische T-Zellen (CTL) (**FISCHER** et al., 1998a; UTKE, **FISCHER** et al., 2007 und 2008; **FISCHER** et al., 2003; **VII** und **XIII**) können allogene und virusinfizierte Zellen lysieren, jedoch in einem MHC Klasse I-restringierten Kontext. CTL sind dem adaptiven Immunsystem zuzuordnen, das phylogenetisch erstmals bei Vertebraten auftrat und die Grundlage für eine gezielte Immunisierbarkeit bildet. Fische besitzen ein antigenspezifisches adaptives Immunsystem, was sowohl durch die Expression entsprechender Gene und Proteine als auch auf funktioneller Ebene bewiesen werden konnte.

Als wichtige Bestandteile der adaptiven Immunreaktion exprimieren Forellen Haupthistokompatibilitätsmoleküle (MHC) der Klassen I und II (DIJKSTRA, **FISCHER** et al., 2001 und 2003; SARDER, **FISCHER** et al., 2003; **FISCHER**, DIJKSTRA et al., 2005; UTKE, **FISCHER** et al., 2007 und 2008 und **V**), TCR (**FISCHER** et al., 2005; **V**; **VIII** und **X**), CD8 (**FISCHER** et al., 2003; **V**; **VII**; **X** und **XII**), CD4 (**FISCHER** et al., 2005; **VII**) sowie Antikörper der Klassen IgM (**FISCHER** et al., 2003; **FISCHER** et al., 2005; UTKE, **FISCHER** et al., 2007 und 2008; **VII, IX**; **XII** und **XIII**) und IgT (**XII** und **XV**), die bei Regenbogenforellen zu großen Teilen bereits während der Embryogenese transkribiert werden (**FISCHER** et al., 2005). Dieses macht einen frühen Immunisierungserfolg wahrscheinlich. Bei Forellen interagieren infizierte antigenpräsentierende Targetzellen mit zytotoxischen T-Zellen (UTKE et al., 2007 und 2008) und B-Zellen (**FISCHER** et al., 2016 unveröffentlicht), was auf einen Infektionsschutz basierend auf zellvermittelter Zytotoxizität bzw. auf neutralisierenden Antikörpern schließen lässt. Bei Ginbuna-Karpfen und Forellen konnte eine MHC Klasse I-restringierte und damit spezifische zellvermittelte Zytotoxizität gegen allogene (**FISCHER** et al., 2003; **FISCHER** et al., 1998a) und gegen virusinfizierte histokompatible Zellen (UTKE, **FISCHER** et al., 2007 und 2008) gezeigt werden. Die Spezifität solcher Effektorzellen konnte anhand von Virusantigen-spezifischem Homing (UTKE, **FISCHER** et al., 2007) und durch Darstellung antigen-spezifischer CTL (SOTO-LAMPE, TAKIZAWA & **FISCHER**, unveröffentlicht) gezeigt werden.

Aufgrund der Expressionsmuster von Masterregulatoren der T-Helferzellrespons *EOMES* (**XIII**) und *GATA-3* (**XIV**) sowie von *Interleukin 4/13A* (**XIV**) bei Forellen konnte geschlussfolgert werden, dass Fische ähnlich wie Säuger über Th1- und Th2-Zellen verfügen, mit denen diese zytotoxische bzw. antikörpervermittelte Immunprozesse triggern.

In der Darmschleimhaut von Salmoniden (Forelle und Atlantischer Lachs) waren Zellen zu finden, die u.a. Eigenschaften von M-Zellen der Säuger aufwiesen und die oral appliziertes Antigen über den Darm aufnehmen konnten (FUGLEM, **FISCHER** et al., 2010). Diese Zellen scheinen für einen wirksamen Impfschutz insbesondere bei solchen Infektionen von Bedeutung zu sein, bei denen die Erregereintrittspforte der Darm ist.

Das Kiemenepithel von Forellen konnte inaktivierte Bakterien aktiv über distinkte Zellen internalisieren, die Eigenschaften von M-Zellen, Makrophagen

oder DCs aufwiesen (**V** und **XI**). Die Kieme von Salmoniden verfügt über ein Interbranchiales Lymphatisches Gewebe (Interbranchial Lymphoid Tissue, ILT) mit hoher Abundanz von T-Zellen (KOPPANG, **FISCHER** et al., 2010; **VIII**). Diese Eigenschaften weisen die Kieme als wichtiges Organ der angeborenen und adaptiven Immunität aus.

Bei Forellen konnten neben Bakterien (**VIII**) auch Viren über die Kieme aufgenommen werden, was die Expression von Immunglobulinen in B-Zellen und Effektormolekülen zytotoxischer T-Zellen induzierte. Die Rekrutierung von Immunzellen in die Kieme wurde durch Chemokine getriggert, was letztere insbesondere als molekulare Adjuvanzien geeignet erscheinen lässt (**XII**).

Kreuzpräsentierende DCs sind bei Säugern wichtig, um extrazelluläre Antigene, die normalerweise eine MHC Klasse II-restringierte Antikörperantwort induzieren, über MHC Klasse I zu präsentieren. Solche DCs konnten erstmals bei Fischen in Forellen beschrieben werden (**V**). MHC Klasse I-restringierte Zytotoxizität erwies sich bei Forellen als ein wichtiger Bestandteil der antiviralen Abwehr, wenn virale Antigene intrazellulär gebildet werden (UTKE, **FISCHER** et al., 2007 und 2008). Inaktivierte Impfstoffe repräsentieren jedoch extrazelluläre Antigene, weswegen inaktivierte antivirale Vakzinen, deren Wirksamkeit MHC Klasse I-restringiert ist, auf Kreuzpräsentation angewiesen sind.

Forellen verfügen über einen Schleimhaut-assoziierten Phänotyp von DCs, der MHC-Klasse-II- sowie CD8-positiv ist (**V**). Dieser Phänotyp könnte ein wichtiges Target insbesondere für Forschungen zu Bad- oder Oralimpfstoffen sein.

Als Ausdruck eines fokussierten Spektrums von antigenspezifischen T-Zellen exprimierten Forellen nach mukosaler Immunisierung (Bad) gegen VHSV ein eingeschränktes T-Zellrezeptor (TCR-)-Repertoire (**X**). Daraus lassen sich wichtige Rückschlüsse auf einen Immunisierungserfolg ziehen.

Da bei der DNA-Immunisierung, ähnlich wie bei einer Virusreplikation, virale Proteine intrazelluär translatiert, prozessiert und über MHC Klasse I den zytotoxischen T-Zellen präsentiert werden, ist diese Form der Antigenapplikation eine probate Methode, um Immunreaktionen gegen diskrete virale Proteine zu vergleichen. Mit einem *in vitro*-System von MHC Klasse I-restringierter Zytotoxizität konnte festgestellt werden, dass die antivirale Abwehr

gegen das Glykoprotein G des VHSV stärker ausgeprägt ist als gegen das Nukleokapsidprotein N (UTKE, **FISCHER** et al., 2008).

Lokale inflammatorsiche Impfreaktionen sind ein wichtiger Trigger, mit dem das angeborene Immunsystem die adaptive Immunantwort befördert. Intramuskuläre DNA-Immunisierung gegen VHSV induzierte an der Injektionsstelle eine lokale inflammatorische Reaktion als Folge der myozytären Expression des entsprechenden viralen Glykoproteins. Dabei war ein chemokininduzierter Influx von Entzündungszellen, insbesondere IgM- und IgT-exprimierenden B-Zellen zu beobachten, während nach intramuskulärer Injektion eines ölbasierten Adjuvans vor allem Granulozyten einwanderten (**XV**).

Die Rotmaulseuche, hervorgerufen durch *Y. ruckeri*, ist für Salmoniden eine ökonomisch bedeutsame bakterielle Erkrankung. Durch Transfer von Leukozyten vakzinierter Spenderforellen auf naive, genetisch identische Rezipientenforellen konnte bei Letzteren ein Immunschutz gegen die Rotmaulseuche etabliert werden, dessen Grundlage in erster Linie den Memory-B-Zellen zuzuschreiben war (YAMAGUCHI & **FISCHER**; 2016).

Putative Memoryzellen, die bei Vertebraten die Grundvoraussetzung für eine aktive Immunisierung (Vakzination) bilden, konnten in Forellen über mehr als 200 Tage nachgewiesen werden. Der wesentliche Teil dieser langlebigen, sich nicht teilenden und damit ruhenden Zellen waren B-Zellen (JAAFAR, YAMAGUCHI & **FISCHER**, unveröffentlicht).

Fische sind wechselwarm, wodurch deren physiologische Leistungen Temperatureinflüssen unterliegen. Bei Ginbuna-Karpfen wurde festgestellt, dass Phänomene der Transplantatabstoßung temperaturabhängig sind (**FISCHER** et al., 1999). Bei Forellen unterlagen antivirale zellvermittelte Zytotoxizität einer jahreszeitlichen Abhängigkeit (UTKE, **FISCHER** et al., 2008). Solche umweltabhängigen Phänomene der Immunantwort sollten bei Vakzinationsstrategien berücksichtigt werden.

Adjuvanzien leisten einen wichtigen Beitrag zur Steigerung der Wirksamkeit von Vakzinen. Einer ihrer Wirkmechanismen besteht darin, dass nach deren Applikation Gewebszerfallsprodukte entstehen, die ihrerseits das Immunsystem triggern. Durch Adjuvantierung eines bakteriellen Impfstoffes mit gezielt *in vitro* hergestellten Gewebszerfallsprodukten konnte bei Forellen ein

erhöhter Immunschutz gegen die Furunkulose erzielt werden (**FISCHER**, KOTTERBA, unveröffentlicht).

6 Schlussfolgerungen und Ausblick

Die kostenintensive parenterale Verabreichung von Impfstoffen an Fische per Nadel, entweder per Hand oder Impfautomat, sollte schrittweise durch mukosale Impfstoffe abgelöst werden. Das erfordert auch in der Zukunft Forschungsarbeiten zur Schleimhautimmunität, die dem tieferen Verständnis der mukosalen Abwehr dienen. Es bleibt zu postulieren, dass orale und Badvakzinen nur dann ihre Wirksamkeit entfalten, wenn eine effektive Aufnahme der entsprechenden Antigene über die Schleimhäute gewährleistet ist. Das setzt voraus, dass dem Impfantigen Eigenschaften verliehen werden, die es vor Umwelteinflüssen schützen, die einen Transport bis an immunrelevante Strukturen ermöglichen und die eine effektive Aufnahme durch antigenpräsentierende Zellen erlauben. Eine Impfung über das Futter erfordert beispielsweise eine sichere Passage des Impfstoffes bis in den Darm. Wichtiges Hemmnis bei mukosalen Vakzinen ist Toleranzinduktion, wo es noch erhebliche Wissenslücken gibt. Zurzeit bearbeitet der Verfasser dieser Arbeit zusammen mit japanischen Kollegen ein Projekt, das die bereits durchgeführten Untersuchungen zur Antigenaufnahme über die Kieme weiterführt.

Da nur inaktivierte Vakzinen eine Chance auf Zulassung haben werden, muss der Antigenpräsentation große Aufmerksamkeit zuteilwerden. Hierbei kommen professionelle APCs ins Spiel, weswegen sich der Verfasser dieser Schrift zusammen mit spanischen Kollegen mit einem weiteren von der spanischen Regierung geförderten Projekt zu mukosalen DCs beschäftigt.

Die Wirksamkeit von Impfstoffen kann durch Adjuvanzien erhöht werden. Auch hier gibt es noch großes Entwicklungspotenzial. Zum einen gilt es, insbesondere für parenterale Impfstoffe, aggressive Adjuvanzien durch besser verträgliche aber dennoch effektive Alternativen abzulösen. Zum anderen müssen wirksamere Adjuvanzien für zukünftige mukosale Impfstoffe entwickelt werden. Da Adjuvanzien adaptive Komponenten des Immunsystems indirekt über angeborene Immunmechanismen stimulieren, repräsentieren adjuvantierte Vakzinen ideale Modelle für die Untersuchung des Zusammenspiels dieser beiden Immunkomponenten.

Große Potentiale zur Stimulierung des Immunsystems eröffnen molekulare Adjuvanzien. Hier bieten sich Chemokine und Interleukine an, mit denen Immunzellen an den Ort der Impfantigenpräsentation gelenkt bzw. mit denen gezielte Immunantworten gesteuert werden, die einen effektiven krankheitsbezogenen Immunschutz induzieren. Ein von der DFG bis 2019 gefördertes Projekt des Verfassers dieser Arbeit beschäftigt sich mit dem Einfluss von Interleukinen aus der IL-2-Familie auf die T-Zellantwort und insbesondere die Memory-Respons.

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Teleost T and NK cell immunity

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ABSTRACT

The main function of the immune system is to maintain the organism's homeostasis when invaded by foreign material or organisms. Prior to successful elimination of the invader it is crucial to distinguish self from non-self. Most pathogens and altered cells can be recognized by immune cells through expressed pathogen- or danger-associated molecular patterns (PAMPS or DAMPS, respectively), through non-self (*e.g.* allogenic or xenogenic cells) or missing major histocompatibility (MHC) class I molecules (some virus-infected target cells), and by presenting foreign non-self peptides of intracellular (through MHC class I-*e.g.* virus-infected target cells) or extracellular (through MHC class II-*e.g.* from bacteria) origin. In order to eliminate invaders directly or by destroying their ability to replicate (*e.g.* virus-infected cells) specialized immune cells of the innate and adaptive responses appeared during evolution. The first line of defence is represented by the evolutionarily ancient macrophages and natural killer (NK) cells. These innate mechanisms are well developed in bony fish. Two types of NK cell homologues have been described in fish: non-specific cytotoxic cells and NK-like cells. Adaptive cell-mediated cytotoxicity (CMC) requires key molecules expressed on cytotoxic T lymphocytes (CTLs) and target cells. CTLs kill host cells harbouring intracellular pathogens by binding of their T cell receptor (TCR) and its co-receptor CD8 to a complex of MHC class I and bound peptide on the infected host cell. Alternatively, extracellular antigens are taken up by professional antigen presenting cells such as macrophages, dendritic cells and B cells to process those antigens and present the resulting peptides in association with MHC class II to $CD4^+$ T helper cells. During recent years, genes encoding MHC class I and II, TCR and its co-receptors CD8 and CD4 have been cloned in several fish species and antibodies have been developed to study protein expression in morphological and functional contexts. Functional assays for innate and adaptive lymphocyte responses have been developed in only a few fish species. This review summarizes and discusses recent results and developments in the field of T and NK cell responses with focus on economically important and experimental model fish species in the context of vaccination.

1. Introduction

The aquaculture industry is a very important factor in world economy and a valuable protein source. In Europe this sector employs about 65,000 people gaining a turnover of more than 3 billion €. However, the limited number of licenced fish vaccines and disease outbreaks considerably decrease production values of farmed fish.

Efficient vaccines are based on efficient immune responses, and modern vaccines can only be designed and optimized if the underlying immune response elements and their interrelationships are well understood.

In mammals, immune response elements are historically divided into cell-mediated vs. humoral (humor from Greek χυμός, chymos: juice or sap = liquid), and into innate vs. adaptive. However, these components cannot exist and act independently. Humoral factors are produced by cells to trigger, modify or suppress other cells. Cells of the innate immune system interact with those of the adaptive immune system, and immune cells interact with non-immune cells.

Cell-mediated immune responses are executed by leukocytes where the main players are granulocytes, macrophages (monocytes), dendritic cells and lymphocytes. Lymphocytes other than B cells can roughly be divided into natural killer (NK) cells and T cells, the latter composed of CTLs, T helper cells (T_h) and regulatory T cells (T_{reg}). CMC is a process in which immune cells recognize and kill altered, tumorous, foreign or infected cells thus maintaining homeostasis of the body. Some bacteria and all viruses replicate intracellularly and the invaded host has a vital interest in eliminating

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infected cells before they mass-produce new infectious particles. The main actors in CMC are NK cells and CTLs belonging to the innate and the adaptive arms of the immune system, respectively.

Although the knowledge on the immune system of several fish species has considerably increased during the last few years, the scientific community is still lacking many of the tools and experimental systems to properly study fish immune responses. This has hampered progress in vaccine development in the past making it difficult to characterize immune responses to vaccines and based on those responses predict immune memory and understand protection mechanisms. However, progress in the discovery of immune related genes and expression of the encoded proteins has resulted in the establishment of specific antibodies against immune related proteins such as CD3, CD4, CD8, and MHC class I and II. In general, these antibodies reveal expression patterns and distributions similar to those of immune cells of mammals. However, some unique features concerning the mucosal immune system have been detected and novel immunological structures have been discovered in the gill, suggesting opportunities for unique strategies using mucosal vaccination.

T_h, NK and CTL responses in fish have been summarized by [91]. The aim of the present article is to provide an update on the knowledge about teleost T cell and NK cell responses to infection and vaccination with focus on their morphological, cellular and molecular equivalents to those described in mammals.

2. Molecules involved in lymphocyte responses

Both, mammalian CTLs and NK cells lyse target cells by two mechanisms, namely granule exocytosis and FasL/Fas interaction, both of which require membrane contact with the target cell. Exocytosed granules contain pore-forming substances, granzymes and other mediators. Pore formation in the outer membrane of target cells is induced by perforin, granulysin and NK-lysin allowing entry of granzymes into the cytoplasm ultimately resulting in apoptotic cell death [102,125,145]; respectively.

Perforin genes have been cloned in Japanese flounder [53], channel catfish [106], rainbow trout [3] and ginbuna [142]. mRNA encoding perforin is strongly expressed in CD8α^+ cells of ginbuna and rainbow trout [138,142]; respectively. When treated with PHA, a mitogen that induces cytotoxic functions in mammals [92] trout CD8α^+ cells respond with strong perforin expression and significant upregulation of IFN-γ genes [142]. Catfish NCCs were shown to express both perforin and granulysin [106] genes. Although in mammals perforin is thought to be primarily produced by NK cells and CTLs it is also expressed by human alveolar macrophages [12]. Similarly, the rainbow trout macrophage cell line RTS-11, which exhibits spontaneous cytolytic activity against xenogeneic cells, showed an increased expression of perforin and granzyme genes after VHSV infection [95].

Another observation suggesting that antiviral effector mechanisms are similar to those in mammals is granzyme gene upregulation in lymphoid organs during spring viraemia of carp SVC [52].

In mammals, FasL can be expressed in a membrane bound form and a soluble cytosolic form. In fish, only soluble cytosolic forms have been detected thus far (tilapia, channel catfish [8] catfish [64] gilthead sea bream [24], respectively) although [80] identified a putative FasL protein by Western blotting using an anti-human FasL antibody. However, they were not able to detect the corresponding protein on the surface of intact cells. Soluble putative tilapia and catfish FasL proteins are released by NCCs upon stimulation and anti-human FasL antibodies can inhibit NCC-mediated lysis of target cells further suggesting FasL mediates killing mechanisms in fish [8]. FasL homologues were also detected in autonomous channel catfish T cell clones and fibroblasts [80] using cross-reactive anti-FasL antibodies, and Japanese flounder [75]. T-cell-like lymphocytes were also shown to express FasL genes [73]. On the target cell level, FasL proteins induce apoptotic cell death of FasR positive target cells in catfish [64], and [31] succeeded to induce apoptotic cell death in zebrafish embryos by ectopic expression of Fas.

2.1. Molecules involved in NK cell activities

The first piscine NK cell equivalents were described in channel catfish as NCCs bearing a vimentin-like surface molecule [55] and expressing a type III membrane protein termed NCC receptor protein 1 (NCCRP-1) [56]. Crosslinking of NCCRP-1 leads to receptor tyrosine and serine phosphorylation [57] suggesting downstream innate immune response signalling. NCCRP-1 type genes have also been cloned in a number of other fish species, as well as in axolotl, mouse and man [18,118]; gene bank numbers HQ388293.1, GU075705.1, HQ388293.1 [62,109]; respectively. Another molecule that is involved in NK cell responses is the so called NK cell enhancing factor (NKEF) which has been sequenced in several fish species (reviewed by Chen et al. [19]). In mammals *NKEF* is contained in red blood cells [120] and upregulation of *NKEF* in rainbow trout during viral haemorrhagic septicaemia (VHS) was suggested to be a result of a compensatory activation and proliferation of erythrocyte precursors [146]. Elevated expression of *NKEF* has also been shown in a VHSV-infected trout cell line [95]. Using recently established antibodies, upregulation of NKEF has been demonstrated *in vitro* and *in vivo* after stimulation with bacteria, LPS and ectoparasites [5].

In mammals, the main NK cell activities are regulated by receptors that recognize MHC class I or MHC class I-related proteins [79]. The first family of receptors belongs to the immunoglobulin superfamily (IgSF) called killer cell Ig-like receptors (KIRs). The second group of receptors also belongs to the IgSF and has been named immunoglobulin like transcripts (ILT). The third family of receptors is formed by C-type lectin receptors. These C-type lectin receptors are composed of heterodimers formed by the transmembrane-anchored glycoprotein CD94 which is covalently linked to members of the NKG2 family of molecules reviewed by Lanier [169] and Borrego et al. [9]. In humans, this third family consists of seven members: NKG2-A, -B, -C, -D, -E, -F and -H. While NKG2-A, -B, -C, -E, -F and -H show high sequence homologies among each other NKG2-D represents a separate class of lectin-like receptors and only NKG2-A, -B, -C, -E and -H can be covalently linked with CD94. Heterodimerisation of CD94 with NKG2A or NKG2B results in inhibitory receptors, while complexes of CD94 with NKG2C, NKG2E or NKG2H function as activating receptors reviewed by Hoare et al. [49]. To our knowledge, sequences encoding homologues to mammalian C-type lectin receptors have only been discovered in cichlid fishes [66,114] thus far.

In primates, non-rearranging KIRs (the Ly49 family molecules in rodents) enable NK cells to discriminate target cells with “missing self signals”. Novel immune-type receptors NITR; reviewed by Yoder [157] and novel immunoglobulin like transcripts NILT [69,96,132] have been recently reported in fish. Both gene families encode non-rearranging receptors with molecular structures similar to those of KIRs.

Mammalian NK cell receptors utilize adaptor molecules such as DNAX-activating proteins (DAP). While NK receptors (KIRs and Ly49s) partners with the adaptor protein DAP12, the lectin-type-activating NK receptor NKG2D partners with DAP10 [54,137]. DAP homologues were found in zebrafish by [156]. In that species, NITR9 signals via the adaptor DAP12 [153]. From receptor binding studies using recombinant channel catfish NITRs [14] it has been further suggested that bony fish NITRs mediate allogeneic recognition. We have recently shown that both DAP12 and FcR mRNA are strongly expressed in rainbow trout CD8$^-$ mucosal lymphocytes (including

putative NK cells) while CD8$^+$ cells were negative for these two molecules [139]. In non-mucosal organs CD8$^+$ cells showed a moderate expression of DAP12 and FcR suggesting the presence of NKT-like cells.

NILTs of teleosts have structural similarities to the natural cytotoxicity receptor NKp44 [132] which is a marker for activated human NK cells reviewed by Marras et al. [84].

In mammals, FcγRs are key molecules in antibody-dependent CMC (ADCC) reviewed by Sun, [136]. In channel catfish, alloreactive cytotoxic cells have been shown to be armed by IgM to execute ADCC, suggesting the existence of an FcμR [122]. Subsequently FcR homologues were found in channel catfish [130] and zebrafish [156].

2.2. Molecules involved in T cell immune responses

The most important molecules in the recognition of infected, antigen presenting cells by T cells are CD3, TCR, CD4 and CD8 (on T cells) as well as MHC class I and II (on antigen presenting cells). CD3 and TCR together form the T cell receptor complex by which T cells recognize MHC associated peptides that are expressed on infected target cells. MHCs are highly polymorphic among individuals of the same vertebrate species and thus antigen presenting cells are only recognized by T cells if both the MHC and the antigenic peptide fit to a given TCR. Thus, recognition of infected cells by T cells is highly specific and only certain TCR species recognize certain peptides requiring highly diverse TCRs. This diversification, like in immunoglobulins, is based on V-(D)-J gene rearrangement. CD4 and CD8 are co-receptors in MHC/TCR interactions. CD4 is associated with the TCR of T helper cells during interaction with cells presenting peptides of extracellular origin (e.g. bacteria) through MHC class II while CD8 is a co-receptor of cytotoxic T cells that interacts with MHC class I during presentation of antigenic peptides of intracellular origin (e.g. viruses). While MHC class II is only expressed by professional antigen presenting cells such as B cells, dendritic cells and macrophages, MHC class I is expressed by all nucleated cells which also includes erythrocytes [28] and thrombocytes [37] in fish. Issues related to teleost fish TCR, CD4 and CD8 have been comprehensively reviewed by Castro et al. [17]; recently.

Disease resistance to both viral and bacterial infections in fish has been associated with MHC class I and II haplotypes in cold water disease [59], furunculosis [44,68], ISA [67], KHV-I [108] and vibriosis [30,155,161]. However, the direct induction of T cells, as the effector cells responding to MHC peptide presentation in association with disease resistance, can only be speculated.

MHC genes have been published in many fish species. Surprisingly however, MHC class II (and CD4) genes have not been found in Atlantic cod thus far and are probably missing, which may have consequences for vaccine design and use in this species [131].

In mammals, many viruses provoke MHC class I expression and peptide presentation followed by an efficient CMC response. Induction of MHC class I pathway genes has been shown in acute infection of salmonids with IHNV [45] and ISAV [60]. DNA vaccination mimics a viral infection in the sense that antigenic proteins are produced intracellularly. In accordance, red sea bream DNA-vaccinated against RSIV [13] respond with an upregulation of MHC class I pathway genes.

When mammalian CTLs bind to target cells they release cytotoxic effector molecules into an immunological synapse [2]. On the effector cells, the synapse forms so- called supramolecular activation clusters (SMAC) where TCR, adhesion and/or signalling molecules concentrate (reviewed by Friedl et al. [40]). While the most important surface-associated molecules in the immunological synapse are TCR and CD4 or CD8 (reviewed by Jenkins and Griffiths [58]) there are several other molecules involved. For example, the intercellular adhesion molecule-1 (ICAM-1) binds to the leukocyte function associated molecule (LFA-1) on the target cells and the co-stimulatory molecules CD28 and CTLA4 (cytotoxic lymphocyte antigen 4) interact with CD80/86 (B7.1/B7.2). The existence of an LFA-1-like molecule has been proposed in channel catfish by [158] but there is no information on ICAM-1 in fish to the best of our knowledge. The signal 2 co-stimulatory receptors CD28 and CTLA4 belonging to the IgSF possess activating and inhibitory functions, respectively. Mammalian CD28 has a recruitment motif for the signalling adaptor growth factor receptor bound protein 2 (GRB2) which is conserved in the cytoplasmic region of fish CD28. When the extracellular domain of human CD28 is fused to the cytoplasmic region of the rainbow trout CD28, TCR-induced interleukin (IL)-2 production can be promoted in a human T cell line, suggesting similar functions of fish and mammalian CD28 (reviewed by Castro et al. [17]). Homologues of the CD28 ligands B7.1/B7.2 were also found in fish and members of this receptor family were described to have inhibitory functions in terms of T cell proliferation and cytokine expression (reviewed by Castro et al. [17]).

CMC-related responses may be different depending on the virulence of the respective pathogen. [48] observed a general upregulation of MHC class I in ISA infection and a depletion of CD8$^+$ cells in tissues regardless of the virulence of the virus. However, highly virulent strains provoked a much higher degree of depletion when compared to low virulent strains suggesting a role of CTLs in protection against ISAV. A downregulation of mRNA encoding both CD8α and CD8β was also observed in spleens of halibut infected with nodavirus and *Vibrio anguillarum*. However, it should be noted here that CD8α can be transiently downregulated in activated CTLs in response to peptide/MHC recognition in mammals [21,82,149].

Some mammalian viruses downregulate MHC class I responses in order to escape from the host's immune responses (reviewed by Horst et al. [51]). Virus-associated blocking of the MHC class I pathway may be initiated by different mechanisms: inhibition of peptide formation in infected cells, blocking of intracellular peptide trafficking, loading on MHC class I, and inhibition of MHC class I transport to the surface of infected cells. To determine whether piscine viruses also can suppress or upregulate MHC class I expression we infected RTG-2 cells with CIV or VHSV. While VHSV upregulated the de novo synthesis of MHC class I shortly after infection, this synthesis was suppressed after CIV infection [38].

Not only viruses but also bacteria may provoke cell-mediated immune responses. Rainbow trout bath vaccinated against *Yersinia ruckeri*, a facultative intracellular pathogen, showed high mRNA expression levels of TCR, CD8α and CD4 in protected fish. Transfer of plasma from immune fish to naïve fish did not confer protection upon challenge suggesting that T cells are more important in protecting fish than humoral factors [107].

Mammalian activated CD8$^+$ CTLs act by two major effector functions, cytokine expression and cytolytic activity, and both mechanisms interact with each other.

Toll like receptors are pattern recognition receptors (PRRs) that interact with PAMPs, resulting in activation of immune cells and the production of pro-inflammatory cytokines. Although there is no information on direct involvement of PRRs in lymphocyte responses in fish they might be of some importance in these processes. TLR22, which is unique in fish, has been suggested to be the functional equivalent of mammalian TLR3 since it recognizes dsRNA (reviewed by Matsuo et al., Palti et al. [85,100]). β2m which is associated with the MHC class I molecule is upregulated in rainbow trout by poly I:C, an immunostimulant that mimics double-stranded viral RNA [65] thus serving as a model PAMP for TLR3. This observation further exemplifies the interrelationship between innate and adaptive immune responses. TLR2, a PRR that is engaged in T cell polarization in mammals (reviewed by Borello et al. [165]), was shown

to be activated by several PAMPs in different fish species (reviewed by Palti et al. [100]). Activation of protective lymphocyte responses by PAMPs is still an unexplored field in fish vaccinology. Considering that many adjuvants function through pattern recognition receptors there is an urgent need to dedicate work force and financial resources for research in this field.

3. Cytokine regulation of lymphocyte responses

3.1. Generic regulations

Cell-mediated immune responses are complex and include components of both the innate (macrophages, NK cells etc.) and the adaptive (T-cells) immune systems. During an immune response against viral infection NK cells react spontaneously and immediately while T cells require activation and clonal proliferation signals to react.

A central role is played by IL-2 in the activation of all mammalian T cell subpopulations. Il-2 is produced by dendritic cells upon antigen presentation but also by T_h cells. Since IL-2 also has an impact on T_{reg} cells it is also very important in terms of immune tolerance and prevention of autoimmunity (reviewed by Malek and Bayer [83]). A homologous teleost IL-2 gene was first described in fugu, and fish injected with the TLR3 ligand poly I:C showed increased levels of mRNA encoding IL-2 in the gills and gut [6]. IL-2 mRNA expression in T-cell cultures from the same species can be stimulated with B7 (CD80/86) fusion proteins and PHA [134]. IL-2 genes have also been cloned in rainbow trout [25] in which its expression can also be stimulated by the CD28 and CTLA-4 ligands CD80 and 86 (respectively), but also by PHA, Y. ruckeri [46] and Tetracapsuloides bryosalmonae infections [152], as well as in mixed leucocyte reactions [160].

Another ubiquitous cytokine in cell-mediated immune responses is IL-6. IL-6 is mainly produced by monocytes, fibroblasts, and endothelial cells, but also by macrophages, T- and B-cells. While IL-6 is known as a B cell differentiation factor, it is also highly important in T cell responses such as T cell survival and proliferation, as well as in Th responses (reviewed by Dienz and Rincon [26]). In the presence of IL-2, it also induces the differentiation of T cells into CTLs [87]. The first teleost IL-6 homologue was cloned in fugu by [7]. Later IL-6 homologous genes were published in a number of other fish species, in which gene expression with respect to organ distribution and regulation by immune stimulants was inconsistent depending on the species (reviewed by Secombes et al. [117]). More recently [23] have shown *in vitro* that rainbow trout recombinant IL-2 triggers CD4 expression in trout pronephrocytes while IL-6 preferentially stimulated B lymphocytes which usually diminished in control pronephrocytes when cultured without a stimulant.

3.2. T_h responses

In the mouse system T helper cell responses are primarily provided by Th1, Th2 and Th17 cells. Th1 responses are characterized by an activation of CTLs, while Th2 responses result in B cell activation and antibody production. Th17 responses are characterized by the recruitment of neutrophils and Th17 cells play a role in autoimmune disease. Th1 development is promoted by the transcription factors STAT1, STAT4 and T-bet resulting in the production of IFN-γ as their 'signature' cytokine. Another important cytokine in Th1 responses is IL-12 which is mainly produced by professional antigen presenting cells such as dendritic cells and macrophages. While IFN-γ and IL-12 promote the development of cytotoxic responses they simultaneously interfere with Th2 driven immune responses. In contrast, the cytokine IL-4, together with the transcription factors STAT6 and GATA3, trigger the development of Th2 cells that in turn produce IL-4, IL-5 and IL-13 (reviewed by Zhu et al. [163]) and in a strong Th2 environment Th1 and inflammatory responses are downregulated.

3.2.1. T_h1 responses

In accordance with many viral infections in mammals, common carp infected with SVCV show a Th1 skewed response through upregulation of CD8 and IL-12 along with type I IFN genes [39]. Similarly, high expression levels of the Th1 cytokine genes *IFN-γ* and *IL-12* were recorded in goldfish infected with the extracellular parasite *Trypanosoma carassii* [94]. In addition *T. carassii* triggers IL-17 expression which is associated with a marked increase in the number of splenic neutrophilic granulocytes [110]. This observation may explain a reduced Th2 cytokine expression and subsequent inhibition of anti-parasite antibody responses which is essential for *T. carassii* clearance [63]. Also, in halibut experimentally infected with nodavirus, TCR, CD8α, CD4 and IFN-γ were upregulated, suggesting a Th1 skewed environment and a CTL response as well [98].

During infection of Atlantic cod with the facultative intracellular bacterium *Francisella noatunensis*, IL-12 and IL-17 expressions are upregulated, suggesting both a Th1 and Th17 immune environment [4]. However, since cod lack MHC class II, CD4 and invariant chain genes they most likely also do not possess T_h cells. It has been therefore suggested that this unique cold water adapted fish species compensates that "lack" of T_h responsiveness by their highly expanded number of MHC class I genes and a unique composition of TLRs [131]. Otherwise, like in mammals, cod CTLs and NK cells may be directly stimulated by IL-12 (reviewed by Sun and Lanier [135]).

There are three types of mammalian IFNs (I, II, III) that suppress viral replication in infected cells. They are most efficiently produced by professional antigen presenting cells. Type I interferons, e.g. IFN-α, -β directly activate $CD8^+$ CTLs but also trigger T_h1, T_h2 and T_h17 responses (reviewed by Sin et al. [124]). Both type I and type II interferons, e.g. IFN-γ also stimulate NK cell activity (reviewed by Seya et al. [119]).

Two of the three IFN families have been identified in teleost fish thus far. Both fish IFN families consist of two members each (reviewed by Zou and Secombes [164]). Fish type I IFNs induce expression of IFN stimulated genes: Mx, viperin, ISG15, PKR and TRIMS, with subsequent antiviral properties (reviewed by Verrier et al. [148]). Grass carp injected with recombinant type I IFN protein showed enhanced MHC class I expression and cytotoxic killing activity of $CD8^+$ CTLs against virus-infected cells [20].

In fish, the type II interferon IFN-γ has been shown to have similar functions as mammalian IFN-γ. Trout macrophages treated with recombinant IFN-γ show enhanced expression of both MHC class I and II molecules, but also of the MHC class I pathway genes LMP-2, LMP-7, MECL-1 and tapasin, thus indirectly contributing to CTL responses. IFN-γ enhances the phagocytic and nitric oxide activities of fish leukocytes and modulates their cytokine and chemokine expression. It induces the expression of pro-inflammatory cytokines including the Th1 cytokine IL-12 and several chemokines (reviewed by Zou and Secombes [164]. Recently, a non-mammalian cytokine IL-21 has been discovered which triggers the expression of both Th1 (IFN-γ) and Th2 (IL-10, and IL-22) cytokines [151].

3.2.2. T_h2 responses

Until recently it has been difficult to characterize Th2 responses in fish although some genes of the Th2 master cytokine IL-4/13 family, the master regulator of Th2 responses GATA-3, the IL-4/13 receptors IL-4Rα, IL-13Rα1, IL-13Rα2 and the signal transducer and activator of transcription 6 (STAT6) have been described (reviewed by Takizawa et al. [139]). We have recently reported the identification of IL-4/13A genes in rainbow trout and Atlantic salmon. When compared to mouse skin and lung, salmonids showed

constitutively high expression levels of IL-4/13A in the corresponding tissues skin and gills. IL-4/13A was mainly expressed by IgM-negative cells and was inducible by the T cell stimulating lectin PHA. IL-4/13A was expressed in reciprocal proportion to the Th1 cytokine gene IFN-γ [139]. Since in mammals IL-4 and IL-13 suppress Th1 and Th17 responses the high levels of these cytokines in gill and skin suggest a balanced T_h2/T_{reg} environment that protects fish from parasite invasion as well as from overwhelming inflammatory reactions induced by the continual contact with waterborne antigens. This observation might have utmost importance for vaccines designed for mucosal delivery.

3.2.3. T_h17 responses

IL-17 homologous genes have been reported in several fish species (reviewed by Kono et al. [70]). Many intraperitoneally injected vaccines cause granulomatous peritonitis in fish, e.g. in Atlantic salmon for which oil-based vaccines are widely used. In a micro-array-based study granuloma formation was associated with a Th17 skewed immune response. While Th1 and Th2 genes were not significantly regulated, pro-inflammatory cytokines (TGF-β), chemokines and also IL-17A and its receptor were significantly upregulated, which explains the attraction of inflammatory cells to the injection site [89]. Interestingly, IgM responsive genes were also upregulated suggesting that effective humoral immunity is not dependent on a Th2 environment.

Another important cytokine in the Th17 immune response is IL-23. IL-23 shares the p40 subunit with IL-12. In zebrafish, IL-23 homologous genes are upregulated after treatment with LPS and during mycobacterial infection suggesting that this cytokine has pro-inflammatory properties [50].

4. Anatomical distribution of lymphocytes

All T cells harbour the CD3 complex attached to the T cell receptor, and identification of CD3 has proved to be a reliable method for identifying T cells in a number of species [168]. Cloning and sequencing of the CD3 complex in the Atlantic salmon [78] made it possible to raise T cell-specific antisera for this species. The tissue distribution of T cells in salmon could thus be investigated [71]. Previously, morphological studies on T cell distribution in the zebrafish had been addressed by injecting transgenic T cells into isogenic recipients [76]. Also in the seabass, T cell distribution has been investigated in a number of studies using population specific monoclonal antibodies (mabs) with special focus on the thymus and the intestinal tract (reviewed by Picchietti et al. [105]). Anatomical studies strongly suggest that the teleost thymus, just like that of mammals, is a major site of T cell development. The thymus of teleost fish shows a great variety compared to that of mammals with respect to anatomical zone organization [22] (reviewed by Rombout et al. ; Ge and Zhao; Piecchetti et al. [111,42,104]; respectively) which also was confirmed for salmonid fish when addressed with a T cell marker [71].

In the teleost species thus far investigated, T cells are abundantly present in lymphoid organs including the thymus, head kidney and spleen. In the thymus of juvenile fish, immature T cells, still not $CD3^+$, are found in the medulla regions while numerous $CD3^+$ T cells may be found in the cortical regions and frequently also encircling blood vessels. In sexually mature fish, the thymus tissue is greatly changed, with no signs of medulla. The remaining cortical region appears to contain only mature $CD3^+$ cells and corpuscles of Hassal at this stage [71]. In the head kidney (the teleost permanent pronephros), but also in the mid-kidney (the teleost permanent mesonephros), abundant $CD3^+$ T cells can be seen. They are seemingly not arranged in any particular pattern, but spread out in the tissue. However, it is notable that they have not been described in the glomeruli. In the spleen, T cells are found encircling ellipsoids in the white pulp, but also occasionally may be seen elsewhere, such as in the red pulp. In the head kidney and the spleen of salmon [48] described the distribution of $CD8^+$ cells and noted an association of such cells with blood vessels and melanomacrophage centres.

T cells are also abundant in mucosal tissues which in fish arguably include the skin in addition to the gastrointestinal tract and the respiratory epithelium of the gills. In the skin, T cells are embedded in the epithelium at all investigated sites apart from the limbus region of the eye, where they are only sparsely present, and in the cornea, a structure of the optical axis, where they are absent [71]. In the gastrointestinal tract, T cells are encountered throughout the intestines, in salmon seemingly more abundant in the hindgut compared to the other parts [71]. They are embedded between the enterocytes and appear to be infrequent beneath the basal membrane. This pattern is very similar to the mammalian counterpart; however, no aggregates of T cells or B cells resembling cryptopatches or Peyer's patches have thus far been reported in teleost intestines. In the respiratory epithelium of the gills, scattered T cells may be seen; however, large T cell aggregates have been reported from other gill locations, which is addressed below. While numerous $CD8^+$ cells can be isolated from the intestine and gills [138] the percentage of such cells among skin leukocytes is as low as among leukocytes isolated from lymphoid organs and peripheral blood (unpublished data).

In non-lymphoid tissues including the liver, heart, central nervous tissue, muscle and eye, T cells are hardly found at all. The distribution of T cells in teleost fish reported to date is highly analogous to that of mammals, as it not surprisingly reflects the functions of the respective tissues. Some of the obvious and surprising differences between the mammalian and teleost immune systems from an anatomical perspective include the lack of lymph nodes and organised mucosal-associated lymphoid tissues [159], and the presence of pigment-producing leukocytes possessing features of classical melanocytes [141]. The existence of lymphatic vessels is still a matter of debate [150]. However, recently reported findings may change our view regarding the putative lack of organised mucosal-associated tissues in teleost fish, particularly in the gill.

There is great variety in the construction of teleost gills. However, common features include a set of four paired gill arches and a more or less developed interbranchial septum. In salmonid fish, the interbranchial septum originates at the base of the gill arch and terminates between the primary lamellae at approximately 1/3 of their length. At this site, a hitherto non-described lymphoid tissue was discovered by [167]. Combined with further investigations [71], it is now evident that the described lymphoid tissue, hereafter referred to as the interbranchial lymphoid tissue (ILT), consists primarily of T cells. [48] showed the presence of $CD8^+$ cells in lymphocyte-rich epithelium of the gills as did [138] in the ILT. [71] demonstrated that the T cells are embedded in a meshwork of epithelial cells, and are demarcated from the underlying tissues by a solid basal membrane. This finding also explains why the structure is devoid of vessels—vessels are not present within epithelium. Nevertheless, there seems to be an organisation into different portions of the ILT as most T cells are found in the centre of the structure, whereas very few are seen at its base near the basal membrane and near its surface, where it is covered by more flattened epithelial cells and mucous cells. Thus, the previous assumption stating that teleost fish are devoid of organised mucosa-associated tissue must be revised. So far, the presence of ILT has only been reported from salmonid fishes. However, with the present findings in mind, it should not be ruled out that other fish families also possess ILT, and with respect to the immense variety found in teleost species, where only a few have been subjected to thorough anatomical investigations, it is also highly premature to rule out the possible existence

of more organised lymphoid structures in the intestinal tract than hitherto reported. Only recently, M-like cells were identified in the teleost gut [41], and together with the recent reports on the ILT, along with the ongoing discussions regarding the secondary vascular system of teleosts [150], such findings indicate the need for a stronger focus on anatomical studies.

5. Functional studies

5.1. Mixed leukocyte reaction (MLR)

The MLR is a standard method to measure T_h lymphocyte proliferation resulting from allogenic diversity of two leukocyte populations that differ in MHC class II. The MLR can be a two-way reaction when MHC class II mismatched cells of one individual stimulate T_h lymphocytes of the second individual and vice versa. When the proliferation of lymphocytes of the stimulator individual is artificially suppressed by chemicals or radiation there is a one-way reaction where only the responder cells proliferate. A one-way MLR is also induced when antigen presenting cells and B cells are the stimulator cells.

The MLR has been reported for several fish species [16,61,77,86,88,128,172]. However, the relatively low number of papers on MLR in fish suggests that this method is rather complicated to establish. This might be related to the high polymorphism in fish MHC and the lack of fish with a defined MHC background.

In mammals, the MLR invokes the generation of FOXP3$^+$ CD4$^+$ regulatory T cells (T_{reg}), keeping lymphocyte proliferation at a certain level. When the stimulator and responder leukocytes are MHC class I matched T_{reg}s strongly suppress MLR [77]. A similar suppressive effect of a putative T_{reg} population on MLR has been recently reported for pufferfish [154].

5.2. Transplantation

Graft rejection and graft-versus host reaction (GVHR) are serious problems in human transplantation medicine. Like MLR, graft rejection and *in vitro* killing of allogeneic and xenogeneic cells provide valuable models to understand immunological phenomena associated with CMC.

Graft rejection has been described in many fish species (reviewed by Fischer et al. [38]), and is present in early life stage skin grafts of 14 day old rainbow trout larvae [140]. As in mammals (reviewed e.g. by Nelson [93]), a relationship between MHC class I alleles and allograft (skin grafts) rejection is present even in the phylogenetically primitive cartilaginous fish [97]. In teleosts (rainbow trout) it has also been proven that MHC class I linkage is decisive for the degree of allograft (erythrocytes) rejection [112] while [15] reported a correlation between allograft (scales) acceptance and MHC class II match. However, even in MHC class II matched donor/recipient combinations grafts were rejected which could have been a result of MHC class I mismatch but also due to the involvement of minor histocompatibility antigens as described for mammals (reviewed by Dierselhuis and Goulmy [27]).

As in mammals, rejection of allografts in rainbow trout is accompanied by lymphocyte infiltration [140] and in seabass subcutaneously grafted with muscle tissue the lymphocytes were identified as T cells [1]. Recently, cells infiltrating scale allografts in ginbunas were shown to be CD4$^+$ and CD8$^+$ [91]. This explains why allografting is a widely used method to induce allospecific cytotoxic cells in fish. Grafting has been done by transplantation of allogeneic scales or skin (e.g. [37]), but also by injection of allogeneic erythrocytes [33,34] or cultured cells (e.g. [37]). Interestingly, repeated anal and oral intubation of allogeneic cells induces allospecific tolerance in recipients [115,116]. This phenomenon may be of importance in repeated booster vaccinations by the mucosal route (oral, spray or bath).

In humans, grafting of haematopoietic tissue (bone marrow) from an immunocompetent donor to an allogeneic immunosuppressed recipient often results in graft-versus-host reaction (GVHR) leading to graft-versus-host disease (GVHD). The complex pathogenesis of GVHD involves both major and minor histocompatibility antigens. More recently, it was demonstrated that polymorphism in cytokines, KIR, and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) between donor and recipient also influences the outcome of grafting. Initial damage of host tissues by immune cells of the donor induces inflammatory T cell-specific cytokines resulting in the proliferation of allospecific CTLs and NK cells. Killed recipient cells are taken up by macrophages which produce even more inflammatory cytokines but also chemokines, integrins and their ligands, thus attracting more cells into target organs such as the intestine. Leakage of bacterial LPS through the damaged intestinal mucosa then triggers additional macrophages through TLR4 binding (reviewed by Penack et al. [103]). Thus, in GVHD tissue destruction is a result of macrophage activation, cytokine release and allospecific CMC (reviewed by Paczesny et al. [99]). GVHR is an established model of CMC in a number of mammalian laboratory animals but also two fish species, ginbuna crucian carp [90] and amago salmon [170]. As with *in vitro* CMC, GVHR was found to be temperature dependent in ginbuna crucian carp [36].

More recently, donor-derived CD4$^+$ and CD8$^+$ T cells have been reported to play important roles in ginbuna GVHD. In this teleost, the severity of pathological changes caused by immune cells in recipient fish coincided with an increase in T cells in the main target organs mesonephros, spleen, skin, liver and intestine [171].

5.3. In vitro CMC

In vitro CMC assays using allogeneic and xenogeneic target cells have been widely applied to prove the existence of cytotoxic cells. As in higher vertebrates foreign cells are killed by NK-like cells, by CTLs, but also by neutrophilic granulocytes and monocytes/macrophages in fish.

Common carp [74], ginbuna [72] and rainbow trout [113] neutrophils were described to exhibit NK-like ("non-specific") cytotoxicity against xenogeneic cells and highly concentrated ginbuna neutrophilic granulocytes may even kill isogeneic cells [35]. Recently, the rainbow trout monocyte/macrophage cell line RTS-11 was shown to kill xenogenic cells and cytotoxicity was enhanced after *in vitro* infection of effector cells with virus [95].

NK-like cells have been extensively described in channel catfish but also in other fish species (reviewed by Fischer et al. [38]). When NK-like cells were first described in fish [43] they were given the name non-specific cytotoxic cells (NCCs). Channel catfish NCCs isolated from lymphoid organs are morphologically distinct from mammalian large granular lymphocytes (LGLs) but also from channel catfish NK-like cells found in PBL. However, as with NCCs the latter spontaneously kill allogeneic cells [158]. A number of alloreactive cytotoxic cell clones were established from channel catfish PBL and a subset of those clones spontaneously kill different allogeneic cells, while others show allospecificity and express TCR [121,133]. These alloreactive TCR$^+$ CTL clones kill their targets via perforin/granzyme mechanisms [162]. CTL-like killing of allogeneic target cells (erythrocytes and cell lines) has also been shown in ginbunas ([33,34] and [47]; respectively) and rainbow trout [37]. In rainbow trout, it has been demonstrated that allospecific cytotoxic cells are of the CTL phenotype since immunomagnetically purified IgM$^-$ lymphocytes expressed *TCR* and *CD8α* genes, while B cells, monocytes, granulocytes and thrombocytes did not kill. Moreover, CD8 expression of IgM$^-$ lymphocytes was elevated after secondary

in vitro contact with homologous allogeneic targets suggesting CTL memory. Finally, the availability of CD8-specific mabs [143] has shown that ginbuna $CD8^+$ cells are the effectors of allospecific CMC, and more recently perforin and granzyme activities have been confirmed as their effector mechanisms ([142] and [144]; respectively).

In fish, transplantation models and alloreactive *in vitro* assays represent valuable experimental systems to study cell-mediated immune responses. However, this has no practical relevance for infection or vaccination. It is well known that intracellular pathogens, particularly virus infections (along with humoral responses) are largely controlled by CMC.

According to the concept of Doherty and Zinkernagel [29] CTLs can only kill virally infected cells if effector CTLs and virus-infected target cells possess identical MHC class I molecules (MHC class I restriction). These conditions are fulfilled in individual animals thus ensuring that the TCRs of effector cells can specifically recognize viral peptides loaded into the MHC class I of virus-infected cells *in vivo*. Experimental *in vitro* systems fulfilling these requirements have been established in two fish species so far, rainbow trout and ginbuna. We have established an effector/target cell system of clonal rainbow trout and susceptible MHC class I matched target cells to show CTL-like killing of virus-infected cells in viral infections and DNA vaccination ([146,147]; respectively). In the same species, homing of inflammatory cells isolated from the injection site of a DNA vaccine has been demonstrated [147]. In rainbow trout, acute VHSV infection also induces a public and private T cell expansion which is characterized by changes in the TCR repertoire [10] and [11] and in ginbuna crucian carp expression of TCRβ and CD8α levels correlate with virus-specific CMC activity [129]. Kidney leukocytes isolated from VHSV survivors can be re-stimulated by homologous viral antigens indicating immune memory. Continuous homologous antigen stimulation of those cells resulted in TCR-expressing cell cultures [32].

There are several reports from mammals indicating that CTLs are induced not only against viral envelope proteins but also against other viral proteins (reviewed by Fischer et al. [38]). DNA immunization is a useful tool to dissect immune responses to specific viral proteins. We could show that leukocytes from rainbow trout DNA immunised against the N-Protein of VHSV preferentially killed MHC class I matched infected target cells, suggesting killing by CTLs. On the other hand, leukocytes from fish DNA immunized against the VHSV G-protein killed both MHC class I matched and mismatched infected target cells, suggesting the involvement of both CTLs and NK cells, respectively [147]. The G protein of not only VHSV but also that of other rhabdoviruses provokes a strong CTL- and NK-like response, induces virus-specific antibodies and confers protection upon challenge (reviewed by Lorenzen and LaPatra [81]). The probable reason for this high effectiveness is that DNA vaccination mimics a viral infection in terms of endogenous antigen expression and processing, thus inducing effective MHC class I restricted responses. However, as with live attenuated viral vaccines they are difficult to register as commercial vaccines due to potential safety issues. As in mammals, a number of vaccination strategies using exogenous antigens that also stimulate MHC class I restricted immune responses have been described for fish (reviewed by Dijkstra et al. [166]).

CTL-like antiviral responses have also been shown in ginbunas infected with CHNV. Leucocytes isolated from infected fish show cytotoxic activity only towards infected isogeneic target cells and not towards CHNV infected allogeneic cells or isogeneic targets infected with a third party virus [126]. Recently, the same antiviral cytotoxic cells were also generated *in vitro* [127].

Mammalian CTLs kill their targets by cell-to-cell contact releasing effector molecules (see above) into the so called immunological synapse where TCRs bind to MHC/peptide complexes on antigen presenting cells (reviewed by Jenkins and Griffiths [58]). The need for effector cell contact with allogenic targets has been shown in channel catfish (reviewed by Shen et al. [123]), and in ginbuna, aggregate formation of $CD8\alpha^+$ cells with allogeneic [142] and virus-infected target cells [127] is crucial for successful killing.

Interestingly, it has recently been suggested that mammalian NK cells have immune memory and thus may execute adaptive immune functions (reviewed by Paust and Andrian [101]). Since fish, as the evolutionary oldest vertebrates, are do likely rely to a large extent on innate immune responses, this information might be highly important for vaccine design considering pattern recognition and missing self mechanisms.

6. Concluding remarks

In conclusion, fish possess many of the immune defence mechanisms that are found in higher vertebrates. The presence of an adaptive immune system and consequently immune memory allows for their vaccination which has been proven successful for several diseases of economically important fish. However, some differences from the immune system of higher vertebrates exist. These include a changing body temperature, a skin that is composed of mucosal tissue exposed to an aqueous environment, the unique ILT, lack of lymph nodes and germinal centres, lack of Ig class switching, the presence of the unique immunoglobulin IgT, high expression levels of IL-4/13A in skin and respiratory tissue, some unique cytokines and PRRs to name the most important differences.

Along with the need for inexpensive delivery strategies for fish vaccines these features of the fish immune system must be kept in mind when developing new fish vaccines for aquacultured fish. The description of cell-mediated immune responses to vaccination is particularly lagging the available data on protection capacities and humoral responses. Thus, there is still an urgent need for tools to understand cell-mediated immune responses to vaccination which is the most appropriate method for disease control, both on economical and ethical grounds. The availability of these tools will provide the basis for our understanding of the immune mechanisms triggered by vaccination and will help us to design new vaccines that induce effective immune response mechanisms.

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Review

Along the Axis between Type 1 and Type 2 Immunity; Principles Conserved in Evolution from Fish to Mammals

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Abstract: A phenomenon already discovered more than 25 years ago is the possibility of naïve helper T cells to polarize into T_H1 or T_H2 populations. In a simplified model, these polarizations occur at opposite ends of an "immune 1-2 axis" (i1-i2 axis) of possible conditions. Additional polarizations of helper/regulatory T cells were discovered later, such as for example T_H17 and T_{reg} phenotypes; although these polarizations are not selected by the axis-end conditions, they are affected by i1-i2 axis factors, and may retain more potential for change than the relatively stable T_H1 and T_H2 phenotypes. I1-i2 axis conditions are also relevant for polarizations of other types of leukocytes, such as for example macrophages. Tissue milieus with "type 1 immunity" ("i1") are biased towards cell-mediated cytotoxicity, while the term "type 2 immunity" ("i2") is used for a variety of conditions which have in common that they inhibit type 1 immunity. The immune milieus of some tissues, like the gills in fish and the uterus in pregnant mammals, probably are skewed towards type 2 immunity. An i2-skewed milieu is also created by many tumors, which allows them to escape eradication by type 1 immunity. In this review we compare a number of i1-i2 axis factors

between fish and mammals, and conclude that several principles of the i1-i2 axis system seem to be ancient and shared between all classes of jawed vertebrates. Furthermore, the present study is the first to identify a canonical T_H2 cytokine locus in a bony fish, namely spotted gar, in the sense that it includes *RAD50* and bona fide genes of both *IL-4/13* and *IL-3/IL-5/GM-CSF* families.

Keywords: immunology; evolution; fish; T_H1; T_H17; T_{reg}; T_H2; i1-i2 axis; cytokines; IL-5

1. Introduction

General Principles of the i1-i2 Axis as Exemplified by Major Polarizations of Mammalian Helper and Regulatory T Cells

Depending on the stimuli, largely in primary immune organs, hematopoietic stem cells can develop into a large array of morphologically and functionally different leukocyte populations [1–3]. At the sites of activation, these mature but "naïve" immune cells can then further polarize towards phenotypically distinct cell populations depending on the conditions. The polarized phenotypes can be more or less fixed by epigenetic changes, including chromatin folding, DNA methylation and histone modification [4–6]. Very important for polarization of immune cells is a loosely defined "axis" of conditions that favor type 1 or type 2 immunity, and which we call here the "i1-i2 axis". In helper T (T_H) cells the i1-i2 axis end conditions induce pronounced polarizations, T_H1 and T_H2 respectively, which are rather stably imprinted in the cell clones by heritable epigenetic changes [5,7–11]. The pronounced and stable character of T_H1 and T_H2 polarizations allowed their discovery already more than 25 years ago [7–9,12]. The type 1 end of the i1-i2 axis is represented by conditions which stimulate expression of interferon γ (IFNγ) and are enhanced by this cytokine, while for the type 2 end of the i1-i2 axis a self-stimulatory marker cytokine is interleukin 4 (IL-4; [5]). Important transcription factors for T_H1 cells are T-bet and STAT4 [5,13,14], and important transcription factors for T_H2 cells are GATA-3 and STAT6 [5,15–19]. The i1-i2 axis affects polarization of various types of leukocytes, and shifts along the axis are not only determined by IFNγ and IL-4 concentrations, but are also affected by other cytokines, pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), the strength and nature of cell-cell interactions, and physiochemical variables such as the concentrations of nucleotides and their derivatives, glucocorticoids, and oxygen [5,20–24]. In this review we will only discuss a few relevant factors, mainly concentrating on several important cytokines and transcription factors.

The term "type 1 immunity" relates to a milieu skewed towards cytotoxic functions including enhanced natural killer (NK), T_H1, and $CD8^+$ T cell activities. The major function of type 1 immunity is to kill cancer cells or cells with intracellular pathogens. Cell killing processes can be expected to be in relative disregard of damaging host tissue, but many tissue damaging inflammations for which originally T_H1 cells were blamed are actually mediated by T_H17 cells [25]. T_H17 cells are only partially shifted towards the i1-end of the i1-i2 axis (Figure 1A), and are representative for what can be called "type 3 immunity" ("i3") [26]. Characteristic for type 3 immunity is the involvement of transcription factors RORα and/or RORγt, secretion of the cytokines IL-17A, IL-17F and IL-22, and the activation of

neutrophils [26–28]. Type 3 immunity has an important function in protection against extracellular bacteria and some fungi. In the healthy intestine, T_H17 cells form an important role in a complex network of interactions between commensal bacteria and immune cells and help to maintain tissue homeostasis and barrier integrity [29–31]. Although phenotypically T_H17 polarizations are often considered to be more plastic than T_H1 and T_H2 polarizations, some epigenetic modifications acquired during T_H17 polarization are rather stable [32].

The use of the term "type 2 immunity" can somewhat differ between researchers and research fields, but tends to encompass both milieus with dominant immunosuppressive functions, for which TGF-β and IL-10 are marker molecules, and inflammatory milieus with dominant functions of cytokines IL-4, IL-5 and/or IL-13. Characteristic for type 2 inflammation are anti-parasite activities involving the activation of mast cells and eosinophils, and the secretion of IgE by B cells. In allergy diseases, these types of reactions are triggered by allergens. Generally, i2-skewed immune milieus may be more protective of tissues than i1-skewed milieus [33,34], which also agrees with type 2 immunity being important in wound healing [23,35]. However, also type 2 immune reactions can cause considerable tissue damages (e.g., [36]), amongst which tissue fibrosis [37]. The stimulation of T_H2 polarization by the alarmin IL-33, which is released from damaged tissue, can be understood from the importance of type 2 immunity in tissue regeneration and wound healing [23,35,38]. Similarly, the expression of chitinase-like proteins (CLPs), which is enhanced by helminth infection or injury, also induces T_H2 responses, although CLPs can also stimulate IL-17 release [39–42].

Figure 1 is our attempt to summarize some principles in cell polarization as they have been described for mammals. The horizontal axis relates to the concentrations of some important factors, whereas the vertical depiction of "energy valleys" relates to the relative stability of a cell polarization; the depiction with energy valleys only serves to explain a model, and the depicted valley depths have no absolute meanings. Some of the molecules characteristically expressed by the respective polarized cells are listed within those valleys, with blue arrows highlighting the cytokines that help fixing the cell phenotype as part of self-stimulatory loops [5,13,43–46]. White arrows refer to studies that described how some already polarized phenotypes are plastic in that they can be modified towards other polarizations; our figure is a simplification in the sense that the cell types produced by this type of route can be somewhat different from those directly produced from naïve T cells [47–53]. Our choice of the white arrows in Figure 1, with numbers for references described in the figure legend, represents our attempt to summarize major literature, and these possible conversions highlight similarities between polarizations in the order that they are depicted as "neighbors" in Figure 1. Readers should, however, realize that also conversions between "non-neighboring" (defined by Figure 1) polarizations have been reported possible (not shown in Figure 1; e.g., [54]), underlining that the Figure 1 depiction is only a model which explains some but not all principles of immune polarization. Importantly, though, the continuous axis-nature of the polarizations as depicted in Figure 1 is also supported by shared expressions of some marker molecules between "neighbors", with typically in one of the populations the expression being considerably lower or restricted to subpopulations (indicated by italic font in Figure 1). Naturally, Figure 1 is an enormous simplification, describing only a few major factors and categorizing only a few major cell populations. Especially T lymphocyte types with regulatory functions are a complex set of cells widely distributed along the i1-i2 axis [55], and the single regulatory T cell valley in Figure 1A is only representative for major sets of T_{reg} cells. The stability of T_{reg} polarizations is believed to differ between subtypes [56].

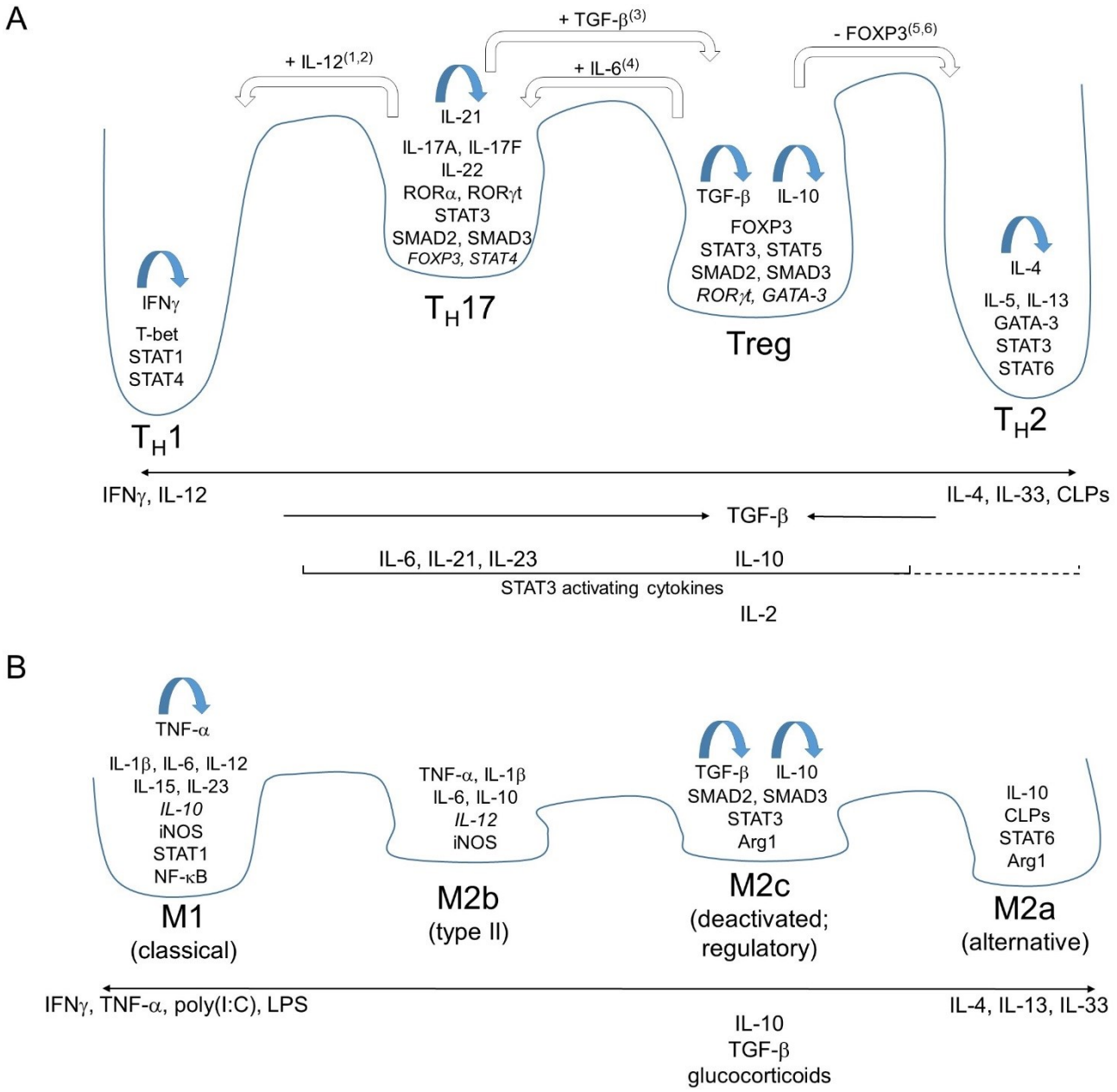

Figure 1. Schematic depiction of the i1-i2 axis affecting the polarizations of mammalian T_H/T_{reg} cells (**A**) and macrophages (**B**). Only some relevant factors, and not all known polarizations, are summarized. The figure organization and purpose is explained in the main text. Italic font relates to molecules that have been described as molecules especially expressed by that respective polarized cell population, but are present in lesser amounts than in other polarized populations for which they are more characteristic. Our depictions of factors relevant to T_H/T_{reg} cell polarizations are the summaries of mainstream ideas, with most references given in the main text. For GATA-3 expression in T_{reg} cells see [57]. For RORγt expression in early T_{reg}, and FOXP3 expression in early T_H17, see review [58]. For STAT4 expression in T_H17 cells see [59]. The macrophage polarization figure (**B**) is importantly based on a figure by Mantovani *et al*. [60], while modifications were made based on additional literature as referenced in the main text. The names between brackets are alternative designations that have been used for the respective macrophage polarizations. The blue arrows relate to

self-amplifying loops as described by: For interferon γ (IFNγ) in T_H1 cells, see [5,13]; for IL-21 in T_H17 cells see [45]; for TGF-β in T_{reg} see [44]; for IL-10 in T_{reg} see [43,46]; for IL-4 in T_H2 see [5]; for TNF-α in M1 macrophages see [61,62]; for IL-10 and TGF-β in M2c macrophages see [63] and [64], respectively. The white arrows relate to experiments that described how addition (+) or repression (−) of factors could push already polarized T_H/T_{reg} cells into another polarization state, with superscript numbers indicating the respective literature: 1, [49]; 2, [50]; 3, [53]; 4, [48]; 5, [47]; 6, [51].

In Figure 1, the actual biological situation of polarizations would probably be better represented by a three-dimensional energy landscape with many hills, ridges and valleys [65,66], where the 1-2 axis might be something like the East-West axis and with multiple possible routes between the East and West sides, and further addition of dimensions would further improve the figure; however, that would need a lot more information than currently available.

In Figure 1A we did not include T_H9 and T_{FH} cells because currently it is impossible to discuss their possible presence in the context of fish because relevant genes (e.g., IL-9) or tissues (e.g., functional equivalents of lymph nodes) have not been found/clarified in fish yet. It is very likely that fish do not have the exact same immune cell polarizations as found in mammals, although the major principles of the i1-i2 axis appear to be the same as we argue in this article. If we somewhat freely interpret the work by Kaplan and co-workers [67], those authors arranged mammalian T_H9 and T_{FH} cells in the order T_H1-T_{FH}-T_H17-T_{reg}-T_H9-T_H2 along the i1-i2 axis. However, others have found similarities between T_{FH} and T_H2 cells [68] or stressed the heterogeneity and the existence of subpopulations among T_{FH} cells [69]; probably the adaptation of cells to T_{FH} function should not be understood as a unique polarization along the i1-i2 axis.

TGF-β limits how far cells polarize along the i1-i2 axis in either direction, and it is an important cytokine for the development of T_H17 and T_{reg} cells. A higher concentration of TGF-β favors development of T_{reg} over that of T_H17 [70,71]. Whereas STAT3 activation in T_H17 cells is especially enhanced by IL-6, IL-21 and IL-23, the STAT3 activation in T_{reg} cells is especially enhanced by IL-10 [46]. It is tempting to speculate that the relatively common transcription factor STAT3 [72] blocks development of the "more extreme" axis-end phenotypes T_H1 and T_H2. However, although STAT3 is known to suppress expression of T_H1 marker genes [73], it was reported necessary for T_H2 development [74]. Because the dependency of T_H2 cells on STAT3 has not been studied intensively, we used a dashed line in Figure 1A for the yet better to be clarified main factors that stimulate STAT3 in T_H2 cell development.

Although STAT5 activity can stimulate survival and proliferation of different sets of lymphocytes [75], it represses T_H17 differentiation and shifts the development of common T_H17/T_{reg} precursor cells towards T_{reg} [76]; the important inducer of STAT5 activity in T_{reg} is IL-2, a cytokine which can also stimulate other lymphocyte populations [77]. The Figure 1 model does not include the STAT5 activity enhancers IL-9 and thymic stromal lymphopoietin (TSLP), which both stimulate type 2 immunity [38], because these two genes have not been found in fish (yet). STAT5 activities in NK cells and $CD8^+$ T cells (not shown in Figure 1), important for type 1 immunity, can be induced by IL-15 that is expressed by dendritic cells or monocytes/macrophages [77].

Although most researchers will agree that polarizations of immune cells depend both on various gradients of factors as well as on more discrete sets of conditions, to try to catch that in a figure with

only a single axis as in Figure 1 could be righteously considered presumptuous, overly simplified, and misleading. However, we argue that in such it doesn't stand out negatively from more popular figures trying to summarize leukocyte polarizations. Furthermore, we argue that if we wish to compare immune polarizations of different cells and tissues, of healthy *vs*. diseased conditions, and among species as diverged as mammals and fish, we need a kind of articulated bird-view of the i1-i2 axis as attempted in Figure 1. In the current study we use the Figure 1 model for analyzing published data in fish, and conclude that the immune systems of mammals and teleost fish seem to obey to at least some similar i1-i2 axis principles.

2. Polarizations along the i1-i2 Axis of Mammalian Leukocytes Other than Helper and Regulatory T Cells

Polarizations towards type 1, type 3 and type 2 immunity, which are very reminiscent of the ones found for T_H cells, have been described for innate lymphoid cells (ILCs) (reviews [26,78,79]). Marker molecules expressed by ILC1 cells are transcription factor T-bet and cytokine IFNγ, marker molecules expressed by ILC3 cells are transcription factor RORγt and cytokines IL-17 and IL-22, and marker molecules for ILC2 cells are transcription factor GATA-3 and cytokines IL-5 and IL-13. The intermediate position of ILC3 along the i1-i2 axis, similar to as found for T_H17 cells, is supported by sharing of some marker transcription factors and cytokines with either ILC1 or ILC2 cells, while ILC1 and ILC2 cells appear to lack unique overlaps with each other [26]. ILC3 cells can be converted into ILC1 cells by stimulation with IL-12, resulting in downregulation of RORγt and upregulation of T-bet [80]. Some difficulties in classification of ILCs are caused by the existence of multiple ILC1-type populations, and by differences in their regulation between human and mouse [78]. Most researchers do not distinguish a separate "ILC_{reg}" population, but besides aiding type 2 inflammation, ILC2 cells are known to have important functions in tissue homeostasis and tissue repair [81,82]. Very interestingly, recently also ILC3 subsets were found to have T_{reg}-like functions in the sense that they could negatively select antigen-specific T cells [83]. Thus, like found among T cells, among ILCs there is an overlap between type 1 and type 3 immunity, between type 3 immunity and regulatory functions, and between regulatory functions and i2 inflammation.

Except for regulatory/helper T and ILC populations, i1-i2 polarizations similar to the ones listed above because involving at least several of the same marker molecules have been reported for $CD8^+$ T cells [84], B cells [85], neutrophils [86] and dendritic cells [87]. However, it is beyond the scope of this article to discuss those polarizations. Macrophage populations, on the other hand, will be discussed here, because macrophage polarizations have been studied relatively intensively and are of major importance in the creation of immune milieus and in tissue modeling. Furthermore, there are some functional data on macrophage polarizations in teleost fish (see further below).

In Figure 1B we made an attempt to characterize major polarizations of mammalian macrophages along the i1-i2 axis. The figure is a modified version from a distribution figure by Mantovani *et al*. [60], and as in Figure 1A, the depths of the "energy valleys" only serve to explain a model and have no absolute meanings. Very importantly, what emerges as a general impression from literature is that macrophage populations have less pronounced self-amplifying loops (although for an autocrine TNF-loop see [61] and [62], for an autocrine IL-10 loop see [63], and for an autocrine TGF-β loop see [64]) than known for T_H/T_{reg} polarizations, and that macrophage polarizations appear to be rather unstable and

hence a rather direct reflection of their immune environment [88,89]. This makes sense since in contrast to T cells which are antigen-specific and whose epigenetic modifications contribute to immune memory [19,90], macrophages interact with a large number of antigens. Their plasticity probably is an important reason why macrophage polarizations were discovered later and remain poorer characterized than T_H/T_{reg} polarizations. Many researchers only distinguish between M1 and M2 macrophages, without further subdivisions.

Macrophages are sensitive to DAMPS and PAMPs. LPS is an important PAMP for shifting macrophage polarizations towards the i1-end of the i1-i2 axis, and can stimulate the development of both M1 and M2b macrophages [60,89]. Viral dsRNA mimic poly(I:C) also induces M1 polarization [91,92]. M1-skewed macrophages express IL-12 which is important for the initiation of T_H1 polarization [89], and they also express IL-15 [93,94] which is especially important for the stimulation of NK and $CD8^+$ T cells [77,95]. Both M1 and M2b macrophages express inducible nitric oxide synthase (iNOS) and are active in clearance of bacteria through NO production [94,96]; the big difference of M1 *vs*. M2b cells is the abundant production of IL-12 *vs*. IL-10, so that M1 cells support type 1 immunity and M2b cells are able to support type 2 immunity [96–98]. The expression (-pathways) of iNOS and arginase affect each other negatively [99–101]. Expression of arginase in M2c and M2a cells leads to production of ornithine, a precursor of extracellular matrix components that contributes to wound healing [60,102]. M2a and M2c macrophages appear to participate in tissue regeneration following tissue injury [103], which explains why M2a polarization can be enhanced by alarmin IL-33 ([104,105]. We did not include alarmin IL-25 (alias IL-17E) in Figure 1, although it also supports type 2 immunity, because of difficulties to find an orthologue in fish [106,107]. M2a polarization involving i2 cytokines induces macrophages to express CLPs [40,108,109]. M2c macrophages have anti-inflammatory properties and are stimulated by glucocorticoids, IL-10 and TGF-β [110,111]. It is of note that in human, different from the mouse situation, some M2 polarizations may not be typified by high levels of arginase expression [112,113].

In many tumors the tumor cells attract monocytes/macrophages and skew their development towards type 2 immunity; the M2 macrophages then in reciprocal interaction with the tumor cells remodel the tumor microenvironment, which aids the tumor cells and protects them from type 1 immunity [114,115]. The current successes in cancer immunotherapies are largely based on shifting the tumor milieu from type 2 towards type 1 immunity, and one of the focuses of investigation concerns macrophage polarizations (e.g., [116,117]).

3. Fish Orthologues of Mammalian genes for i1-i2 Axis Functions

For all the mammalian genes encoding the proteins shown in Figure 1, homologues could be found in ray-finned and/or in cartilaginous fish, in most cases including probable orthologues. Examples are shown in Figure 2 plus supplementary file 1.

For teleost fish now a relatively large number of "whole genome" sequences have been published. However, for cartilaginous fish, the only species for which the sequence of a large part of the genome has been published is the chimaera elephant shark (*Callorhinchus milii*; [107]); hence, gaps in the published elephant shark genome can't be "filled in" with information from other cartilaginous fish.

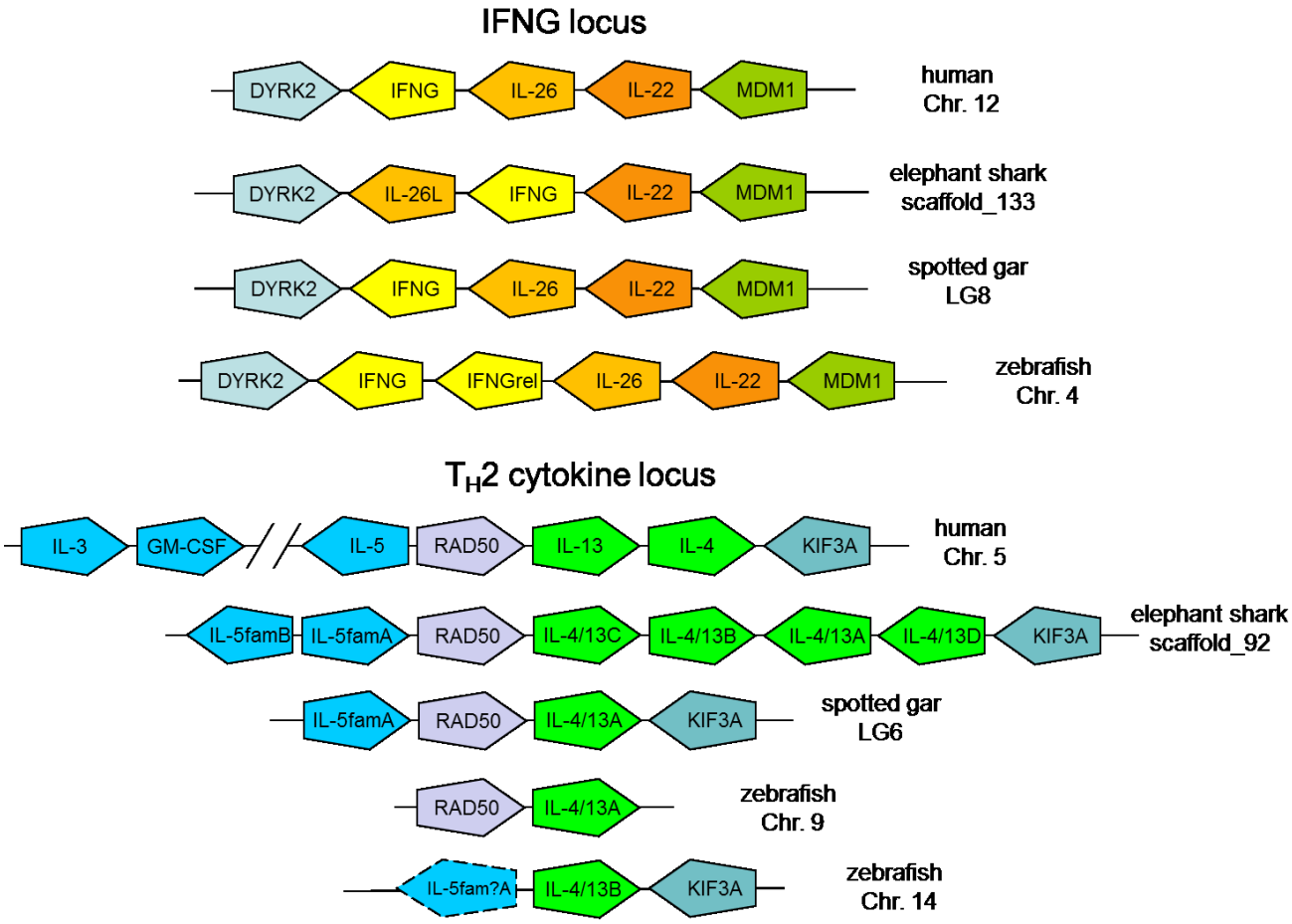

Figure 2. Schematic depiction of the conservation of the IFNG and T_H2 cytokine loci in fish and mammals. The pentagon orientations correspond with gene directions. Depicted gene organizations are based on analysis of genomic sequence information available for elephant shark (*Callorhinchus milii*) provided by the Elephant Shark Genome Project ([118] and GenBank accession number AAVX02000000) and for the other species in the following datasets of the Ensembl database [119]: human (*Homo sapiens*), GRCh38.p2; spotted gar (*Lepisosteus oculatus*), LepOcu1; zebrafish (*Danio rerio*), GRCz10. Between human IL-5 and GM-CSF lays a 465 kb stretch with a number of genes which are not shown in this figure. Most of the depicted gene organizations have been described before [107,120–125]. The deduced elephant shark IL-26L amino acid sequence is MRCAAACLLVSLGVCVVRTSTA TCKPKVSDRLIQDFIRCVGNVMNASQHYWGSSWSDGKGYRFLPKPVKMTKHGKC TVVKKALEFYLIFLKQYRPMPDGFKQDLIKVKHYLEEMYAKTRCDECKSSKDLNAE RAIKRLEKEICKARCSKHTSVTKKSIIFQLYILRNLITNMA. For the deduced encoded elephant shark T_H2 locus cytokine sequences see Table S3 (except for IL-4/13D these are also described in [124]). For the spotted gar IL-4/13A sequence we refer to [125]. The deduced spotted gar IL-5famA sequence is MSMYLVLLILGVHYSGVRTQHYHFISEIISHIENAK QGVVHTILLTPQNVLNANCTASYSKIFLKGIKHLSVHSEHGSQEELKLIIHNMERMD VICPNLKHQVPDCEVQDTSTFQFLRQFTKFLQKIKRSDCFRLRSEYPFSA, which is compared with other sequences in Figure 3.

Nevertheless, for most of the molecules depicted in Figure 1 probable gene orthologues can be found in the published elephant shark genomic sequences, and for the exceptions the incomplete nature of the published sequences might be to blame. It has been argued that elephant shark does not have an *RAR-related orphan receptor* (*ROR) gamma* gene [107]. That may be true, although such conclusion would need full genome sequence information, and in our preliminary phylogenetic tree analyses (data not shown) the molecule encoded by the elephant shark "*RORA-like"* (*RORAL*) gene at scaffold 2358 (supplementary file 1) clusters with RORγ sequences. A serious analysis of the evolution of the ROR family of transcription factors would need a more serious effort than feasible within the scope of this article. Regardless, because in mammals not only *RORC* but also *RORA* can contribute to T_H17 development [28], the question on *C* or *A* identity might not be so relevant across these wide species borders when addressing the possibility of T_H17 polarization.

An early ancestor of all extant teleost fishes experienced a whole genome duplication event [126], and several teleost fish lineages experienced an additional genome duplication event (e.g., [127]). This was frequently followed by gene losses, lineage specific gene duplications and/or translocations, causing a tendency for the analysis of orthologous relationships between mammals and teleost fish to be more complicated than between tetrapods, cartilaginous fish and non-teleost primitive bony fish. However, overall and in principle, many gene organizations in teleost fish resemble those of mammals and elephant shark [107,126], and it was because of conserved syntenies that we and others could identify teleost fish genes for small cytokine genes despite their poorly conserved sequences (e.g., [120–122,128,129]). A number of the gene syntenies between fish and human depicted in Figure 2 and supplementary file 1 were already described earlier (e.g., [107,130]), but we feel it is convenient for the readers to have the data presented together.

Figures 1 and 2, and supplementary file 1 do not provide information regarding the relevant receptors and neither regarding many of the pathway molecules, because we argue that the currently depicted molecules are sufficiently representative for their functional pathways. However, it is important to realize that also the relevant cytokine receptor and pathway molecules tend to be rather well conserved between fish and mammals (e.g., [107,131–133]). Furthermore, regarding the molecules depicted in Figure 1, we did not analyze the genomic locations of fish genes involved in glucocorticoid pathways and of fish CLP genes; for these genes we refer interested readers to references [134] and [135], respectively.

As a negative exception among the proteins depicted in Figure 1, for IL-33, which is a highly diverged member of the IL-1 family with poor sequence conservation even between mammals and birds (see Ensembl accession ENSGALG00000020558), we could not find a likely gene candidate in any of the investigated fish species. However, in teleost fish multiple IL-1 family members have been found [136,137], and teleost genes have been annotated as *IL1RL1* (alias *ST2* or *ST2L*; [136,138,139]) which in mammals encodes the receptor for IL-33. Since *IL1RL1* maps to a locus with multiple similar genes of the IL-1 receptor family [140], this *IL1RL1* designation in fish probably would need a more intensive analysis than has been published to date or is feasible within the scope for our present study. In short, fish may have IL-33 (-receptor) function, but there is no real evidence to support that.

From the early days that we started to identify i1-i2 axis cytokine genes in fish despite of their very poorly conserved sequences with the help of gene syntenies (e.g., [120,122,129]), we have been fascinated by the high conservation of loci between fish and mammals, often even in simple 1:1 orthologies. The high level of evolutionary conservation of the genomic organization of many of the i1-i2

axis gene loci, as shown in Figure 2 and supplementary file 1, contrasts with the abundant locus turnovers and copy number differences found for other genes of the immune system, like for example genes encoding MHC molecules [141,142], chemokines [143], and type I interferons [144]. For several NK cell receptor families there may not be close relatives in fish at all [145]. That many of the gene loci important for the i1-i2 axis are so well conserved between jawed fish and mammals strengthens the idea that in all jawed vertebrates major principles of the i1-i2 axis system have been conserved as core mechanics of their immune system. Our attempts to find i1-i2 axis genes in published sequences of jawless fish (lampreys, hagfish) and invertebrates proved to be difficult/impossible (data not shown), and future careful analyses should determine if to any extent some principles of the i1-i2 axis might be present in those species. For reviews on the immune systems of jawless fish, which are fundamentally different from those in jawed vertebrates, we refer to [146–148].

4. Conservation of the IFNG and T_H2 Cytokine Loci

Very important in the T_H1 and T_H2 polarizations are their divergent epigenetic modifications of the IFNG and T_H2 cytokine loci [5,149]. Especially the pronounced modifications of the T_H2 cytokine locus, including chromatin refolding, have received a lot of attention [4,18,150–153]. Binding of transcription factor GATA-3 and STAT6 induces chromatin refolding by inducing interactions between (inter-) gene regions of *IL-5*, *RAD50*, *IL-4* and *IL-13* [18,19]. It is fascinating to see how well the IFNG and T_H2 cytokine loci have been conserved between fish and mammals (Figure 2). The name *IL-4/13* is used for genes related to tetrapod *IL-4* and *IL-13*, because it can't be decided to which of the two tetrapod genes the fish genes are closer related, and the gene duplication leading to *IL-4 vs*. *IL-13* may have occurred after the separation between the ancestors of tetrapods and ray-finned fish [122].

Most of the gene organizations shown in Figure 2 have been reported before [107,120–125], but different from previous publications [107,124,125] we found (i) *IL-26-like* (*IL-26L*; for the encoded sequence see the Figure 2 legend) in the elephant shark IFNG locus, (ii) an extra *IL-4/13* copy, *IL-4/13D*, that we had missed [124] but which was properly annotated as an *IL-4/13* gene by automated database gene prediction (XM_007902044) in the elephant shark T_H2 cytokine locus, and (iii) an *IL-5 family* (*IL-5fam*) member in the spotted gar T_H2 cytokine locus (for the encoded sequence see the Figure 2 legend). Our analysis of the spotted gar T_H2 locus constitutes the first identification of a bony fish T_H2 cytokine locus that includes *RAD50* and seemingly bona fide genes of both *IL-4/13* and *IL-3/IL-5/GM-CSF* families.

Expression of elephant shark *IL-26L* was confirmed by sequence read archive (SRA) database reports (data not shown), and the gene has an intron-exon organization typical of the IL-10 family (to which also IL-22 and IL-26 belong). Phyre^2 software [154] predicts that elephant shark IL-26L protein has multiple α-helices, and that its structure is similar to IL-10 (confidence 52%). Although the deduced molecule does not have a typical IL-10 signature motif in the carboxy-terminal α-helix, it shares some specific cysteines with IL-26 (data not shown), and despite the minimal similarity we considered "IL-26L" to be the best possible name.

In a previous paper we depicted the elephant shark *IL-5famA* and *IL-5famB* genes as "*IL-5A*" and "*IL-5B*", upon request of the respective journal who considered the "fam" indication (for family) to be confusing for a general audience ([124]; the "fam" designations were only given as optional in the supplement of that publication). But, although the genes are related to *IL-5*, and are situated at the

expected *IL-5* location, we have no strong arguments for their closer relation to *IL-5* than to *IL-3* or *GM-CSF* (for discussion of the evolution of the T_H2 cytokine locus see also [122]). So in the current article we like to use the nomenclature including "*fam*", although both nomenclatures are defendable. The cytokine family including IL-3, IL-5 and GM-CSF is characterized by extremely poorly conserved sequences, especially among the IL-3 molecules [155], with only a few typical and conserved sequence motifs (Figure 3). Whereas hitherto for bony fish no convincing IL-5-family candidates were reported, we now found such gene at the expected *IL-5* site in the spotted gar T_H2 cytokine locus which we designated *IL-5famA* (Figures 2 and 3). It has the family-typical intron-exon organization, and in contrast to the other detected IL-5fam molecules in fish, gar IL-5famA has a cytokine as top-match upon blastp comparison with the NCBI database (Genbank accession KFO26617); the relevant unknown cytokine gene appears to be correctly predicted for cattle (GenBank accession XP_010796657), maps directly downstream of mammalian *IL-3* and *GM-CSF*, is predicted to encode multiple α-helices according to Phyre[2] software, and appears to have pseudogene identity in humans (data not shown). We may discuss this hitherto unknown mammalian cytokine in more detail in a future publication, and only mention it here as additional evidence that the fish IL-5fam molecules truly belong to the IL-3/IL-5/GM-CSF family.

In teleost fish gene candidates for the common β receptor chain (alias IL-3Rβ) have been known for a long time [131], and accordingly the finding of IL-3/IL-5/GM-CSF family genes has been anticipated. However, the best that we could do so far was a zebrafish gene with unclear signature which we designated "*IL-5?*" [122] and which we now in Figure 2 designate as "*IL-5fam?A*". We actually are still insecure whether this somewhat peculiar zebrafish gene is an intact gene, as it may not have a normal exon1 sequence (data not shown), and therefore it is indicated by a dashed line in Figure 2. However, we are now confident that the zebrafish gene is at least related to intact cytokine genes, as orthologous and apparently bona fide cytokine genes can be found in carps and goldfish. Like in zebrafish, in common carp the gene is also linked with *KIF3A* (GenBank accession LN591230). Figure 3 shows four cyprinid IL-5fam? sequences, namely the LN591230 encoded common carp sequence, a goldfish sequence encoded by GenBank TSA accession GBZM01010380, and two very similar golden mahseer sequences assembled from SRA reads (data not shown; we don't show the individual SRA accessions). The question mark in the "IL-5fam?" nomenclature expresses our insecurity about the molecule identities, because whereas in our opinion the gar and elephant shark molecules have a convincing IL-3/IL-5/GM-CSF family signature, the only partially conserved signature in cyprinid sequences fails to convince us (Figure 3). Nevertheless, because of genomic location and lack of better matching candidates, the most likely hypothesis appears to us that these cyprinid sequences are highly diverged members of the IL-3/IL-5/GM-CSF family. To our frustration, even with the knowledge of the gar and cyprinid IL-5fam(?) sequences, we have been unable so far to find any IL-3/IL-5/GM-CSF family gene candidates in non-cyprinid teleosts. Although negative findings for gene members of these small cytokine families with poorly conserved sequences shouldn't be overvalued (see [107] and [124]), it might be speculated based on the lack of convincing gene candidates that the importance of the IL-3/IL-5/GM-CSF family was reduced in teleost fish compared to other classes of vertebrates. Functional analyses of the fish molecules, including their possible interaction with the common β receptor chain, should clarify these matters.

As a general statement based on our many years of experience in identifying genes of the immune system, we feel that at the genetic level the immune systems of elephant shark and gar have more

similarities with the mammalian immune system than found between teleost fish and mammals. Slow evolution towards the elephant shark genome and rapid evolution towards the extant teleost fish genomes have been noted before [107,126,156]. When fish research is performed with the aim to deduce the ancestral features of the human immune system, it might be worth considering to move research away from teleosts to for example gar (*Lepisosteus oculatus*).

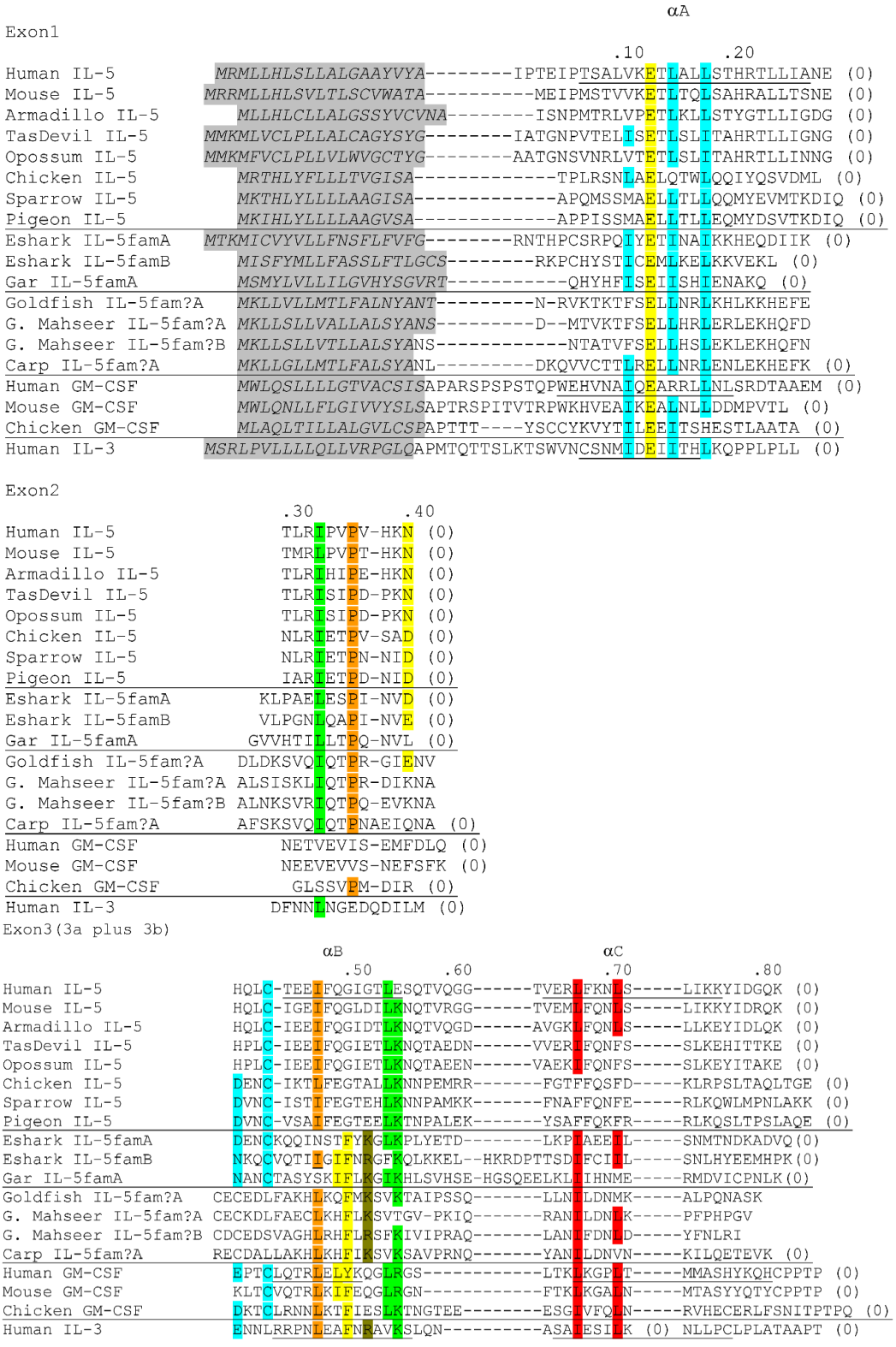

Figure 3. *Cont.*

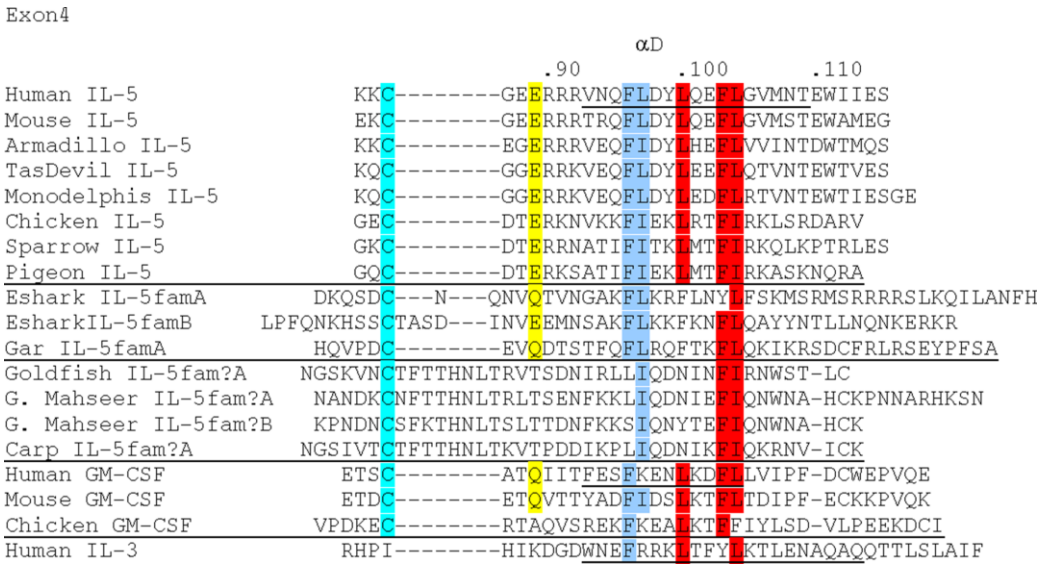

Figure 3. Alignment of (deduced) IL-3/IL-5/GM-CSF family member amino acid sequences. (Predicted) leader peptides are indicated with gray shading; for predictions SignalP software was used (http://www.cbs.dtu.dk/services/SignalP/). The alignment is organized according to the matching exons, and brackets relate to intron positions with the number indicating the intron phase. The α-helices αA-to-αD, of human IL-5, GM-CSF and IL-3 are indicated by underlining following [157], [158], and [159], respectively. Sequences were aligned by hand, based on considerations regarding structure and evolution (as in [122], although we made some different choices now). For the alignment among the core regions of human IL-3, IL-5 and GM-CSF we mostly followed the structural alignments by [159] and [160], with as notable exception the α-helix B sequences of IL-5 in which we introduced a gap for a better match of sequence identities with the other cytokines. Readers should realize that alignments of such highly differentiated sequences remain discussable. Conserved motifs are highlighted by different color shading in a somewhat instinctive and random manner. Some of the highlighted motifs can also be found in cytokines not belonging to the IL-3/IL-5/GM-CSF family, but not in this combination (compare with [122,160,161]. The yellow shaded glutamic acid in α-helix A is important for function [162–164], and, at least for GM-CSF, for binding the common β receptor chain [165]. Aligned, in that order, are the following sequences: Human (*Homo sapiens*) IL-5, GenBank accession NP_000870; mouse (*Mus musculus*) IL-5, NP_034688; nine-banded armadillo (*Dasypus novemcinctus*), XP_004456511; Tasmanian devil (*Sarcophilus harrisii*) IL-5, XP_003756529; gray short-tailed opossum (*Monodelphis domestica*) IL-5, XP_001371840; chicken (*Gallus gallus*) IL-5, ADL28818; white-throated sparrow (*Zonotrichia albicollis*) IL-5, XP_005483812; rock pigeon (*Columba livia*) IL-5, EMC79983; elephant shark (*Callorhinchus milii*) IL-5famA and IL-5famB, see supplementary file 2 and [124]; spotted gar (*Lepisosteus oculatus*) IL-5famA, see the Figure 2 legend; goldfish (*Carassius auratus*) IL-5fam?A, GBZM01010380; golden mahseer (*Tor putitora*) IL-5fam?A and IL-5fam?B, see supplementary file 2; common carp (*Cyprinus carpio*) IL-5fam?A, compare LN591230 or LHQP01003280 with the goldfish or golden mahseer sequences; human (*Homo sapiens*) GM-CSF, NP_000749; mouse (*Mus musculus*) GM-CSF, CAA26192; chicken (*Gallus gallus*) GM-CSF, NP_001007079; human (*Homo sapiens*) IL-3, AAH66275.

5. Investigation of Tissue-Specific Co-Expressions of T_H1, T_H17, T_{reg} and T_H2 Signature Genes in Fish; Gills Consistently Express High Levels of T_H2 Signature Genes

Previously we showed that in salmonid fishes the expression of *GATA-3* and *IL-4/13A* are high in gill, skin and thymus, also in relation to other genes of the immune system, and we assumed that these tissues are skewed towards type 2 immunity [166]. Similar findings for high GATA-3 and IL-4/13A expression in teleost gill were also made by others [121,167,168]. In the current study we extended this type of investigation with database mining, in which we blasted sequences against tissue-specific single SRA datasets available for healthy individuals of several fish species (http://www.ncbi.nlm.nih.gov/sra) (data not shown) as summarized in Table 1. We found our previous observations of high *IL-4/13A* and *GATA-3* expression in rainbow trout and Atlantic salmon gills confirmed by transcriptome analyses for these two species, but also for pike (like salmonids member of *Protacanthopterygii*) and golden mahseer (a cyprinid fish) (Table 1). Excitingly, even in elephant shark the *GATA-3* and *IL-4/13* expressions appear to be particularly high in gill, coinciding with high *IL-5fam* gene expression, which suggests that the elephant shark IL-5fam/RAD50/IL-4/13 locus is a similar GATA-3 driven T_H2 cytokine locus as present in mammals, and that the i2-skewage of the gill immune milieu is ancient. More analysis would be necessary to determine how the i2-skewage is distributed over the gill, and to what extent it maps to interbranchial lymphoid tissues (ILT; [169]). Interestingly, in Golden mahseer, the expressions of *IL-4/13B* and *IL-5fam?* genes are not tightly associated with those of *IL-4/13A* and *GATA-3*, and are not consistently although often high in gill (Table 1 and data not shown). This suggests that only one of the two teleost copies of the T_H2 cytokine locus resulting from the teleost ancestral whole genome duplication, namely the *RAD50* + *IL-4/13A* locus (Figure 2; see also [122]), retained the expression mode of the ancestral T_H2 cytokine locus. In accordance, in the promoter regions of teleost *IL-4/13A* genes and not in those of teleost *IL-4/13B* genes we found a rather well conserved GATA-3 binding motif [122]. In elephant shark *STAT6* expression is highest in gill, as expected from an i2-skewed tissue, but for unknown reason in teleost fish it does not tightly associate with the high *GATA-3* and *IL-4/13A* expression found in gill (Table 1).

Besides a clustering of high expressions of T_H2 signature genes in gill, the investigated elephant shark individual displays such clustering for T_H1 signature genes in its spleen and for T_H17-signature genes in its intestine (Table 1). Although this appears very interesting, and may be indicative of ancient tissue-specific immune biases, these data do need confirmation in other cartilaginous fish individuals before allowing conclusions.

In the investigated teleost fish individuals, besides the consistent link between *GATA-3* and *IL-4/13A* expression, we also found a consistent link between high *STAT1* and *STAT4* expression. The highest *STAT1* and *STAT4* expressions correlated relatively well with the highest *T-bet* (alias *TBX21*) expression, but the tissue of highest expression differed among the investigated teleosts and there was no clear correlation with *IFNG* expression (Table 1). Whether the lack of consistencies seen in Table 1 represent genuine differences between species or are due to random differences between fish individuals or between sampling techniques, can't be decided without further investigation. However, it is of note that, for example, in another study comparing among healthy trout and salmon individuals we also found considerable variation regarding the tissue of highest *IFNG* expression [166].

In the teleost fish turbot the highest expression of both *IL-17A/F* and *IL-22* was found in the intestine [170], which would agree with the findings in elephant shark shown in Table 1. However,

before concluding that the fish intestinal immune milieu—or part of it—tends to be i3-skewed, more research is needed, and the data of the teleost fish individuals summarized in Table 1 argue against it.

For signature genes of regulatory functions such as *FOXP3*, *IL-10* and *TGF*-β, we could not distinguish any notable expression patterns among healthy tissues of either elephant shark or teleost fish (Table 1).

Table 1. Expression levels of immune signature genes in various tissues determined by BLAST analysis against cartilaginous and teleost fish sequence read archive (SRA) datasets. Read numbers per 5×10^7 reads of various immune signature genes of cartilaginous and teleost fish species were determined by similarity searches against tissue-specific SRA datasets (from http://www.ncbi.nlm.nih.gov/Traces/sra/ (data not shown); see Table 1C) using the BLAST search function at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ORF sequences (see supplementary file 2) were subjected to "Megablast" analysis (blastn) using default software settings except that the "max target sequences" number was changed to 20,000 and that the "word size" number was changed to 64. To ensure specificity of the Megablast analysis, only matches with "score" values ≥128 for elephant shark, ≥251 for golden mahseer, ≥168 for northern pike and Atlantic salmon, and ≥169 for rainbow trout, were counted. Colored backgrounds highlight the tissues with the highest relative expression of the respective gene, and the red frames highlight the consistent high expression of *IL-4/13A* and *GATA-3* in teleost gills.

A. Cartilaginous fish

		Elephant Shark (*Callorhinchus milii*)			
		Gill	**Kidney**	**Spleen**	**Intestine**
T_H1-signature	T-bet	4	1	121	3
	STAT1	6006	701	5263	2168
	STAT4	726	131	3873	738
	IFNγ	17	1	78	6
T_H17-signature	IL17A/F1	1	0	0	7
	IL17A/F2	21	0	2	66
	IL-21L	0	0	3	2
	IL-22	4	0	0	15
T_{reg}-signature	Foxp3	10	1	51	3
	IL-10	7	4	185	13
	TGFβ1	72	36	66	3
T_H2-signature	GATA3	2287	92	575	95
	STAT6	290	41	199	102
	IL-4/13A	3	0	0	0
	IL-4/13B	8	0	0	1
	IL-4/13C	0	1	0	0
	IL-4/13D	0	0	0	0
	IL-5A	0	0	0	0
	IL-5B	3	0	0	0

Table 1. *Cont.*

B. Teleost fish (bony fish)

		Golden mahseer (*Tor putitora*)			Northern pike (*Esox lucius*)					Rainbow trout (*Oncorhynchus mykiss*)				Atlantic salmon (*Salmo salar*)			
		Gill	Kidney	Spleen	Gill	Kidney	Head Kidney	Spleen	Intestine	Gill	Kidney	Spleen	Intestine	Gill	Kidney	Spleen	Intestine
T_H1-signature	T-bet	4	47	54	91	114	663	899	28	12	20	22	11	134	320	1434	66
	STAT1_1	523	1174	42	2589	1555	3200	2579	930	2041	2219	5794	2422	5608	5386	10317	2530
	STAT1_2									1990	1870	3563	1878				
	STAT4_1	1525	1714	686	1866	1375	3702	2708	785	822	964	1122	679	1611	729	2273	1916
	STAT4_2									484	37	62	88				
	IFNγ_1	85	47	31	8	0	4	11	1	19	0	189	84	6	6	32	2
	IFNγ_2									17	0	211	99				
T_H17-signature	IL-17A/F1a	14	21	13	13	15	2	0	2	14	5	1	9	0	5	0	0
	IL-17A/F1b									1	6	0	4	5	11	0	0
	IL-17A/F2a	11	6	4	10	0	0	0	0	4	5	0	2	8	39	0	0
	IL-17A/F2b									3	0	0	2	1	0	0	2
	IL-17A/F3	6	10	6	75	7	7	4	1	3	0	2	0	2	1	2	1
	IL-21	8	28	19	53	35	26	14	123	1	0	0	6	0	0	0	0
	IL-22	2	4	1	15	2	0	5	2	13	0	11	5	15	5	7	4
T_{reg}-signature	Foxp3_1	11	12	9	100	62	257	138	32	61	11	28	35	230	143	666	149
	Foxp3_2									115	25	43	63				
	IL-10	32	179	130	23	127	111	109	8	0	2	7	1	1	2	2	0
	TGFβ1a	98	279	59	1374	891	1549	1330	270	177	659	888	211	66	122	125	7
	TGFβ1b									220	410	910	500				
T_H2-signature	GATA3	>20590	472	113	10801	409	365	202	141	2669	98	13	24	16724	219	334	84
	STAT6	460	870	17	2354	2183	2650	2781	875	872	827	1274	827	1719	1313	1688	788
	IL-4/13A	1147	56	36	25	7	4	3	4	29	9	5	5	112	42	33	15
	IL-4/13B1	13	3	2	3	0	1	0	2	6	0	1	1	31	7	8	14
	IL-4/13B2				1	6	5	3	8	5	2	2	7	7	3	0	7
	IL-5fam?A	29	13	9	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	IL-5fam?B	6	29	12	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

Table 1. *Cont.*

C. Accession numbers of the SRA datasets and their number of total reads

	Elephant shark (*Callorhinchus milii*)			
SRA dataset	SRX154852	SRX154856	SRX154860	SRX154855
Tissue	Gill	Kidney	Spleen	Intestine
No. of reads	71430454	118965654	83369382	147745918

	Golden mahseer(*Tor putitora*)			**Northern pike (*Esox lucius*)**					**Rainbow trout (*Oncorhynchus mykiss*)**				**Atlantic salmon (*Salmo salar*)**			
SRA dataset	SRX768559	SRX768561	SRX767362	SRX514237	SRX514263	SRX514240	SRX514270	SRX514238	ERX297522	ERX297511	ERX297523	ERX297509	SRX608399	SRX608574	SRX608599	SRX608567
Tissue	Gill	Kidney	Spleen	Gill	Kidney	Head Kidney	Spleen	Intestine	Gill	Kidney	Spleen	Intestine	Gill	Kidney	Spleen	Intestine
No. of reads	41751362	34023336	51857480	58499888	60694314	61054936	61731442	60466858	39064840	32103778	41714660	40271788	59793962	61054936	60203316	59806348

6. Evidence Supporting the Existence of T_H Cells in Fish

In this and the following chapters with "fish" we refer to teleost fish if not mentioned otherwise. Readers should realize though that the relatively little information available for sharks suggests that in essence they have immune systems similar to those found in other jawed vertebrates [147,171]. It is of note, however, that despite the overall similarities, there are also some aspects of the fish immune system that importantly differ from the mammalian situation, such as the poikilothermic conditions and the absences of lymph nodes, of mammalian-type haematopoietic bone marrow, and of antibody class switching [147,171]. It is also of note that the general pattern of basic similarity does not involve all jawed fish species, like for example gadoid fish do not have an MHC class II presentation system [172]. In the below we only try to summarize the (teleost) fish consensus situation.

Formally, helper T cell function in fish probably cannot be considered proven. However, multiple lines of evidence indicate that fish T_H cells similar to their human counterparts do exist. Fish have B cells and macrophages, which like antigen presenting cells in mammals express MHC class II molecules [173–176], and fish have T cells which express somatically rearranged TCR-α and -β genes that are expressed and selected in a clonal manner [177,178]. Furthermore, teleost fish have CD4 molecules with a motif for signaling capacity (CD4-1 and CD4-2; [179–182]), as well as sets of CD3 and signaling pathway molecules necessary for T cell function [183,184]. Fish CD4 and MHC class II molecules are expressed at high levels in the thymus in a similar tissue organization as in mammals [174,180,185–189], suggesting that, like in mammals, the fish thymus generates T_H cells that have been negatively selected against self-antigens. Early thymectomy results in a decreased antibody response against "T-cell dependent antigens" [190], and anti-hapten B cell responses were found to be supported by carrier-specific aid of non-B cells in hapten-carrier immunized fish [191–193]. More recently, adaptive transfers of CD4-positive (CD4-1 positive) lymphocyte fractions of immunized ginbuna crucian carp to syngeneic non-immunized individuals were found to aid antigen-specific antibody and cell-mediated cytotoxic responses *in vivo* [194]. In a zebrafish model support of CD4-1 positive cells to an antigen-specific immune reaction was suggested by their enhanced cytokine gene expression profiles after zebrafish booster immunization [195]. Many teleost lymphocytes that express CD4-1 also express CD4-2 [182,194–197], but within the detection ranges of the applied assays it appears that teleost lymphocytes can also be single-positive for only CD4-1 or CD4-2 [182,196–199]. It is not sure yet which, if any, of the fish CD4 molecules can confer mammalian-type CD4 function. Definite evidence for T_H functions in fish may need experiments involving immunizations with different combinations of haptens and carriers (to reduce the chance of misleading results because of nonspecific immune stimulation by the antigens), purification of CD4-1^+ and/or CD4-2^+ lymphocytes, and the ability to manipulate MHC class II-presentation or -matching by antigen presenting cells; since recently those experiments appear to be possible, but they haven't been done yet.

7. T_H1-like Responses in Teleost Fish

In this paragraph and the following ones, we try to summarize principle similarities between data published for i1-i2 axis functions in fish and in mammals, and we will for example not discuss alternative functions encoded by fish-specific paralogous genes. We also try as much as possible to only reference

those fish studies which allow straightforward conclusions in regard to polarization models, leaving out for example most studies that only investigated expression of i1 markers, or in which i1 markers were up-regulated together with markers of other types of immune responses as part of an inflammation reaction.

Important for the discussion of possible type 1 immunity in fish is that they appear to have perforin and granzyme containing *TCRαβ*$^{+}$CD8α^{+} T cells that can kill virus-infected cells in a specific manner, as well as "natural killer" cells that display less specificity for their cellular targets (reviewed by [200,201]. Our definition of fish NK (-like) cells refers to perforin and granzyme containing non-B non-T lymphocytes with cell-killing ability, although their surface markers may be substantially different from those of mammalian NK cells [145]. Type 1 immunity pivots around the cytokine interferon γ (IFNγ), and T-bet is the most important transcription factor. Fish *T-bet* and *IFNG* can be expressed by CD4-1 positive cells [195,202]. The involvement of fish IFNγ in self-amplifying loops, as known in mammals, was suggested by the observation that in flounder systems recombinantly expressed IFNγ was able to induce *IFNG* expression in whole kidney leukocytes and in a permanent cell line [203].

In trout systems, the expression of *IFNG* by splenic leukocytes and head kidney cells could be stimulated by recombinant IL-15 [204] and recombinant IL-12 [205], respectively. It was speculated that the trout *IFNG* induced by IL-15 was expressed by NK and/or CD8^{+} T cells [204], because in mammals these cell types are known to be particularly stimulated by IL-15 and to be able to release considerable amounts of IFNγ [77,95,206,207]. In its turn, recombinant trout IFNγ was found to enhance the expression of the i1 cytokine genes *IL-15* and *IL-12A* in trout macrophage and fibroblast cell lines [204] and in Atlantic salmon head kidney cells [208], respectively. Because under similar conditions some other trout or Atlantic salmon cytokines were not upregulated [204,205,208], this suggests that also in fish the IFNγ, IL-15 and IL-12 molecules cooperate in type 1 immunity. Moreover, fish *IFNG* was found upregulated by viral dsRNA mimic polyI:C, under conditions in which some other cytokine genes were not upregulated [202]. In mammals poly(I:C) is known to specifically stimulate type 1 immunity [209]. In zebrafish spleen and head kidney poly(I:C) was found to enhance transcription of *T-bet*, while in the same experiments *STAT6* appeared to be downregulated [130]. Direct correlations between fish IFNγ addition and a transcription factor were reported for STAT1, with Zou *et al*. [120] reporting upregulation of *STAT1* expression in a trout macrophage cell line, and Yabu *et al*. [210] reporting the induction of human STAT1 phosphorylation in a human cell line transfected for expression of ginbuna crucian carp IFNγ receptor chain. STAT4 has hardly been investigated in fish. In summary, possible existence in fish of a mammalian-like T$_{H}$1 transcriptional regulation network has not been clarified yet, but the available fragmentary data do agree with its existence.

A very important feature of type 1 immunity is that it suppresses the other types of immunity, and *vice versa*. Indirect indications for such suppression is that, similar as in mammals, expression of fish *IFNG* and *IL-4/13A* can be found up- or down-regulated in opposing manners [166,202]. Furthermore, under conditions in which recombinant trout IFNγ enhanced the expression of *IL-12A* in Atlantic salmon head kidney cells, the expression of some *IL-17A/F* family genes was slightly downregulated [208].

In conclusion, specific co-regulation of factors important for mammalian type 1 immunity suggests the existence of a basically similar i1-system in fish. However, because with the establishment of antibodies recognizing CD4 only recently it became more accessible to isolate (putative) fish helper T cells, available evidence supporting the existence of fish T$_{H}$1 cells is still very limited. Definite evidence of T$_{H}$1

cells will require the establishment of long-term proliferation assays for fish helper T cells and investigations of to which extent they can be polarized.

8. T_H17-Like Responses in Teleost Fish

Important for the possibility of type 3 immunity is that fish do have heterophilic-neutrophilic granulocytes (neutrophils). In mammals, IL-17A and IL-17F molecules induce the release of abundant cytokines and chemokines from leukocytes and other cell types, which amongst others attract neutrophils [211]. Mammalian neutrophils form an important and first line of defense against infiltrating bacteria [212]. Like their mammalian counterparts, fish neutrophils have granules with the enzyme peroxidase, they can phagocytose bacteria, they rapidly and extensively migrate to bacterial infection sites, and have high bactericidal and respiratory burst capacities [213–215]. Furthermore, like mammalian neutrophils, after their stimulation fish neutrophils can release DNA in the form of neutrophil extracellular traps (NETs) [216].

Fish have several genes of the *IL-17A + IL-17F* family, which are called *IL-17A/F* followed by a number [217]. Fish *IL-17A/F* genes can be expressed by CD4-1 positive cells [195,202]. To our knowledge there is only one study that investigated fish IL-17A/F at the protein level, namely in grass carp by Du *et al*. [218]. Du *et al*. found that recombinant grass carp IL-17A/F stimulates expression of the genes for the cytokines IL-1β, IL-6 and TNF-α and the chemokine CXCL-8 (alias IL-8) in head kidney cells. Other studies have shown that in fish CXCL-8, like in humans, can recruit neutrophils [219,220]. Hence, it is likely that fish IL-17A/F can induce neutrophil recruitment, although there is no direct evidence at the protein level for that in fish yet.

However, a study by Ribeiro *et al*. [221] provides supportive evidence at the gene expression level that also in fish IL-17A/F probably is involved in neutrophil recruitment. Ribeiro *et al*. [221] compared the infections in common carp of the related protozoan parasites *Trypanoplasma borreli* and *Trypanoplasma carassii*, which cause quite different patterns of disease development. At a time-point of infection in which *T. borreli* induced higher levels of *IFNγ* expression than induced by *T. carassii*, only *T. carassii* induced enhanced *IL-17A/F* expression, which was accompanied by a marked neutrophil infiltration into the spleen of only *T. carassii*-infected fish. Ribeiro *et al*., furthermore showed that factors derived from these parasites could *ex vivo* stimulate expression of *IL-23A* in head kidney leukocytes from parasite-infected fish, and that these same factors could efficiently interact with carp toll-like receptor 2 (TLR2) molecules expressed on a human cell line [221]. Hence, for their *T. carassii*-infected carp model, the authors postulated a "T_H17-like immune response" model involving the stimulation cascade *T. carassii* -TLR2-IL23-T_H17-IL17A/F-neutrophil. We are not aware of other studies in fish that suggest a correlation between IL-23 and IL-17A/F expression.

In Atlantic salmon head kidney cells, under conditions in which recombinant trout IL-1β induced large increases in expression of the pro-inflammatory cytokine genes *IL-1B* and *TNFA*, the expression of *IL-17A/F* genes remained unchanged [208]. In the same study recombinant trout IFNγ stimulated the expression of i1-signature gene *IL-12A*, while slightly reducing the expression of *IL-17A/F* genes [208]. In contrast, in this type of experiments, recombinant trout IL-21 was found to enhance expression of *IL-17A/F* genes [208], as well as of *IL-22* [222], suggesting specific involvement of IL-21 in T_H17 polarization as known in mammals.

An antibacterial function of fish IL-22 was indicated by *IL-22* upregulation induced in several fish species through bacterial agents, by the stimulation through recombinant IL-22 of antimicrobial peptide synthesis in trout and mullet, by the protection of mullet and turbot against bacterial challenge after injection with recombinant IL-22, and by a decreased resistance against bacterial challenge after *IL-22* knockdown in zebrafish embryos [170,223–226]. In zebrafish *IL-22* can be expressed by CD4-1 positive cells [195], and at the tissue level there are a few studies suggesting some correlation between fish *IL-22* and *IL-17A/F* expression ([170]; Table 1).

In our opinion, there are no convincing reports in fish yet linking expression of T_H17 signature cytokine genes to *RORC* expression.

In summary, from the available evidence it seems likely that also in fish the molecules IL-17A/F, IL-21 and IL-22 (and possibly IL-23) can be orchestrated in an anti-bacterial defense response that involves recruitment of neutrophils by IL-17A/F-induced chemokine expression. But it is unclear whether this response also involves RORγ(t) and/or TGF-β molecules, and/or the T_H17-like polarization of helper T cells. We expect that research of fish T_H cell polarizations will first concentrate on the possibility of i1-i2 axis end polarizations, T_H1 and T_H2, because in mammals these are so pronounced and well-defined, before possible T_H17 polarization will be convincingly addressed.

9. T_{reg}-like Responses in Teleost Fish

Studies have shown that if fish are fed or otherwise treated with antigen preparations that lack PAMPS or DAMPS, their immune system can develop some level of tolerance against these antigens (e.g., [227,228]). In mammals immune tolerance is importantly mediated by natural or induced T_{reg} cells, for which FOXP3 is a master transcription factor and which are immunosuppressive by a variety of means, including the release of the cytokines IL-10 and TGF-β [229,230].

Fish *FOXP3*, *TGFB1* and *IL-10* genes can be expressed by CD4-1 positive cells [202]. Wen *et al*. [231] showed that an unknown percentage of the Tetraodon lymphocyte population positive for CD4-2 and IL-15Rα molecules also expressed *TCRα* and *FOXP3*. Fish IL-15Rα is a receptor chain that can bind IL-2 as well as IL-15 and, although it looks like mammalian IL-15Rα, it corresponds to the evolutionary precursor form of both mammalian IL-2Rα and IL-15Rα [161,231]. Under non-stimulated conditions, in mammals, T_{reg} cells are the cell type expressing the highest amount of IL-2Rα and are the most sensitive to IL-2 [232,233]. The fact that Wen *et al*. [231] didn't detect *FOXP3* expression in the CD4-2^+IL-15Rα^- cells suggests that also in fish IL-2 may have an important function in a negative feedback loop of immune reactions through activation of T_{reg} (-like) cells [161,234]. Wen *et al*. [231] reported immunosuppressive functions of Tetraodon CD4-2^+IL-15Rα^+ cells observed during *in vitro* and *in vivo* experiments. The *in vitro* experiments showed that if CD4-2^+IL-15Rα^+ cells were depleted from Tetraodon spleen and head kidney leukocytes, the remaining cell population became more effective in executing non-specific cell-mediated cytotoxicity and in inducing mixed lymphocyte reactions in the respective assays [231]. Repeated *in vivo* treatment of Tetraodon with rabbit antibodies binding to Tetraodon IL-15Rα resulted in bowel inflammation [231], which the authors interpreted as deriving from a depletion of T_{reg} cells, a model for which they provided some evidence but which may need more investigation.

Quintana *et al*. [235] found that zebrafish *FOXP3* (*FOXP3a*) was expressed by lymphocytes, and that in zebrafish embryos overexpression or knockdown of *FOXP3* resulted in decreased *vs*. increased

IL-17A/F expression, respectively [235]; this perfectly agrees with FOXP3 involvement in immunosuppressive functions as known in mammals. Quintana *et al*. [235] also showed that zebrafish FOXP3 retained its capacity to induce T_{reg}-like features upon expression in mammalian cells, because murine T cells transfected with zebrafish *FOXP3* were found to suppress activation of other murine T cells.

In goldfish monocytes recombinant IL-10 suppressed the immune response induced by bacterial agents as indicated by reduced increase of expression of genes for TNF-α, IL-10, CXCL-8, IFNγ and several NADPH oxidase subunits, as well as by a reduced increase in production of reactive oxygen intermediates [236]. Similar results were found for carp, where recombinant IL-10 was shown to reduce the expression increase induced by LPS in neutrophils and/or macrophages of genes for TNF-α, IL-1β, IL-6, IL-12A, MHC class I, and MHC class II molecules [237]. Piazzon *et al*. [237] also found that carp IL-10 could induce STAT3 phosphorylation, implying similar signaling cascades as in mammals. It is of note that Piazzon *et al*. [237] also revealed that carp IL-10 does not only have immunosuppressive functions, but, like known for multifunctional mammalian IL-10, also has some stimulatory and modifying effects on the immune system, like the stimulation of proliferation, differentiation, and antibody secretion by IgM^+ B cells.

In regard to TGF-β, it is difficult to distinguish clear regulation patterns from the number of studies that investigated fish *TGFB1* expression. We therefore decided not to try to summarize those studies. However, it is important that an immunosuppressive function was found for recombinant goldfish TGF-β, as it was shown to down-regulate the nitric oxide response of TNF- α-activated macrophages [238].

In summary, FOXP3 in fish has been associated with immunosuppressive functions, and, at least in Tetraodon, FOXP3 is expressed by CD4 positive T cells that constitutively express high levels of IL-2 receptor chain. This suggests the existence of a T_{reg} system in fish similar to that found in mammals. Both fish IL-10 and TGF-β were found to have immunosuppressive functions. It will need further investigation whether in fish, as known in mammals, IL-10 and TGF-β are associated with FOXP3 positive cells.

10. T_H2-like Responses in Teleost Fish

For the possibility of raising T_H2-like responses, it is relevant that fish do have granulocytes other than neutrophils which have anti-parasite functions. Whether (some of) these cells can be called eosinophils, basophils and/or mast cells depends on the chosen definition and on the fish species. Our use of the terms eosinophils and mast cells in the text below is based on the definitions in the indicated references. Similar to mammalian eosinophils, fish eosinophils express transcription factor GATA-2, and can migrate to sites of parasite infection and release their peroxidase containing granules upon stimulation by parasite agents [239,240]. Fish mast cells, which are abundant in the gill and the intestine, can also accumulate and degranulate at the site of parasitic infection [239–243]. Similar but not identical to mammals, the granules of fish mast cells contain phosphatases, peroxidase, proteolytic enzymes, arylsulfatase, 5'-nucleotidase, lysozyme, antimicrobial peptides, and, depending on the species, they can contain serotonin or histamine [241–244].

Fish *GATA-3*, *IL-4/13-A* and *-B* genes can be expressed by CD4-1 positive cells [195,199,202]. Chettri *et al*., found that if rainbow trout skin was infected with the parasitic flagellate *Ichthyobodo necator*, locally there was substantial increase in *GATA-3* but not of *FOXP3* or *T-bet* expression, concomitant with a substantial decrease in the number of $CD8\alpha^+$ cells and a substantial increase in IgM^+

B cells [245]. This might represent a T_H2 response, as suggested by the authors, although all investigated cytokine genes including *IFNG* were found upregulated. More convincing of a T_H2 response are conditions in which *IL-4/13A* expression increases while *IFNG* expression decreases, as could be found in experiments analyzing (cells of) trout gill [166,246]. Very interestingly, because it suggests that fish T_H2 responses are involved in anti-parasite immunity, the infection of salmon with the parasite *Paramoeba perurans* enhanced the expression in infected gill of T_H2 signature genes *IL-4/13A* and *IL-4/13B* while the expression of signature genes for T_H1, T_H17 and T_{reg} like *IFNG*, *IL-17A/F*, *TGFB1* and *IL-10* were downregulated [246]. An opposite regulation of T_H1 and T_H2 signature genes was also found by Zhu *et al*. [247] who showed that injection into zebrafish of recombinant IL-4/13A resulted in an increase in expression of *GATA-3* and *STAT6* in the spleen, while concomitantly the expressions of *T-bet* and *IFNG* were decreased; curiously, the authors did not check the effect on *IL-4/13-A* or *-B* expression. Regarding opposite regulations an important observation is also that injection into zebrafish of recombinant zebrafish "IL-4" (probably IL-4/13A) induced expression of CD209 in blood leukocytes, while addition of LPS to the IL-4 preparation caused a reduction in the CD209 increase [248]. For carp we established a clonal (semi-) permanent *CD4-1*$^+$*TCR*$\alpha\beta^+$ T cell line that expresses readily detectable amounts of *GATA-3* but not of *T-bet*, thus has a T_H2-like profile in regard to its transcription factors [199]. In agreement with T_H2-like polarization, this cell line lost the ability to increase its *IFNG* expression after suitable stimulation while it retained an ability for upregulation of *IL-4/13B*; curiously, we were unable to increase *IL-4/13A* expression in this cell line [199]. It remains to be determined whether the carp cell-line phenomenon represents an artefact introduced by prolonged *in vitro* culture, or that not all fish T_H2-like cells can make significant amounts of IL-4/13A.

An important finding by Zhu *et al*. [247] analyzing recombinant zebrafish IL-4/13A was that the cytokine can bind to receptor chain IL-4Rα. The same study also provided evidence that zebrafish IgM^+ B cells specifically express IL-4/13Rα and that they can be stimulated by recombinant zebrafish IL-4/13A, although the extent of the specificity was not investigated [247]. In mammals the stimulation of B cell activity is not restricted to i2-skewed conditions, but mammalian IL-4 is one of the molecules that can efficiently stimulate B cell proliferation and the molecule was originally named "B cell growth factor" [249]. Thus although the B cell stimulation by zebrafish IL-4/13A does not provide direct evidence of a T_H2 function, it does provide additional evidence that IL-4/13 functions in fish are similar to those of their mammalian counterparts.

In summary, there is accumulating evidence that in fish the expressions of *GATA-3* and *IL-4/13A* are correlated, and that their expression suppresses the expression of T_H1 signature genes. Although the regulation mechanisms in fish have not been elucidated yet, it seems likely that fish T cells can polarize into a T_H2 phenotype by mechanisms similar to those in mammals. Fish IL-4/13A has been shown to stimulate B cells, but it still needs to be investigated whether fish IL-4/13A can stimulate typical i2 functions such as anti-parasite activities of eosinophils and mast cells.

11. M1-like *vs*. M2-Like Macrophage Polarizations in Fish

Like mammals, fish have macrophages with potent phagocytic and bactericidal abilities that make use of reactive oxygen and nitrogen intermediates (reviewed by [250]). Also, like in mammals, zebrafish macrophages are found in healing wounds [251–253] and are important for normal tissue regeneration [254].

The involvement in both M1-like and M2-like functions opens the possibility of differential polarization towards those functions. As listed below, there is some evidence that fish macrophages can polarize towards M1- or M2-like phenotypes through similar pathways as known in mammals. The best review on that has probably been published by Forlenza *et al*. [255], who importantly realized that also in studies on fish M2 macrophages it is necessary to conceptually distinguish between M2a (alias "alternatively activated") and M2c (alias "deactivated", or, as Forlenza *et al*., not unreasonably call it, "regulatory") polarizations. For lack of solid polarization data, Forlenza *et al*. [255] developed a working definition, based on how fish macrophages were stimulated, to divide them into four "polarization states", akin to as how this has been accepted by some researchers studying mammalian macrophages (e.g., [92]) as a simplification of the classification system by Mantovani *et al*. [60]. Forlenza *et al*. [255] defined the above mentioned M2a and M2c polarizations as those deriving from stimulation with IL-4/13 and from stimulation with microbial agent + IL-10, respectively. At the far i1-end of the polarization spectrum, Forlenza *et al*. [255] conventionally considered macrophages stimulated by both microbial agents plus IFNγ as "classically activated" (M1). The definition by Forlenza *et al*., which may not hold in the long term, but which is practically convenient and seems to define macrophages only somewhat shifted towards the i1-end (not unlike the mammalian M2b macrophages; [60,92]), concerns "innate activated macrophages" ("iaM") which are stimulated with only microbial agents and not with IFNγ. Similar to mammalian M1 and M2b, fish M1 and iaM express iNOS (reviewed by [255]).

Although not in all cases studied as one among more possible polarizations, there is evidence that M1 phenotypes can be induced by similar agents as in mammals. In synergy with LPS, carp IFNγ was found to stimulate carp macrophages into expressing higher levels of *IL-12A* and *TNFA* [256]. Furthermore, recombinant rainbow trout IFNγ plus some LPS enhanced respiratory burst activity of rainbow trout macrophages [120], and recombinant goldfish IFNγ, said to be without LPS contamination, primed goldfish monocytes/macrophages for enhanced respiratory burst, phagocytic and nitric oxide responses, while it also stimulated their expression of genes for TNF-α, IL-1β, IL-12α, IL-12β and iNOS [257,258]. Likewise in agreement with M1 differentiation as known in mammals, recombinant goldfish TNF-α could also prime goldfish monocytes/macrophages for enhanced respiratory burst, phagocytic and nitric oxide responses [257,259].

There appears to be little direct evidence for the existence of M2c ("M_{reg}") polarization of fish macrophages, but recombinant IL-10 or TGF-β were found able to reduce M1-type macrophage activations [236,238].

In carp differential polarizations of macrophages using LPS *vs*. cAMP stimulation have been shown [260]: while neither LPS nor cAMP stimulated *IL-10* expression and both stimulated *IL-1B* expression, only LPS stimulated *NOS2* (the gene for iNOS) and only cAMP stimulated *ARG2* (the gene for arginase 2) expression. However, although in mammals cAMP is one of the factors contributing to an i2 environment [21], we are somewhat hesitant to accept the isolated addition of cAMP as an inducer of a natural M2 (-like) polarization. We would also like more research to be done before concluding that in fish arginase 2 and not arginase 1 is a major marker for M2 differentiation (for a discussion on fish arginase genes see [260] and [255]).

One of the tasks of macrophages is the removal of cell debris, and in mammals apoptotic bodies are known to stimulate an M2 phenotype [261,262]. Zymosan is a glucan-rich particle prepared of the surface of fungi, which in mammals can induce pro-inflammatory responses and in synergy with other

factors can stimulate M1 polarization [263,264]. *In vitro* analysis of goldfish macrophages showed that their respiratory burst activity induced by PMA treatment was enhanced by incubation with zymosan and reduced by incubation with apoptotic bodies [265]. Injection of zymosan or apoptotic bodies in the goldfish peritoneal cavity, followed by isolation of myeloid cells and analysis of their ability to generate respiratory burst responses, showed that the *in vivo* treatment had a similar effect on priming for respiratory burst activity as the above described *in vitro* treatments [265].

An interesting study was recently published by Nguyen-Chi *et al*. [253], who used a double fluorescent labeling system for zebrafish macrophages (using the *MPEG1* promoter) and TNF-α expression (using the *TNFA* promoter). They found at the population level that *TNFA* expression by zebrafish macrophages positively correlated with their expression of *IL-1B* and *IL-6*, and negatively correlated with their expression of *TGFB1* and *CXCR4*. In mammals both *TGFB1* and *CXCR4* have been used as markers for M2 macrophages [266], and Nguyen-Chi designated the *TNFA*-high macrophages as "M1-like" and the *TNFA*-low macrophages as "M2-like". Nguyen-Chi *et al*. [253] also found that in wounded fin of zebrafish larvae the *TNFA*-high macrophages tended to display a flattened and lobulated morphology, whereas the *TNFA*-low macrophages tended to be elongated and dendritic. Other than creating a wound by fin amputation, Nguyen-Chi *et al*. [253] also inoculated zebrafish larvae with *E. coli*. Based on the abundances of the different macrophages at the relative sites in these experiments, Nguyen-Chi *et al*. [253] concluded that zebrafish M1-like macrophages are important in anti-bacterial combat and initial stages of wound healing and that M2-like macrophages are important in especially the later stages of wound healing. Nguyen-Chi *et al*. [253] furthermore showed that in later stages of wound healing the *TNFA*-positive (M1-like) macrophages changed towards a phenotype which they call intermediate to M1 and M2 and which shows high *TGFB1* expression besides lowered *TNFA* expression.

In summary, fish macrophages can be stimulated towards several phenotypes, and at least the M1 phenotype seems to be defined by similar pathways and characteristics as in mammals. The fish macrophage non-M1 polarizations are not well characterized and appear to be predominantly defined by reduced M1 features and maybe by the upregulation of *ARG2*. Hopefully this gap in knowledge on possible non-M1 polarizations can be closed by future inclusion of recombinant fish IL-4/13 cytokines in the macrophage polarization assays.

12. I2-Skewed Tissue Milieus in Healthy Mammals and Fish

For fish this paragraph has an overlap with paragraph 5, but we nevertheless like to dedicate a special paragraph to the comparison between fish and mammals.

Previously we reported that trout and salmon gill and skin appear to have i2-skewed milieus since we observed rather consistent high ratios of *IL-4/13A* plus *GATA3 vs*. *IFNG* expression [166]. We found similar high ratios for the thymus of trout, salmon and mouse, but it is discussable whether a primary immune organ with its unique immune functions can be classified as "i2-skewed" [166]; on the other hand, it was found in mammals that recent thymic emigrants have a bias towards T_H2 polarization [267], thus at least in some sense the thymus can be seen as "i2-skewed". High levels of *IL-4/13A* and/or *GATA3* in fish gill were also found by others [121,167,168], and we are glad that in the present paper we could additionally confirm these findings by SRA dataset analysis for several teleost fish and also for elephant

shark (see paragraph 5). We speculate, as before [166], that i2-skewage of the fish gill helps to protect it against parasites, but also against possible i1- or i3- type inflammation that might harm this delicate tissue. There are some data indeed that indicate that it is hard to induce an i1-response in fish gill [166,268], but other studies suggest that it is possible to induce i1- or i3- responses in this tissue (e.g., [269]); more research will be needed to clarify the degree of i2-skewage of fish gills.

Our idea that a sensitive fish tissue like gills may be i2-skewed for its protection from other types of immune reactions actually derives from similar claims made in mammals for the immunity of pregnancies [270–273] and neonates [33,34,274]. Whether these claims for mammals are actually true, however, has, at least in a general sense, been disputed [275], and precise locations, conditions and measured parameters should probably be acknowledged.

We did some preliminary analysis of tissue-specific transcriptomes for mammals available in public databases to assess the expression levels of i1-i2 axis marker genes, similar to the method followed to make Table 1, but could not distinguish notable expression patterns (data not shown). However, in mammals pronounced tissue-specific distinctions were observed by others who investigated the i1-i2 axis positioning of individual cell types. It is intriguing, for example, that IL-13 secreting ILC2 cells can readily be found in the mouse lung and skin, while these tissues have hardly any ILC3 cells [276]; in a way this is reminiscent of the i2-skewage observed in fish skin and respiratory tissue (gill). For the mouse healthy intestine an opposite ratio is found, with abundant ILC3 cells and relatively few ILC2 cells [276]; this agrees well with the facts that also in fish this mucosal tissue is not i2-skewed, and that in some fish including elephant shark the intestine may be i3-skewed.

Overall, however, it is surprising to us how little work there seems to have been done in mammals to analyze non-diseased tissues for their immune biases along the i1-i2 axis. It seems to us that this should be important information when considering how and where to administer vaccines or therapeutic agents.

13. I2-Skewed Tissue Milieus of Tumors in Mammals and Fish

The only mammalian "tissues" for which i2-skewage has been intensively studied and generally accepted as proven are a variety of tumors. The i2-skewage protects the tumors from eradication by type 1 immunity, and immunotherapy shifting the tumor immune milieu towards type 1 immunity has been shown helpful in fighting the cancer. The champion results so far are obtained by antibodies that can block the immunosuppressive functions of the molecules PD-1 and CTLA-4, but many other methods to induce a shift towards i1-immunity are being tried [116,117,277].

It is important to realize that in tumor studies the term "type 2 immunity" tends to be used for a combination of immunosuppressive ("T_{reg}-type") and i2 inflammation ("T_H2-type") conditions, and that among these the immunosuppressive conditions probably are more pronounced. The difficulty with changing this immune milieu by therapy is that the cancer cells and the i2-skewed immune cells reciprocally attract/support each other, so that effects of therapeutic i1-stimuli tend to be undone once the stimuli fade out after administration.

An example of a situation in which tumor cells and infiltrating immune cells support each other is found in human and rodent pancreatic ductal adenocarcinoma (PDA). In rodent models, expression of mutant *KRAS* oncogene in pancreatic ductal epithelial cells is sufficient to induce their cancerous proliferation and their expression of factors like STAT3, NF-κB and IL-6 that form part of a (self-)

amplifying loop [278–280]. These tumor cells release abundant amounts of chemokine CCL2 which attracts monocytes that within the tumor stroma develop into M2 macrophages [281,282]. The tumor is also invaded by abundant lymphocytes, including many T_H2 and T_{reg} cells, while few are T_H1 or $CD8^+$ T cells [282–284]. Despite abundant infiltration with immune cells, in a rat PDA model the expressions of the i1-markers *CD8B*, *IL-15* and *granzyme-C* were found to be 2-, 5- and 5-fold lower than in healthy rat pancreas [285]. Meanwhile, in these rat PDA samples, the expressions of i2 markers *TGFB1* and *IL-33* were found to be 14- and 18-fold higher compared to healthy rat pancreas [285]. Experiments in a mouse PDA model have shown that the cancer cells and M2 macrophages reciprocally support and stimulate each other [286].

Unfortunately in fish the immunology of tumor microenvironments has hardly been studied yet, but there is a nice study by Yan *et al*. [287] which indicate that also in fish the progress of tumors can be enhanced by i2 conditions. Yan *et al*. [287] found that when they induced mutant KRAS expression in zebrafish hepatocytes, this resulted in rapid recruitment of (fluorescently labeled) neutrophils to the liver area and in hepatocarcinogenesis. The experimental results of independent knockdowns of *GCSFR* and *IRF8* let the authors conclude that the infiltrating neutrophils enhanced carcinoma growth. By using specific stimulators or inhibitors of neutrophils, they concluded that the neutrophils stimulated proliferation of the mutant KRAS expressing hepatocytes, while reducing their apoptosis. They found that the nucleus morphology of the infiltrating zebrafish neutrophils resembled that of tumor associated neutrophils in mammals and that they displayed a modified cytokine gene expression profile, which they speculated, based on high *IL-1B* expression, to support angiogenesis. Yan and co-workers [287] also found that the mutant KRAS expressing hepatocytes expressed increased amounts of *TGFB1* (*TGFB1a*), and that blocking of TGF-β pathways reduced both the number of neutrophils and carcinoma growth, and changed the cytokine gene expression pattern of the tumor-associated neutrophils. While some important questions still remain to be answered in the zebrafish model provided by Yan *et al*. [287], their study allows the important conclusion that also in fish tumor development can be supported by TGF-β expression and by infiltration of immune cells that adapt their phenotypes under influence of TGF-β.

In summary, considering the enormous medical importance of the immune milieus of tumors, it is surprising how little this matter has been studied in fish. However, the first results appear to confirm that like in mammals, tumor growth in fish can be stimulated by i2 cytokines and by interaction with leukocytes. We expect that soon many more studies on the immune milieus of tumors in fish will be performed.

14. Conclusions and Future Prospects

In the present study we have tried to summarize the i1-i2 axis and its effect on leukocyte polarizations in mammals in a model (Figure 1) that reflects our interpretation of literature consensus. Although not unique, the difference from most existing models is the stressing of the continuity between the different conditions and their associated leukocyte polarizations, in combination with the placement of the i3 and "T_{reg}-type" conditions/polarizations between the i1 and "T_H2-type" conditions/polarizations. We feel we need this type of model to be able to compare between polarizations of different cell types and beyond species borders, and that at the very least our model is a good starting point for discussion. The Figure 1 model automatically leads to the question whether it wouldn't be better if the term "type 2 immunity"

would be split up into T_{reg}-type ("regulatory immunity" alias "i-reg"?) and a narrower definition of type 2 immunity (T_H2-type). However, such change of nomenclature would need a thorough discussion on how entangled T_{reg}-type and T_H2-type conditions are, and whether the change of nomenclature would reduce or increase the confusion.

The strongest evidence that the fish immune system obliges to similar i1-i2 axis principles as known in mammals comes from the remarkable conservation of many of the most important gene loci. But beyond that, recent years have also seen an accumulation of functional data that support that fish leukocytes respond to i1-i2 axis factors in a similar way as they do in mammals. Although these functional data are still fragmentary, when considered together they are rather convincing. In future research of the i1-i2 axis regulation of the fish immune system it hopefully will become more commonplace to simultaneously investigate multiple possible polarizations, so that they can be compared directly. Furthermore, it should be attempted to maintain long-term T cell cultures under polarizing conditions, followed by analysis of epigenetic modifications and of the stability of the polarized phenotype. In the short term we expect the biggest breakthroughs from the research of fish macrophage polarizations, because a lot of good work has been done already. For research of the tumor microenvironment (transparent) fish appear to be a great model, and like in mammals, it can be expected that a lot of i1-i2 research in fish will be dedicated to tumor tissues.

Besides the general discussion on the evolution of the i1-i2 axis, very interesting points of the present study are the findings that throughout bony as well as cartilaginous fish the gills appear to be i2-skewed, and that with analysis of the spotted gar genome sequence a canonical type T_H2 locus was found in bony fish that harbors *RAD50* as well as genes of both *IL-4/13* and *IL-3/IL-5/GM-CSF* families. Future functional research should help to clarify the identity of the IL-5fam? sequences in teleost fish.

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Author Contributions

Takuya Yamaguchi and Fumio Takizawa analyzed transcriptome databases. Johannes M. Dijkstra wrote the manuscript scaffold. Takuya Yamaguchi, Fumio Takizawa, Uwe Fischer and Johannes M. Dijkstra all investigated literature on the fish immune system and together wrote the current form of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Review

Antiviral functions of CD8$^+$ cytotoxic T cells in teleost fish

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ABSTRACT

Cytotoxic T-cells (CTLs) play a pivotal role in eliminating viruses in mammalian adaptive immune system. Many recent studies on T-cell immunity of fish have suggested that teleost CTLs are also important for antiviral immunity. Cellular functional studies using clonal ginbuan crucian carp and rainbow trout have provided *in vivo* and *in vitro* evidence that in many respects, virus-specific CTLs of fish have functions similar to those of mammalian CTLs. In addition, mRNA expression profiles of CTL-related molecules, such as CD8, TCR and MHC class I, have shown that in a wide range of fish species, CTLs are involved in antiviral adaptive immunity. These findings are a basis to formulate possible vaccination strategies to trigger effective antiviral CTL responses in teleost fish. This review describes recent advances in our understanding of antiviral CTL functions in teleost fish and discusses vaccination strategies for efficiently inducing CTL activities.

Contents

1. Introduction

Because viruses cannot replicate by themselves, they reproduce by invading viable cells of the host and hijack their cellular machinery, which forces these host cells to express and assemble viral proteins. Thus, one of the immune mechanisms for preventing further viral spread in the host is eliminating these virus-infected cells by cytotoxic cells. The major cytotoxic cells are natural killer (NK) cells and cytotoxic T-lymphocytes (CTLs). While NK cells can spontaneously recognise altered target cells, including virus-infected cells (recently reviewed by Fischer et al., 2013), CTLs need to be recruited through an adaptive immune response until sufficient numbers are available to effectively eliminate virus-infected cells (Williams and Bevan, 2007). These generated CTLs remarkably contribute to eliminating viruses in many infections. Thus, in order to mount an efficient immune response, CTL memory induction is crucial for effective vaccination.

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Table 1
The studies on generation of virus-specific CTL in fish.

Fish species	Virus	Immunogen	Refs.
Induction of virus-specific CTL activity			
Ginbuna crucaian carp	CHNV (rhabdovirus)	Live virus	Somamoto et al. (2002, 2006, 2009, 2013)
	CHNV (rhabdovirus)	Folmaline inactivated virus	Sato and Okamoto (2010)
	EVA (rhabdovirus), IPNV (birnavirus)	Infected syngeneic cells	Somamoto et al. (2000)
Rainbow trout	IHNV (rhabdovirus)	DNA vaccine	Fischer et al. (2006)
	VHSV (rhabdovirus)	Live virus	Utke et al. (2007)
	VHSV (rhabdovirus)	DNA vaccine	Utke et al. (2008)
Grouper	NNV (nodavirus)	Live virus	Chang et al. (2011)
CD8-positive cells bind MHC class I with viral peptide			
Grass Carp	GCHV (reoviruses)	Live attenuated virus	Chen et al. (2010)
Reguration of CD8 mRNA expression			
Ginbuna crucian carp	CHNV (rhabdovirus)	Live virus	Somamoto et al. (2006)
Common carp	KHV (herpesvirus)	Live virus	Rakus et al. (2012)
	SVCV (rhabdovirus)	Live virus	Forlenza et al. (2008)
Atlantic halibut	Nodavirus	Live virus	Patel et al. (2008), Øvergård et al. (2012, 2013)
Flounder	VHSV (rhabdovirus)	Live virus	Avunje et al. (2012), Kato et al. (2013)
Rainbow trout	VHSV (rhabdovirus)	Live attenuated virus	Adelmann et al. (2008)
	VHSV (rhabdovirus)	Live virus	Utke et al. (2007)
	VHSV (rhabdovirus)	DNA vaccine	Utke et al. (2008)
Atlantic salmon	SAV (alphavirus)	Live virus	Grove et al. (2013), Xu et al. (2012)
	ASRV (reovirus)	Live virus	Mikalsen et al. (2012)
	IPNV (birnavirus)	Live virus	Maisey et al. (2011), Ingerslev et al. (2009)
	ISAV (Isavirus)	Live virus	Hetland et al. (2010, 2011)
Grouper	NNV (nodavirus)	Live virus	Chang et al. (2011)

CTLs recognise specific peptides that bind to self-major histocompatibility complex (MHC) molecules, which is a fundamental feature of antigen recognition by CTLs. To establish assays for CTL activity, this unique characteristic of antigen recognition by CTLs requires preparation of syngeneic or autologous target cells. Assay systems for virus-specific CTLs that used clonal fish and syngeneic cell lines have been established for rainbow trout and ginbuna crucian carp (Nakanishi and Okamoto, 1999; Nakanishi et al., 2011; Fischer et al., 2013). Furthermore, CTL-related molecules, such as MHC class I, CD8 and T-cell receptor (TCR), have been identified in many fish species (Laing and Hansen, 2011). These studies demonstrated that fish possess cytotoxic cells that are functionally equivalent to their mammalian homologues. However, there remain considerable gaps in our understanding of their role in controlling viral infections. Two recent reviews (Nakanishi et al., 2011; Fischer et al., 2013) discussed the current knowledge on NK cells and CTL activities against various target cells in teleost fish (allogenic, xenogenic and infected). In this review, we will focus on virus-specific CTL functions and discuss these functions within the framework of antiviral vaccination strategies.

2. Characterisation of virus-specific CTLs in teleost fish

Historically, most research on antiviral responses in teleost fish focused on humoral immunity (Kaattari and Piganelli, 1996) because, until the late 1990s, there were no assay systems available for studying antiviral cell-mediated immunity. However, during the last 15 years, studies on specific cell-mediated cytotoxicity (CMC) in fish have remarkably advanced because of increased information at the molecular level and by establishing cell function assays using clonal fish (Nakanishi et al., 2011; Fischer et al., 2013). Here we describe the progress regarding virus-specific CTLs in teleost fish (the primary achievements are summarised in Table 1).

2.1. Virus-specific CTL activity in fish

Because this review describes our current understanding of antiviral CMC responses, we must mention one of the pioneers, Dr. Bill Clem and his group at the University of Mississippi Medical Center, Jackson, MS, USA, in the study of CMC in teleosts. Dr. Clem's group was the first to demonstrate the antiviral CMC in a channel catfish model (*Ictalurus punctatus*) (Hogan et al., 1996). They reported that catfish leukocytes could spontaneously kill channel catfish virus (CCV)-infected autologous and allogeneic cells. However, because of a lack of clonal catfish strains and infectable MHC class I-matched cell lines, they were unable to demonstrate anti-viral CTL responses.

This situation changed when experimental systems using clonal fish and infectable MHC class I-matched cell lines became available for the ginbuna crucian carp (*Carassius auratus langsdorfii*) and rainbow trout (*Oncorhynchus mykiss*). Syngeneic cell lines derived from naturally occurring clonal ginbuna crucian carp were shown to be useful as MHC class I-matched target cells for anti-viral CTLs. In these studies, CTLs were induced in these clonal fish by the crucian carp haematopoietic necrosis virus (CHNV) infection, and target cells were infected *in vitro* (Hasegawa et al., 1997a,b; Somamoto et al., 2000). Although nonsensitised leukocytes from naïve ginbuna carp could not kill CHNV-infected cells, cells isolated from fish that were sensitised by a sublethal virus infection displayed a killing activity against the infected target cells (Somamoto et al., 2002, 2006). Similar to CTL activities in mammals, this killing activity was induced only against the infected syngeneic cells but not against infected allogeneic ones. In addition, the secondary CMC response was stronger than the primary response, which implied the presence of memory CTLs in the teleost fish.

Viral antigen-driven expansion of ginbuna lymphocytes was demonstrated not only *in vivo* but also *in vitro* using co-cultures with putative antigen presenting cells (Somamoto et al., 2009). The effector cells generated *in vitro* expressed TCRβ and CD8α mRNA and suggested that CHNV-specific CTLs expanded in a manner that is similar to their mammalian counterparts. A recent study using an anti-ginbuna CD8α-monoclonal antibody demonstrated that $CD8\alpha^+$ cells were anti-viral cytotoxic cells, which further supported the theory that there were virus-specific CTLs in teleosts (Somamoto et al., 2013). As previously shown in a study on alloantigen-specific CTLs of ginbuna carp (Toda et al., 2011a), $CD8\alpha^+$ lymphocytes kill virus-infected targets by utilising perforin

and granzyme. In addition to these studies, adoptive transfer of sensitised cytotoxic effectors, including CD8α+ CTLs, to naïve syngenic recipients provided protection against CHNV infection, suggesting that fish CTLs play an important role in antiviral immune responses (Somamoto et al., 2002, 2013).

Although studies on CMC against CHNV have considerably contributed to our understanding of fish CTL functions, infections with this virus do not pose a threat to the global cyprinid aquaculture like the *Office International des Epizooties* (OIE)-listed diseases, spring viraemia of carp (SVC) and Koi herpes virus (KHV) infection. However, CHNV infection of ginbuna crucian carp could be an excellent model for studying host–pathogen interactions in cyprinids. This model can also be applied while studying other viruses because the target cell lines available from ginbuna carp are also sensitive to infection by other viruses. For example, we recently confirmed that a cell line, CFS, derived from the ginbuna carp can be infected with KHV (unpublished data). Although some researchers have investigated carp immune responses against KHV infection (Rakus et al., 2012; Sunarto et al., 2012; Syakuri et al., 2013), no information on CMC against KHV is currently available. Thus, further studies using the experimental system employing ginbuna carp may shed light on how fish eliminate viruses or how certain viruses like KHV escape the host defence.

Based on the rule that CTL responses are MHC class I restricted, effector and target cells must be MHC class I matched to enable demonstration of antiviral CTL responses. This condition is fulfilled by the ginbuna carp CMC system, for which syngenic target cells are genetically identical (including MHC class I genes) to effector cell donors. However, more intensive investigations on the role of MHC class I molecules are difficult to perform because of insufficient sequence data. Recently, four different MHC class I sequences (caauUA-S3n, caauUF-S3n, caauZE-S3n and caauZB-S3n) were identified in S3n strains of the ginbuna crucian carp (Urabe et al., 2011). However, that study did not show any polymorphisms for these MHC class I sequences because they were analysed only in one strain. The ginbuan crucian carp are triploid fish ($3n = 156$) and are considered to have originated from the diploid ginbuna crucian carp or putative ancestor of the goldfish (Murakami et al., 2001). Therefore, more sequence data need to be generated for these *Carassius* species, including additional triploid ginbuna crucian carp, diploid crucian carp and goldfish.

Considerably more information is available on MHC class I molecules in the rainbow trout than those in the ginbuna crucian carp. It was found that the homozygous C25 strain of the rainbow trout and RTG-2 cell line (rainbow trout gonad; fibroblast-like cells) share the classical Onmy-UBA*501 allele (now renamed Onmy-UBA*15010; Dijkstra et al., 2001). A CMC assay system using a combination of this cell line and C25 clonal fish provided information on virus-specific CTL functions in another teleost fish species (Fischer et al., 2006; Utke et al., 2007, 2008). For this species, specific CMC activities were induced against hematopoietic necrosis (IHNV)-and viral hemorrhagic septicaemia virus (VHSV)-infected cells both by DNA vaccination against the glycoprotein of the respective rhabdoviruses and viral infection. Upregulation of CD8α mRNA expression suggested a major role for CTLs during this process. For the same species, homing was demonstrated in teleosts for the first time using inflammatory cells isolated from the DNA vaccine injection site (Utke et al., 2008). Although these studies have contributed to our understanding of CMC responses in salmonids, we still do not have sufficient information on antiviral CMC responses for other economically important salmonid fish species, such as the Atlantic salmon, and other virus infections, such as infectious pancreas necrosis (IPN), pancreas disease and infectious salmon anaemia (ISA), which pose serious problems in the Americas and Europe. Because the importance of antiviral CMC responses is obvious, understanding the mechanisms for inducing CTLs is crucial for developing effective, targeted antiviral vaccines.

Similar to the MHC class I matched CMC system in ginbuna carp and rainbow trout, an antiviral CMC assay using autologous target cells was established for the grouper (Chang et al., 2011). In that study it was shown that CD8α+ cells from nervous necrosis virus (NNV)-infected groupers killed NNV-autologous cells but not allogeneic and RSIV-infected autologous cells. As previously demonstrated for channel catfish target cells (Hogan et al., 1996), autologous cells were prepared from primary cultured fin cells that were established from individual fish and were subsequently used as effector cell donors. Although this indicated that it was possible to establish CMC assays for fish species that lacked clonal lines in general, similar approaches have not yet been used for other species and viruses.

2.2. Regulation of CTL related genes after viral infection

Draft genome sequence data and a large number of EST sequences have markedly facilitated the identification of immune-related genes in teleost fish. Not surprisingly, important CTL-related genes, such as CD8, TCR and perforin, have been cloned in many species (Castro et al., 2011; Laing and Hansen, 2011). Several reports have shown that these CTL-related genes are regulated by either viral infection or vaccination (Table 1). CD8α mRNA expression was shown to be upregulated after SAV, ASRV, IPNV, VHSV or ISAV infection in the Atlantic salmon and rainbow trout, VHSV infection in flounder, KHV and SVCV infections in common carp, CHNV infection in ginbuna crucain carp, NNV infection in grouper and nodavirus infection in Atlantic halibut. These studies suggested that virus-specific CTLs were generated with most viral infections of fish.

Important evidence that T cells clonally expanded after viral infection was found by spectratyping the TCR repertoire in VHSV-infected rainbow trout. In infected fish, TCR diversity was skewed as reflected by an irregular CDR3 length distribution, whereas the TCR repertoire in naïve fish was polyclonal (Boudinot et al., 2001; Castro et al., 2011). More recently, the same group showed that clonal expansion occurred between both CD8+ and CD8− cells (Castro et al., 2013).

In conclusion, these studies demonstrated at molecular levels, fish CTLs were involved in eliminating viral infections. Thus, it can be suggested that CTLs are the primary players in antiviral adaptive immunity in teleost fish and therefore should be given appropriate attention when designing new vaccines.

3. MHC-restriction of virus-specific CTLs in teleost fish

MHC class I genes have been identified in a wide range of teleost fish species, including the rainbow trout (Aoyagi et al., 2002), Atlantic salmon (Lukacs, 2010), common carp (Kruiswijk et al., 2002), zebrafish (Kruiswijk et al., 2002), grass carp (Yang et al., 2006), medaka (Nonaka et al., 2011) and sea bass (Pinto et al., 2013). However, functional evidence for MHC restriction of CTLs remains to be demonstrated in most species. In studies that used clonal ginbuna carp, effector cells from the infected fish could kill virus-infected syngeneic cells but not infected allogeneic cells, suggesting that effector cell killing is genetically restricted (Somamoto et al., 2002). However, although several MHC class I sequences have been identified in ginbuna crucian carp (Urabe et al., 2011), there is no direct evidence that virus-specific CTLs of ginbuna carp recognise virus antigenic peptides on MHC class I molecules.

Studies that used C25 clonal strain of the rainbow trout have demonstrated that sensitised effector cells can kill only MHC class I-matched infected cells but not mismatched infected cells, sug-

gesting that trout CTLs recognise virus-infected cells based on MHC restriction (Utke et al., 2008). In another study, MHC tetramer technology was used to show that grass carp CTLs recognised virus peptides that were complexed with MHC class I molecules, which provided further strong evidence for MHC-restricted recognition of virus-infected targets by CTLs in teleost fish (Chen et al., 2010). mRNA expression studies have shown that molecules involved in antigen-presentation/processing in the MHC class I pathway were upregulated after viral infection in several fishes. These molecules' upregulation was observed in salmonids infected with IHNV (Hansen and La Patra, 2002; Landis et al., 2008), ISAV (Koppang et al., 1999; LeBlanc et al., 2012; Jørgensen et al., 2006, 2007) and IPNV (Koppang et al., 1999), in grouper infected with Singapore grouper iridovirus (SGIV) (Ou-yang et al., 2012) and in flounder infected with VHSV (Avunje et al., 2012). These results further support that CTLs play an important role in eliminating virus-infected cells in fish.

4. Vaccination strategies for efficiently inducing CTL activity

Antibiotics are ineffective against viruses, and the use of interferon therapy or antiviral peptides does not seem to be applicable for economical reasons. Thus, vaccination in conjunction with sanitary measures remains the only effective strategy for preventing viral diseases in aquaculture. To efficiently trigger the expansion of virus-specific CTLs, vaccination methods should promote the expression of intracellular antigens followed by antigen processing and loading of the resulting peptides onto the host cell's MHC class I molecules. Although a live, attenuated vaccine virus to produce intracellular antigens should induce an efficient CMC response, this could not be easily developed and was not accepted because of serious safety concerns regarding genetic stability and virulence (Foged et al., 2012). This chapter will consider how a CMC response can be effectively induced by vaccinations in teleost fish.

4.1. DNA vaccination

Many studies with mammals have shown that DNA vaccines can induce both humoral and cellular immunity. These vaccines are particularly effective against viral diseases because the encoded immunogenic proteins are synthesised and processed as intracellular antigens in host cells similar to that occurring during an actual viral infection (Donnelly et al., 2005). Experimental DNA vaccines against VHSV, IHNV, IPNV, HIRRV and SVCV have shown considerable efficacy in a number of different fish species (Lorenzen and LaPatra, 2005; Mikalsen et al., 2004; Tonheim et al., 2008; Purcell et al., 2012). A DNA vaccine encoding for the G-protein of VHSV could induce a CMC response by trout leukocytes against VHSV-infected MHC class I-matched cells (Utke et al., 2008). As described in a previous chapter, this model is the only assay system available for determining CMC activity induced by DNA immunisation in teleost fish. Thus, this model can be applied for exploring antigenic epitopes that can induce efficient CMC activity against various viruses.

In addition, the mRNA expression analysis demonstrated that DNA vaccines against several viruses resulted in upregulation of CTL-related molecules, such as CD8, IFN-γ and MHC class I in the rainbow trout (Cuesta and Tafalla, 2009; Cuesta et al., 2010) and Japanese flounder (Byon et al., 2005, 2006). Thus, DNA immunisation is considered to be a simple and effective means to induce an effective antiviral response in fish. However, although DNA vaccines against IHNV have been licensed in Canada (Salonius et al., 2007; Liu, 2010), these are difficult to register because of concerns regarding their safety and consumer acceptance in many countries. Dijkstra et al. (2001) reported that plasmid DNA could persist in fish for long periods of time after the initial injection, indicating risks for gene transfer to a host's genome or to the intestinal bacteria and its residue in the edible parts of a fish. Thus, to overcome these concerns, further studies will be required to determine whether DNA used is circular or integrated into the chromosomes of various fishes.

DNA vaccination may be a good tool for protecting fish species that do not enter the food chain. It was reported that DNA vaccines could protect koi carp against SVCV (Emmenegger and Kurath, 2008), although CTL responses against SVCV were not found. Thus, protective immune mechanisms remain to be explored. Koi carp are exported and exhibited worldwide, which poses a serious threat to the spread of viral infections. Therefore, there is an urgent need for an effective vaccine. Because certain ornamental fish like koi carp are considerably more expensive than aquacultured fish, safety issues regarding human consumption and economical issues such as cost per vaccine dose and cost per individual vaccine delivery are almost negligible.

4.2. Adjuvant inducing CTL activities

Because CTLs can induce the secretion of Th1-cytokines during the recognition of virus-infected cells, vaccines should include an adjuvant to promote Th1 responses and antigen presentation through MHC class I molecules (Malyala et al., 2009; Foged et al., 2012). For mammals, a number of adjuvants for CTL priming have been developed. The use of these adjuvants in conjunction with otherwise less efficient antigens may be more acceptable for commercial use than live attenuated or DNA vaccines. These include lipid-based adjuvants, such as fusogenic liposomes and immunostimulatory complexes (ISCOMs), which consist of cholesterol, phospholipid and saponin (Lovgren Bengtsson et al., 2011; Sun et al., 2009; Sanders et al., 2005; Nordly et al., 2009). Vaccines with these hydrophobic agents aid exogenous antigens to pass through the cellular membrane and enhance antigen delivery to the cytosol. After antigen processing, loading of the resulting peptides onto MHC class I molecules and their subsequent presentation on the cell surface, these presented antigens can be recognised by CTLs, which promotes these cells' clonal expansion. Because lipids are natural constituents of biomembranes and are well-tolerated because of their biodegradability, they are promising candidates as adjuvants.

However, in fish, approaches to implement lipid-based adjuvants are limited compared with those in mammals. Several studies have reported using liposome-based vaccines as oral delivery systems for bacterial diseases (Afonso et al., 2005; Irie et al., 2005; Yasumoto et al., 2006; Ramasamy et al., 2012; Harikrishnan et al., 2012). The only viral fish disease for which oral immunisation with a liposome vaccine was applied was for the KHV disease. In this case, KHV antigens within liposomes conferred efficient protection for carp (Yasumoto et al., 2008), indicating that liposomes can deliver antigens to the immune inductive sites in the fish intestine and that they can induce specific immunity against KHV. However, in that study, it was unclear whether or not KHV-specific CTLs were generated.

CpGs and poly I:C are known to activate immature dendritic cells (DCs) and induce their terminal differentiation/maturation in higher vertebrates (Hartmann et al., 1999; Guermonprez et al., 2002). Because DC-like cells have been reported in teleosts (Lugo-Villarino et al., 2010; Bassity and Clark, 2012; Wittamer et al., 2011), CpGs and poly I:C may also be useful as adjuvants for stimulating CTLs in fish. In addition to studies that used lipid-based adjuvants, CpG oligonucleotides and poly I:C were shown to upregulate IFN-γ expression in salmon, which suggested that CpG/poly I:C could enhance Th1-type immune reactions (Strandskog et al., 2011; Thim et al., 2012). It was also reported that Poly

I:C administration with live viruses provided efficient protection for several fish species and suggested that specific protective immune responses can be induced in these hosts (Nishizawa et al., 2009, 2011; Takami et al., 2010; Oh et al., 2013). Although these studies did not mention whether CTL activity was effectively induced by Poly I:C administration, it can be inferred that this vaccination method activated immature DCs.

A review that was published more than 10 years ago proposed several vaccination strategies using exogenous antigens that may potentially induce CTL activities (Dijkstra et al., 2001). However, very few attempts have been made to develop these vaccines because of a lack of CTL assays for most commercial fish species due to the unavailability of clonal fish and MHC class I-matched effector/target cell systems. A recent study that used ginbuna carp demonstrated that granzyme B-like activity correlated with allospecific CTL activity (Toda et al., 2011b), suggesting that granzyme B activity may be useful as an indicator for CTL activity in those fish species for which MHC class I-matched effector/target cell systems have not yet been developed. Therefore, it is anticipated that vaccine efficacy evaluations based on granzyme B activity will be widely used for many fishes.

4.3. Extracellular antigen cross-presentation

Classical antigen-presentation studies with mammals have shown that MHC class I molecules present peptides that are derived from proteins synthesised within the cell, whereas MHC class II molecules present peptides that originate from exogenous proteins. However, antigen presenting cells (APCs) also have the capacity to process exogenous antigens using the MHC class I pathway, which is known as cross-presentation (Dresch et al., 2012; Joffre et al., 2012). Thus, cross-presentation is an important mechanism for activating $CD8^+$ CTLs during anti-viral immune defence (Flinsenberg et al., 2011; Kurts et al., 2010). This is because APCs can acquire exogenous antigens from virus-infected cells by phagocytosis and present them on MHC class I molecules, even when the APCs are not directly infected. Central players in this process are dendritic cells (DCs), which are specialised for cross-presentation. Because all inactivated vaccines use exogenous antigens, cross-presentation seems to be a crucial mechanism for inducing CTLs by vaccination with inactivated viruses or viral proteins.

Recent studies have demonstrated that DC-like cells, which morphologically resembled mammalian DCs, existed in zebrafish and rainbow trout and have antigen-presenting functions similar to DCs of mammals (Lugo-Villarino et al., 2010; Bassity and Clark, 2012; Wittamer et al., 2011). These DC-like cells in rainbow trout expressed MHC class II molecules and DC markers (CD83, CD209, CXCR4 and CCR7) and had many functional similarities with mammalian DCs. The CD83 gene, which is a marker for mature human DCs, was identified in the rainbow trout (Ohta et al., 2004), Atlantic salmon (Haugarvoll et al., 2006; Pettersen et al., 2008), sea bass (Buonocore et al., 2012), turbot (Hu et al., 2010) and ginbuna crucian carp (unpublished data). Furthermore, some Toll-like receptor (TLR) homologues that are known to be involved in mammalian DC functions were also identified in several fishes (Palti, 2011). However, cross-presentation in teleosts has not been investigated. More efforts to understand DC functions in teleosts are required to determine whether or not cross-presentation can be induced in fish. This will have consequences for the development of inactivated anti-viral vaccines for which cross-presentation seems to be crucial for inducing CTL-mediated protection.

4.4. Mucosal vaccines for generating virus-specific CMC responses

Fishes and mammals independently evolved over millions of years. Thus, it is not surprising that there are several anatomical and physiological differences between mammals and fishes. Because of gills, a mucous skin and no Peyer's patches, the mucosal immune system of fish is unique (Løkka et al., 2013; Rombout et al., 2011; Salinas et al., 2011). Sato and Okamoto (2010) reported that oral administration of formalin-inactivated CHNV (FI–CHNV) can induce a CMC response against CHNV-infected cells in ginbuna crucian carp. Recently, we demonstrated that T cell-related genes, such as those for IFN-γ and TCR, were upregulated, and the proportion of $CD8^+$ cells increased following anal administration of FI–CHNV, which suggested that virus-specific CTLs could be induced by immunisation with inactivated viruses (unpublished data). Furthermore, the anal administration of FI–CHNV provided more efficient protection against CHNV infection than intraperitoneal injection of FI–CHNV. These findings suggest that immunisation with antigens through the intestinal route may effectively induce cross-presentation in teleost fish.

Takizawa et al. (2011b) reported that the proportions of $CD8^+$ cells in the intestine and gills are higher than those in blood and spleen of the rainbow trout, suggesting that the distribution of CTLs in fish is different from that in mammals and highlighted the importance of mucosal surfaces in CTL responses. In addition, constitutive high expression of interleukin 4/13A and GATA-3 in the gill and skin of the rainbow trout suggests that the outer mucosal surfaces in fish represent a Th2-skewed environment (Takizawa et al., 2011a). This may protect fish from overwhelming Th1/Th17-driven inflammatory reactions, although it may also have consequences for bath vaccination for which Th1-driven anti-viral responses must be triggered.

The identification of a unique T-cell-rich intraepithelial structure in salmonid gills indicated its special function in piscine mucosal immunity (Haugarvoll et al., 2008; Koppang et al., 2010), although its function remains to be determined. Because the route of vaccine delivery may be crucial for triggering immune responses at a pathogen's typical port of entry (Belyakov and Ahlers, 2009), it will be necessary to learn more about the interface between mucosal and systemic immunity in teleost fish. The identification of M-like cells in salmonid intestines is promising with respect to targeted delivery approaches (Fuglem et al., 2010).

Oral administration of vaccines against several viruses has been studied in the rainbow trout, Japanese founder and common carp, and vaccine delivery systems have been developed using Poly(D, L-Lactic-Co-Glycolic Acid) nanoparticles, polyethylene glycol and liposomes (Yasumoto et al., 2008; Kim et al., 2011; Adelmann et al., 2008; Adomako et al., 2012; Plant and LaPatra, 2011). These studies showed that efficient protection could be conferred and suggested that oral vaccination can successfully deliver vaccine antigens to the hindgut. Because oral vaccination for fish is one of the most feasible delivery methods with respect to costs and labour involved, these delivery systems may lead to the development of an ideal vaccination system for fish. As described above, it is thought that fish have a mucosal immune system that is different from that of mammals. Thus, it will be necessary to develop vaccines based on this unique system. Therefore, additional studies are required to understand the inductive mechanisms for generating virus-specific CTLs following oral vaccination in addition to developing delivery systems to protect vaccine antigens from digestion.

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T cell immunity in the teleost digestive tract

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ABSTRACT

Fish (along with cyclostomes) constitute the most ancient animal group in which an acquired immune system is present. As in higher vertebrates, both B and T lymphocytes cooperate in implementing an adequate response. Although there is still a debate on whether fish possess a true gut associated lymphoid tissue (GALT), the presence of diffuse B and T lymphocytes throughout all mucosal surfaces has been demonstrated in a wide variety of fish species. The lack of antibodies against T lymphocyte markers has hampered the performance of functional assays in both systemic and mucosal compartments. However, most components associated with T lymphocyte function have been identified in fish through extensive genomic research, suggesting similar functionalities for fish and mammalian T lymphocytes. Thus, the aim of this review is to briefly summarize what is known in teleost concerning the characteristics and functionalities of the different T cell subsets, to then focus on what is known to date regarding their presence and role in the gastrointestinal tract, through either direct functional assays or indirectly by conclusions drawn from transcriptomic analysis.

1. Introduction

B and T lymphocytes mediate the specific responses of adaptive immunity in jawed vertebrates. Both types of lymphocytes undergo the unique characteristic process of somatic DNA re-arrangement of their antigen specific receptors (members of the Ig superfamily) by random combination of the variable gene segments present in the receptor locus. B cells express their antigen receptor on their cell surface as B cell receptor (BCR) and secrete them as immunoglobulin (Ig) or antibody, whereas the T cell receptor (TCR) is always cell-surface bound. In both cases, one of each multiple variable (V), diversity (D) and joining (J) gene segments are randomly combined together with the constant gene segments (C) that define a specific mature functional VDJC antigen receptor in each cell. This process allows a vast repertoire of B or T cells bearing structurally diverse antigen receptors for specific pathogen recognition. Thus, each lymphocyte carries a unique Ig domain-containing receptor and can originate a clone of cells upon induction that will react specifically with only one antigen. The classical B cell function is the production of antibodies (or Igs) to specifically neutralize pathogens and label them to be removed by the immune system. T cells act mainly as coordinators of the responses of other immune cells and as effector cells to directly kill infected or tagged cells. V(D)J recombination is controlled by the recombination activating genes *RAG1* and *RAG2* (Oettinger et al., 1990). V(D)J recombination also occurs in fish and *RAG* genes have been cloned in a number of fish species (reviewed by Flajnik and Kasahara, 2010).

Because the gastrointestinal tract is one of the main portals of pathogen entry, there is an important defense function associated to the gut, in addition to other physiological functions. These defense mechanisms are triggered locally by a gut-associated lymphoid tissue (GALT) that in endotherms is mainly organized into Peyer's patches. Peyer's patches are composed by aggregated lymphoid follicles containing both B and T lymphocytes, as well as follicular dendritic cells (FDCs). Each follicle extends into a dome villus, that is covered by a follicle-associated epithelium (FAE) forming an interface between the GALT and the luminal microenvironment (Jung et al., 2010). Characteristic for the FAE are microfold (M) cells that are responsible for antigen uptake and

Abbreviations: B cell receptor, BCR; days post-fertilization, dpf; follicle-associated, FAE; follicular dendritic cells, FDCs; gut associated lymphoid tissue, GALT; grass carp reovirus, GCRV; infectious pancreatic necrosis virus, IPNV; immunoglobulin, Ig; innate lymphoid cells, ILCs; interferon, IFN; interleukin, IL; lipopolysaccharide, LPS; major histocompatibility complex, MHC; pathogen-associated molecular patterns, PAMPs; pattern recognition receptor, PRR; T cell receptor, TCR; Terminal deoxynucleotidyl transferase, TdT; transforming growth factor β, TGF-β; tumor necrosis factor α, TNF-α; viral hemorrhagic septicemia virus, VHSV.

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translocation. Although the teleost digestive tract lacks such an organized structure, it contains diffusely distributed B and T lymphocytes as reported in many different fish species (Salinas and Parra, 2015). However, there is still a debate on whether these structures fulfill the definition of a lymphoid tissue and particularly of a true GALT. The aim of this review is to briefly summarize what is known in teleost concerning the characteristics and functionalities of the different T cell subsets, to then focus on what is known to date regarding their presence and role in the gastrointestinal tract, through either direct functional assays or by indirect transcriptomic approaches.

2. T lymphocytes in fish

T cells are characterized by the presence of a TCR through which they recognize antigens. In mammals, unlike B lymphocytes, T lymphocytes fail to recognize antigens in the absence of antigen presentation, with the exception of superantigens (antigens that produce a non-specific polyclonal T cell activation with a massive cytokine release), because the TCR of most T cells (except that of γδ T cells) is restricted to recognizing antigens only when exposed in the context of an isogenic major histocompatibility complex (MHC), either class I or II, present on the cell surface. All TCRs transmit their signal through a complex of CD3 signaling chains, including one chain each of CD3-γ, -δ and two chains of CD3-ε and -ζ. In teleost, only three chains have been identified (*CD3*-γδ, -ε and -ζ), suggesting a common ancestor of mammalian CD3-γ, and CD3-δ (Laing and Hansen, 2011).

2.1. Subtypes of teleost T lymphocytes

A first classification of T cells can be based on the TCR chains they express, either αβ or γδ. Mammalian αβ TCR T cells recognize antigen presenting cells through MHC class I or II while γδ TCR T cells do not require interaction with conventional MHC molecules. γδ TCR T cells rather use their TCR as a pattern recognition receptor (PRR) to recognize phosphorylated microbial metabolites and lipid antigens. As a consequence, mammalian γδ T cells are more innate-like immune cells with less dependence on MHC presentation, and are mostly found in epithelial and mucosal tissues, representing around 2% of the total T cell population (Bonneville et al., 2010). All four *TCR* chains have been identified in different teleost species (Criscitiello et al., 2006; Flajnik and Kasahara, 2010; Nam et al., 2003), but it is still not known if γδ T cells exert similar roles as the equivalent mammalian subsets.

Conventional αβ T cells can be divided into T cytotoxic (Tc) or T helper (Th) cells, distinguished by the expression of the membrane bound glycoproteins CD8 or CD4 respectively. These molecules act as co-receptors for the TCR, stabilizing the interaction with the MHC and enhancing TCR activation through the CD3 tyrosine phosphorylation pathway. Tc cells, expressing CD8 on their cell surface, are able to kill infected or cancerous cells after recognizing non-self or cancer-associated antigens, respectively, in the context of MHC class I (Strasser et al., 2009). Different *CD8* (both *α* and *β* chains) have been sequenced in multiple teleost species (Buonocore et al., 2006; Hansen and Strassburger, 2000; Moore et al., 2005; Patel et al., 2008; Pinto et al., 2006; Quiniou et al., 2011; Somamoto et al., 2006; Suetake et al., 2007; Sun et al., 2007) and mature teleost Tc cells have been suggested to express a heterodimer of CD8 formed by α and β chains (Laing and Hansen, 2011), as occurs in mammals (Quiniou et al., 2011). Interestingly, in mammals, a conserved binding motif associated with tyrosine kinase p56 (lck) is only present *CD8α* genes and not in *CD8β* genes, and as a consequence CD8β cannot be expressed on a cell unless is part of a CD8α–CD8β heterodimer. In fish, both *CD8α* and *CD8β* genes contain this motif suggesting that CD8β might have signaling functions in the heterodimeric as well as homodimeric forms (Quiniou et al., 2011). Alternatively, Th cells express CD4 and produce cytokines to regulate the action of other immune cells, mainly B cells. In this case, numerous fish species such as rainbow trout (*Oncorhynchus mykiss*) (Dijkstra et al., 2006; Laing et al., 2006), catfish (*Ictalurus punctatus*) (Edholm et al., 2007), Atlantic salmon (*Salmo salar*) (Moore et al., 2009), tetraodon (*Tetraodon nigroviridis*) (Wen et al., 2011), common carp (*Cyprinus carpio*) (Yamaguchi et al., 2013) and Japanese flounder (*Paralichthys olivaceus*) (Kato et al., 2013) express two distinct *CD4* genes (designated as *CD4-1* and *CD4-2*). In fugu, CD4-1$^+$ cells express transcripts for both *CD4-1* and *CD4-2*, but are negative for *CD8α* (Kono and Korenaga, 2013). Furthermore, another study in pufferfish has documented a specific Treg (regulatory T cell) phenotype for CD25$^+$CD4-2$^+$ T lymphocytes (Wen et al., 2011). Thus, additional antibodies should be developed in order to unequivocally establish whether fish CD4-1$^+$ and CD4-2$^+$ T lymphocytes consistently exhibit a distinct phenotype.

In mammals, CD4$^+$ T lymphocytes can be further classified according to their expression of specific transcription factors and the secretion of representative combinations of cytokines (Fig. 1). Although there is still some controversy as to whether these Th subsets constitute differential cell types or cells in a different state of activation with a certain degree of plasticity (Kleinewietfeld and Hafler, 2013), well-defined subsets in mammals include Th1, Th2, Th17 and Treg. The differentiation of Th cells towards a Th1 profile is controlled by the master transcription factor T-bet (Kanhere et al., 2012). These cells secrete effector cytokines such as interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) to control intracellular infections, and interleukin 2 (IL-2) to induce lymphocyte proliferation. GATA-3, however, is the master transcription factor that mediates the differentiation of Th cells towards a Th2 profile (Kanhere et al., 2012). Mammalian Th2 cells produce IL-4, IL-5, and IL-13 that stimulate B cells to secrete different antibody isotypes and thus control extracellular infections. Th17 cells use the transcription factor RORγ and produce IL-17 together with IL-21 and IL-22 (Ruan et al., 2011). These cells appear to be implicated in the control of extracellular pathogens such as bacteria or fungi, and play a key role in autoimmune diseases. Treg cells, which are regulated through Foxp3, have a crucial role in keeping self-tolerance (Zhang and Zhao, 2007), partially mediated through the secretion of IL-10 (Chaudhry et al., 2011), even though IL-10 can also be produced by other Th subsets such as Th2, for example (Kubo and Motomura, 2012). Additional Th subsets, still not well characterized in mammals, include follicular Th cells (T_{FH}), Th6 and Th9. T_{FH} secrete IL-21 (Fazilleau et al., 2009), whereas Th6 and Th9 are defined by their capacity to produce IL-6 and IL-9, respectively (Azizi-Semrad et al., 2010; Dardalhon et al., 2008). Concerning fish, genomic studies performed in different species have identified most components associated with T cell function, making it possible to speculate that fish have all these different T cell subsets (Laing and Hansen, 2011; Wang and Secombes, 2013), however, whether the functionalities are maintained is something that needs to be further investigated.

Functions similar to those of Th cells can also be driven by innate lymphoid cells (ILCs), a group of lymphoid cells lacking TCR or BCR, that share some functional aspects with both NK and Th cells. ILCs are classified into distinct groups on the basis of their cytokine production, following a similar classification scheme as that used for Th cells (reviewed by Annunziato et al., 2015; Hazenberg and Spits, 2014; Moro and Koyasu, 2015). ILC1 cells, e.g. express T-bet and release IFN-γ, while a signature molecule for IL-5 and IL-13 producing ILC2 cells is GATA-3. Typical marker molecules of ILC3 cells are the transcription factor RORγt and the cytokines IL-17 and IL-22. In the postnatal mammalian gut, ILCs rapidly respond to commensal

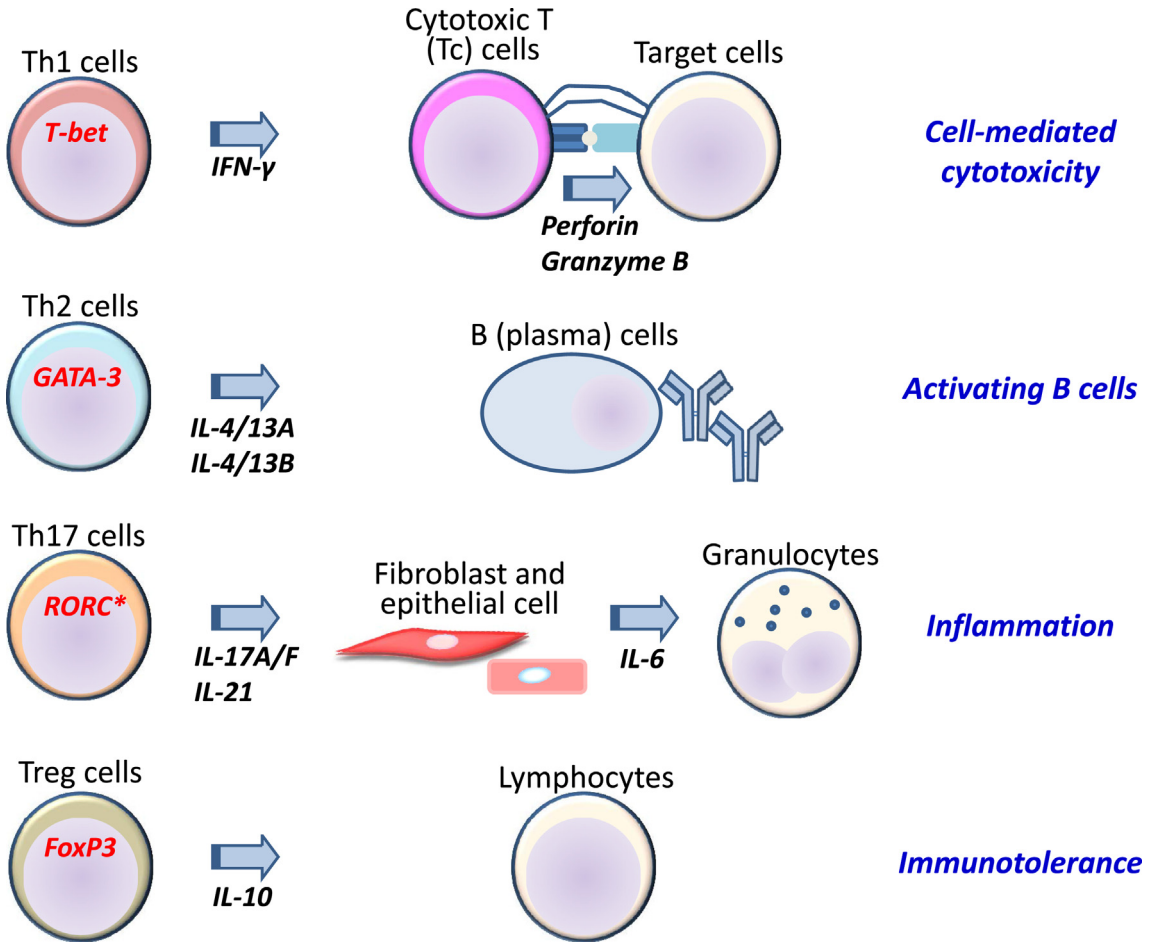

Fig. 1. The regulation of the genes associated with different T cell populations. (*) There are no convincing reports in fish yet linking expression of Th17 signature cytokine genes to *RORC* expression.

and pathogenic intestinal bacteria, parasites and food components by polarized cytokine production (reviewed by Pearson et al., 2012). Although these cells have not been described in fish so far, the fact that teleost fish possess a phylogenetically ancient immune system that relies greatly on innate immunity makes it possible to hypothesize the existence of these innate immune cells.

2.2. Functionality of T lymphocytes in teleosts

The combination of specific anti-CD8α antibodies, clonal fish and MHC class I matching cell lines has permitted the verification that fish $CD8^+$ cells kill virus-infected cells when the TCR matches the MHC class I in which an antigenic peptide is exposed (Fischer et al., 2006). In ginbuna crucian carp (*Carassius auratus langsdorfii*), $CD4^+$ and $CD8^+$ lymphocytes were separated through the use of respective specific antibodies, confirming the cytotoxic activity of $CD8^+$ T lymphocytes and the lack of such functions for $CD4^+$ cells (Shibasaki et al., 2010; Toda et al., 2009). Furthermore, the dependence of $CD8^+$ T lymphocytes on perforin to achieve their cytotoxic activity has been established (Toda et al., 2011a). TCR repertoire studies of $CD8^+$ T cells in trout revealed a highly diverse and polyclonal repertoire in naive fish, while $CD8^+$ T cells from virus infected fish were able to mount a typical response that was characterized by a significant skewing of CDR3 profiles (Bernard et al., 2006). Interestingly, $CD8^+$ cells also showed an antibacterial activity against both intracellular and extracellular bacteria in ginbuna crucian carp at high effector to bacterial cell ratios (Nayak and Nakanishi, 2013). A role for $CD8^+$ T cells in a graft-versus-host reaction (GVHR) has also been established using a model of clonal triploid ginbuna and tetraploid ginbuna-goldfish hybrids, as GVHR was not induced when $CD8^+$ cells were removed from the leukocyte fraction by magnetic sorting (Shibasaki et al., 2010).

In the case of $CD4^+$ T cell subsets, there is some recent evidence suggesting that the functionality of Th cells in fish is similar to mammals. In ginbuna crucian carp, it was demonstrated that $CD4^+$ Th cells play a major role in the regulation of humoral responses rather than cellular responses. This was concluded from experiments where fish transplanted with virus-sensitized donor cells containing $CD4^+$ cells had a more rapid and stronger antibody response than fish transplanted with sensitized cells that did not contain $CD4^+$ cells, while both recipient groups had similar cellular responses that were independent from the presence or absence of $CD4^+$ cells in the transferred virus-sensitized donor cells (Somamoto et al., 2014). Additionally, a polyclonal antibody was recently developed against zebrafish (*Danio rerio*) CD4-1 and used to demonstrate that the corresponding cells transcribe cytokines and transcription factors specific either of Th1 or Th2 lineages in response to different antigenic stimulations (Yoon et al., 2015). Finally, for carp there is a clonal permanent CD4-1^+TCRαβ$^+$ T cell line that expresses detectable amounts of *GATA-3* and *IL-4/13B* (counterpart of mammalian IL-4 and IL-13) but not of T-bet or IFN-γ, thus has a Th2-like profile in regard to its transcription factors and cytokines (Yamaguchi et al., 2013). As already mentioned for ginbuna crucian carp $CD8^+$ cells, $CD4^+$ cells also

showed antibacterial activities (Nayak and Nakanishi, 2013). Therefore, in general, it seems that teleost T cells are capable of conducting most of the immune functions expected from them, as regards to their homology with mammalian T cell subsets.

3. Evidences of T lymphocyte presence in the fish digestive tract

In teleosts, the total number of gut associated intraepithelial T lymphocytes (T-IEL) was found to be comparable to the total number of T cells from all other secondary lymphoid organs, highlighting the importance of the intestine in T cell responses (Boardman et al., 2012; Rombout et al., 1998; Scapigliati et al., 2000; Takizawa et al., 2011a). T lymphocytes in fish have been detected either through the use of anti-CD3ε (Koppang et al., 2010; Boardman et al., 2012), anti-CD8α (Takizawa et al., 2011a), anti-CD4-1 (Toda et al., 2011b) antibodies directed against their corresponding species-specific molecules or in some cases through the use of pan-T antibodies. However, most of these pan-T antibodies were developed against non-IgM expressing lymphocytes and the target epitopes they react with are yet to be specified. For example, a pan-T cell monoclonal antibody (WCL38) developed in carp recognizes an unknown 76 KDa protein that is expressed in 50–70% of the lymphocytes from intestine but in less than 6% of lymphocytes from thymus, spleen or kidney. It is therefore regarded as a pan-T-cell antibody because it did not react with IgM^+ cells, macrophages or non-specific cytotoxic cells. (Rombout et al., 1998; Scapigliati et al., 2000). In sea bass, the pan-T monoclonal antibody DTL15 reacts with most thymocytes (around 75%) and a small proportion of lymphocytes from other tissues, while the recognized target molecule is still unknown (Scapigliati et al., 2000). Despite this, a few studies performed with some of these antibodies have provided interesting data on T cell functionality later described (Abelli et al., 1999; Romano et al., 2011; Scapigliati et al., 2000).

3.1. Ontogeny of T lymphocytes in the digestive tract

In fish, the origin of gut IEL T cells is largely unknown and thymic, extrathymic and intestinal origins have been discussed. Lymphocytes are thought to colonize the carp intestine as early as 4 days post-fertilization (dpf), a time point where cells expressing RAG1 were already detected in the basal membrane (Huttenhuis et al., 2005). When using a monoclonal antibody specific for carp mucosal T cells (WCL38), positive cells were already detected at 3 dpf (Rombout et al., 1998). In fertilized rainbow trout eggs, *CD8α* and *TCRβ* were already expressed at the mRNA level one and two weeks after fertilization, respectively (Fischer et al., 2005). Studies performed in zebrafish pointed however to 9 dpf as the moment when TCR transcription started in the intestine (Danilova et al., 2004), whereas in species such as sea bass TCR is not detected in the intestine until 28 days post hatching (dph) (Boschi et al., 2011). These results, along with the fact that the expression of TdT (terminal deoxynucleotidyl transferase), an enzyme implicated in creating junctional diversity in pre-T cells, is strongly detected in the adult teleost gut (Hansen, 1997), have led many authors to speculate an extrathymic development of T lymphocytes in the digestive tract (reviewed by Salinas and Parra, 2015). Concerning γδ TCR T cells, a recent study in olive flounder (*Paralichthys olivaceus*) in which the expression of the four TCR chains was evaluated in different developmental stages revealed that whereas *TCRα* was expressed already in the fertilized egg, *TCRγ* and *TCRδ* were not expressed until hatching (Lee et al., 2013), thus it can be implied that γδ TCR T cells colonize the intestine at a later stage than αβ TCR T cells.

3.2. Detection of T lymphocytes in teleost digestive tract

Piscine intestinal lymphocytes are embedded between the enterocytes as IELs and in the lamina propria (Ballesteros et al., 2013; Koppang et al., 2010). However, in contrast to mammals, no lymphocyte aggregates similar to Peyer's patches and germinal centers have been reported. In mammals, the majority of lamina propria T cells are $TCR\alpha\beta^+$ expressing either CD4 or CD8αβ. However, the epithelium contains a second unconventional subpopulation of T cells that express CD8αα homodimers and may either be $TCR\alpha\beta^+$ or $TCR\gamma\delta^+$ (reviewed by Peaudecerf and Rocha, 2011). These cells are thought to play a major role in maintaining the integrity of the gut wall; are regarded to have originated from early thymus precursors and have not yet undergone TCR rearrangement and TCR αβ/γδ commitment. Both *CD8α* and *CD8β* transcription can be recorded in the gut of several fish species such as rainbow trout (Moore et al., 2005), Atlantic salmon (Moore et al., 2005), fugu (Suetake et al., 2007), carp (Forlenza et al., 2008), sea bass (Picchietti et al., 2011) and orange-spotted grouper (Xu et al., 2011). However, due to the lack of suitable antibodies against CD8β, it remains unknown whether distinct $CD8\alpha\beta^+$ and $CD8\alpha\alpha^+$ lymphocyte subpopulations coexist in the fish gut. The major subpopulations of T cells in the fish gut are summarized in Table 1 and compared to those found in other secondary lymphoid organs.

In salmonids, $CD3\varepsilon^+$ cells were found to be abundant in all lymphoid tissues of fish with highest percentages in thymus, followed by the intestine and gills, with lowest numbers in spleen and head kidney (Boardman et al., 2012; Koppang et al., 2010). $CD3^+$ T cells were reported throughout the whole gastrointestinal tract but were abundant in the pyloric caeca and in the midgut/hindgut region (Ballesteros et al., 2013). In this case, all $CD3^+$ cells within the pyloric caeca were IELs. $CD3^+$ IELs were also visualized in the carp intestine using a commercial anti-human CD3ε antibody (Uran et al., 2008). A considerable part of these T cells found in the digestive tract have been shown to be $CD8\alpha^+$ (Takizawa et al., 2011a). In sea bass, T cell distribution in the intestinal tract (along with the thymus) was subject to a number of investigations using the pan-T monoclonal antibody DTL15. Using this antibody, T cells appeared to be more abundant in the midgut than in other gut segments. Because most IEL did not show TCRβ transcription, it was hypothesized that these cells were γδ T cells (reviewed by Picchietti et al., 2011). The pan-T cell monoclonal antibody WCL38 developed in carp was also used to label T cells in the digestive tract that were found to be numerous in the intestinal epithelium and less abundant in the lamina propria (Rombout et al., 1998).

Among lymphocytes, the fish gut does not only harbor T cells, but also NK like cells which are often referred to as nonspecific cytotoxic cells (NCC; Graves et al., 1984). Although this review is focused on intestinal T cells some information on intestinal NCCs will also be given. A natural cytotoxic activity has been described for intestinal lymphocytes against xenogenic (mouse tumor) cells in rainbow trout (Martin et al., 2012; McMillan and Secombes, 1997), sea bass (Picchietti et al., 2011) and carp (Rombout et al., 2014). In rainbow trout, NCC activity of intestinal lymphocytes was correlated with the transcription of the natural killer enhancement factor (NKEF) (Martin et al., 2012). In channel catfish, NCCs express the non-specific cytotoxic cell receptor (NCCRP)-1 (Jaso-Friedmann et al., 2004) and this receptor was also found to be highly expressed in the intestine of Nile tilapia (*Oreochromis niloticus*) (Ishimoto et al., 2004).

3.3. Functionality of T cells in the digestive tract

By expressing PRRs such as for instance toll-like receptors (TLRs), human γδ TCR T cells can directly respond to pathogen-

Table 1
Major subpopulations of T cells in teleost fish. Marker molecules, expected and identified genes/molecules in teleost fish are listed, along with main functions expected according to their mammalian counterparts. (*) Note that the existence of TCRαβ/TCRγδ T cells and CD8αα T cells was concluded from gene expression only and is still a matter of discussion.

A. In intestine

Subpopulations		Marker molecules			Expected functions
Gut- associated T-IEL	Conventional T-IELs	CD3	TCRαβ*	CD4-1	T helper functions
				CD4-2	(also abundant in other organs)
				CD8αβ	MHC class I dependent cytotoxic T cell functions
					(also abundant in other organs)
	Unconventional T-IELs			CD8αα*	Maintaining the integrity of the gut wall
					Intestinal tolerance
			TCRγδ*	CD8+/−?	Inflammation
					MHC class I independent cytotoxicity
					Immune suppression

B. In other peripheral lymphoid organs

Subpopulations		Marker molecules			Expected or confirmed** functions
Th cells	Th1 cells	CD3	CD4-1	T-bet, IFN-γ	Against intracellular infections by activating Tc cells
	Th2 cells	TCRαβ	CD4-2	GATA-3, IL-4/13A, IL-4/13B	Against extracellular infections by activating B cells
	Th17 cells			IL-17A/F, IL-21	Against extracellular infections by activating granulocytes
	Treg cells			FoxP3, IL-10	Self-tolerance
Tc cells			CD8αβ		Killing of infected cells in the context of MHC class I**

associated molecular patterns (PAMPs) resulting in the release of pro-inflammatory cytokines as for instance IFN-γ and TNF-α (reviewed by Beetz et al., 2008). However, it has also been shown that depletion of gut γδ T cells disturbs the intestinal tolerance (Frossard et al., 2015; Locke et al., 2006). This observation is supported by the fact that mammalian γδ T cells produce the anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF-β). Furthermore γδ TCR T cells are involved in controlling the movements and functions of key inflammatory effector cells such as neutrophils, macrophages and NK cells (Hayday and Tigelaar, 2003; Zachariadis et al., 2006).

There is strong evidence on the existence of γδ TCR T cells in fish based on molecular data and functional observations. Given the unique features of innate/adaptive immune functions of γδ TCR T cells, their preferred location in the intestine and the first appearance of the corresponding genes in fish, it has been hypothesized that the adaptive immune system of vertebrates evolved in the gastrointestinal region (Matsunaga and Rahman, 1998). Some *TCRγ* homologs have been cloned in several teleost fish species such as sea bass (Buonocore et al., 2012), olive flounder (Lee et al., 2013; Nam et al., 2003), zebrafish (Lam et al., 2004), mandarin fish (*Siniperca chuatsi Basilewsky*) (Tian et al., 2014), carp (Shang et al., 2008; Yamaguchi et al., 2013), channel catfish (Moulana et al., 2014) and Atlantic salmon (Yazawa et al., 2008). In sea bass, *TCRγ* mRNA was constitutively expressed at high levels in thymus and intestine, and intestinal leukocytes stimulated *in vitro* by poly I:C showed even higher transcription levels (Buonocore et al., 2012). Nevertheless, when juvenile sea bass were infected with betanodavirus, *TCRγ* was down-regulated (Buonocore et al., 2012), however, it remains unclear if the virus induced a direct damage, a true down regulation and/or a redistribution of γδ T cells. In carp, highest expression levels of the *TCRγ* gene were detected in the thymus, followed by the spleen, gill, head kidney and intestine (Shang et al., 2008). *TCRγ* up-regulation has been recorded during soy bean meal induced gastroenteritis in Atlantic salmon suggesting proliferation of the corresponding T cells (Marjara et al., 2012). Thus, it would be quite interesting to develop specific antibodies against γδ TCR T cells that allow us to study the functionality of these cells in sites such as the intestine.

IELs of adult humans and mice possess a highly oligoclonal TCRβ repertoire while in young individuals this repertoire is still polyclonal (reviewed by Castro et al., 2013). In rainbow trout, however, the repertoire of naïve young adult fish was found to be highly diverse and polyclonal, suggesting that either the selection of IELs is different from mammals or that these fish have not yet encountered sufficient amounts of antigen to trigger TCR repertoire skewing (Bernard et al., 2006). This suggestion is supported by the observation that infection of rainbow trout with viral hemorrhagic septicemia virus (VHSV) induces significant modifications of the IEL TCRβ repertoire, which was focused on the variable (V) domain 4 of TCRβ corresponding to the public response to the VHSV glycoprotein. In addition, a TCRβV4-(D)-βJ1 junction which has been recorded as the most expanded in the spleen public T cell response to the VHSV glycoprotein, was also amplified in IELs of infected fish (Boudinot et al., 2004). Since the TCRβ repertoire of gut IELs seems to be diverse and polyclonal in fish and since no specific properties of trout IEL TCRβ repertoires could be demonstrated in naïve and virus infected animals some authors hypothesized that there are no distinct IELs in teleosts when compared to non-mucosal lymphoid tissue such as spleen and pronephros (Castro et al., 2013). This is also supported by the fact that trout IELs did not show specific homing to the gut mucosa, something that in mammals defines IELs as a distinctive mucosal population (Bernard et al., 2006).

In zebrafish, it has been recently established that gut T lymphocytes, and not gut B lymphocytes, control microbial composition by regulating the abundance of *Vibrio* sp. (Brugman et al., 2014a). The authors demonstrated this at an early time point of development when adaptive immunity is still not present, but also in *RAG1*-deficient zebrafish. In both circumstances, the abundance of *Vibrio* in the gut microbiota was higher than in adult *RAG1* expressing zebrafish. Control of the gut microbiota could be reestablished in *RAG1*-deficient fish by adoptive transfer of T lymphocytes from *RAG1*-expressing fish, but not by transfer of B lymphocytes. Additionally, intestinal T lymphocytes secrete cytokines that induce the secretion of other inflammatory factors, and for example, the presence of T cells has been shown to be essential for the production of CXCL8-L1 but not that of CXCL8-L2 in the intestine during homeostasis (Brugman et al., 2014b). Despite this, the induction of CXCL8-L1 in response to gut inflammation was independent of T lymphocytes. All these data point to an important role of T cells in the homeostasis of the gut.

Furthermore, a recent work has evaluated the expression of different T cell marker genes in wild, reared vaccinated, and reared non-vaccinated Atlantic salmon. In all intestinal regions, gradually

higher transcription levels of *CD3ζ*, *CD4-2* and *TCRα* were observed in wild fish, moderate levels in reared non-vaccinated and even lower levels in vaccinated post smolts, while *CD8α* and *TCRδ* had lowest transcription levels in the reared unvaccinated group (Lokka et al., 2014). Interestingly, these results suggest that farming conditions (commercial feeds and vaccination) have an important effect on the regulation of T cells in the gut.

4. Immune regulation of proteins associated with different T lymphocyte subsets in the fish intestine

Although the number of assays dealing with T lymphocyte functions is quite limited in teleost, a great amount of transcriptional assays have been performed in the fish intestine in response to different stimuli, in which the transcription of T cell related molecules has been studied. These studies have provided us with additional valuable information that allows us to anticipate several aspects of T cell immunity within the digestive tract. However, care should be taken when extrapolating gene expression data to immune function, since in some cases, other cell types different than T cells can also transcribe T related genes. For example, as already mentioned above, ILCs can secrete cytokines catalogued as "signature cytokines" for certain subpopulations of T cells.

4.1. Immune regulation associated to cytotoxic T cells

Mammalian NK cells and cytotoxic T cells produce the effector molecules perforin and NK-lysin. Both NK cell activities and $CD8^+$ cells have been found in the piscine intestine, in addition to perforin (Athanasopoulou et al., 2009; Hwang et al., 2004) and NK lysine expression (Hirono et al., 2007; Wang et al., 2006b). Due to these observations it has been suggested that subpopulations of teleost intestinal lymphocytes may execute cell-mediated cytotoxicity as in mammals (reviewed by Fischer et al., 2013). However, the regulation of these molecules in the intestine in response to antigenic stimulation has not been studied so far.

4.2. Immune regulation associated to Th1 cells

Mammalian Th1 cells secrete effector cytokines such as IFN-γ and TNF-α to control intracellular infections and IL-2 to induce T cell proliferation (Romagnani, 2000). In teleost, the transcription of *IFN-γ* can be regulated in response to different stimuli in the intestine. For example, Atlantic salmon infected with *Aeromonas salmonicida* had an increased *IFN-γ* transcription in the intestine when compared to non-infected controls (Kumari et al., 2015). In rainbow trout, two different bacterial infections provoked increased *IFN-γ* transcript levels in the intestine (Evenhuis and Cleveland, 2012) and similar results were observed in Atlantic cod (Ellingsen et al., 2011). In large yellow croaker (*Larimichthys crocea*), *IFN-γ* was also up-regulated in the intestine after either bacterial or protozoan infections (Chen et al., 2015). In the case of grass carp (*Ctenopharyngodon idellus*), an infection with grass carp reovirus (GCRV) provoked a significant up-regulation of *IFN-γ* transcription levels, that went along with an increased *T-bet* and a decreased *GATA-3* transcription (Wang et al., 2013). These results were used to hypothesize that the virus provoked a shift from a Th2 profile in naïve fish to a Th1 profile in infected fish.

Concerning TNF-α, intraperitoneal injection of the PAMP lipopolysaccharide (LPS) resulted in a significant increase of transcription levels of this pro-inflammatory cytokine in the intestine of both common (Jiang et al., 2015) and grass (Bo et al., 2015) carp, that was impaired when fish were treated with vitamin D prior to the LPS injection (Jiang et al., 2015) suggesting an anti-inflammatory role of the latter. TNF-α was also induced in the intestine when grass carp were exposed to recombinant IL-1β (Bo et al., 2015). Recently, a recombinant *Aeromonas salmonicida* vaccine administered to grass carp through bath, was also shown to increase *TNF-α* transcription levels in the gut, along with those of *CD8α* (Liu et al., 2015). In turbot (*Scophthalmus maximus*), the administration of probiotic lactic acid bacteria of aquatic origin also produced an up-regulation of *TNF-α* transcription in the intestine (Munoz-Atienza et al., 2014). Similar observations were made after administration of human probiotics to nile tilapia (Pirarat et al., 2011). The induction of a local gut inflammation in zebrafish also provoked a TNF-α up-regulation in this organ (Liu et al., 2014). In rainbow trout, an orally administered DNA vaccine against infectious pancreatic necrosis virus (IPNV) as well as infection with IPNV increased the transcription of *TNF-α* in different gut segments (Ballesteros et al., 2013). A similar effect was observed in response to *Yersinia ruckeri* and *Flavobacterium psychrophilum* bath exposure (Evenhuis and Cleveland, 2012).

In mammals, IL-2 plays a central role in T cell control. This cytokine is produced by Th1 cells and by dendritic cells during antigen presentation. Furthermore, IL-2 is known to activate Treg cells, thus also contributing to immune tolerance and control of autoimmunity (reviewed by Malek and Bayer, 2004). An *IL2* homologue was originally described in fugu (Bird et al., 2005) and since then corresponding genes have also been found in other fish species (Secombes et al., 2011). In fugu, the injection with poly I:C induced the transcription of *IL-2* in both gut and gills (Bird et al., 2005).

The Th1 master transcription factor *T-bet* has been cloned in several fish species (Laing and Hansen, 2011). In trout, *T-bet* expression levels were compared to those of the Th2 master transcription factor *GATA-3* in the intestine, and although *T-bet* was constitutively expressed in naïve fish, its expression levels were lower than those of *GATA-3* (Wang et al., 2010a). Recently, a *T-bet* sequence has been identified in Atlantic salmon and showed to be transcriptionally induced in the intestine in response to infection with *Aeromonas salmonicida* (Kumari et al., 2015).

4.3. Immune regulation associated to Th2 cells

Mammalian Th2 cells produce IL-5, IL-4 and IL-13 to stimulate B cells and control extracellular infections. While genes for IL-5 have not been identified yet in any teleost species (Laing and Hansen, 2011) two paralog genes appear related to a common ancestor of *IL-4* and *IL-13* in teleosts, strengthening the hypothesis that mammalian IL-13 arose from IL-4 by gene duplication (Li et al., 2007; Ohtani et al., 2008). Consequently, these genes have been designated as *IL-4/13A* and *IL-4/13B*. A type 2 skewed environment in the gills of ginbuna carp (Takizawa et al., 2008) has been concluded from the high constitutive expressions of GATA-3. This type 2 skewing was further supported by elevated *IL-4/13A* expression in gills and also in skin of rainbow trout (Takizawa et al., 2011b). This seems also to be the case in the intestine, as demonstrated in rainbow trout (Wang et al., 2010a) and grass carp (Wang et al., 2013), since both species had higher *GATA-3* than *T-bet* transcription levels. Since Th2 cytokines are known to elicit the secretion of different antibody isotypes in mammals, it might be possible that this Th2 skewing in fish mucosal tissues somehow mediates the secretion of natural antibodies in mucus. In a vaccination trial of Atlantic salmon against IPNV using alginate encapsulated antigens, this environment was even more shifted toward type 2 immune responses as concluded from increased *GATA-3*, although in this occasion expression of genes associated with a Treg profile such as *FoxP3*, *TGF-β* and *IL-10* was also detected (Chen et al., 2014).

4.4. Immune regulation associated to Th17 cells

Th17 cells are involved in controlling extracellular bacterial and fungal infections and also play a key role in autoimmune disease in mammals. These cells are defined by their ability to secrete IL-17 although they can also secrete IL-21 and IL-22 (Wei et al., 2007). IL-17A was discovered in 1993 as the first member of the IL-17 family of cytokines that also includes IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F (Pappu et al., 2011). However, mammalian Th17 cells mainly secrete IL-17A and IL-17F. Different forms of *IL-17* have been identified in fish species such as fugu, zebrafish and salmonids, many of them being orthologous to *IL-17A* and *IL-17F* (reviewed by Laing and Hansen, 2011). Th22 constitute an additional mammalian Th subset that secretes IL-22 but not IL-17 (Eyerich et al., 2010). A third group of mammalian adaptive immune cells that produce IL-17 and/or IL-22 are $CD8^+$ T cells. Although not all $CD8^+$ T cells produce IL-17, it is unclear whether these cells represent a functionally distinct subset of $CD8^+$ T cells and if they also secrete IL-22 (Shin et al., 1999). Furthermore, there is some evidence for a subgroup of $CD8^+$ T cells that produces IL-22 but not IL-17 (designated as Tc22 cells) (Nograles et al., 2009).

All *IL-17* forms identified in zebrafish are constitutively expressed in intestine, as well as in kidney, spleen and gills (Gunimaladevi et al., 2006). Among them, *IL17A/F-1*, *F2* and *F3* were transcribed at higher levels in the intestine than in other tissues. Furthermore, *in vivo* stimulation with LPS provoked an up-regulation of zebrafish *IL17A/F-1* specifically in the intestine. Similarly, all IL-17 forms found in salmonids (Wang et al., 2015) are constitutively expressed in the intestine, whereas in the case of *IL-17A/F1*, the intestine is the tissue that has higher constitutive expression levels. Likewise, in turbot, both *IL-17A/F* and *IL-22* showed their highest constitutive expression in the intestine in comparison to expression levels in other tissues (Costa et al., 2012). In the latter study, *in vitro* stimulation with PMA induced a strong up-regulation of *IL-17A/F* and *IL-22* in intestinal cells, whereas *A. salmonicida* only triggered *IL-22* but not *IL-17A/F* expression. On the contrary, most of the *IL-17* genes identified in pufferfish (Korenaga et al., 2010) or tongue sole (*Cynoglossus semilaevis*) (Chi and Sun, 2015) showed very low or even absent transcription in the intestine. Thus there seem to be important species-related differences in the role that Th17 cells (and other IL17-secreting cells) behave in the intestine.

IL-21 has also been identified in different teleost species such as pufferfish (Bird et al., 2005), spotted green pufferfish (*Tetraodon nigroviridis*) (Wang et al., 2006a) or salmonids (Wang et al., 2011), where it is constitutively expressed in the intestine. In spotted green pufferfish, *IL-21* transcription in the intestine was further up-regulated in response to LPS (Wang et al., 2006a) and in fugu, in response to poly I:C (Bird et al., 2005). In trout, functional assays performed with recombinant IL-21 demonstrated that it could induce the transcription of *IFN-γ*, *IL-10* and *IL-22* (Wang et al., 2011). Although mammalian IL-21 is able to modulate the expression of IFN-γ, IL-10 and IL-22 in different cell types, the simultaneous up-regulation of these three genes in head kidney and spleen cells by rainbow trout IL-21 is quite surprising and needs to be clarified.

4.5. Immune regulation associated to Treg cells

FoxP3 is a master transcription factor in Treg cells. Expression of FoxP3 homologous transcripts has been recorded in the gut of several fish species such as rainbow trout (Wang et al., 2010b), grass carp (Yang et al., 2012) and Nile tilapia (Wei et al., 2013) suggesting the presence of Treg cells in the fish intestine. However, relatively high constitutive expression levels of *FoxP3a* in trout muscle suggest some differences in the functionality of mammalian and fish FoxP3 (Wang et al., 2010b).

IL-10 is an important regulator of the mammalian Th1/Th2/Th17 balance and it is known to mediate the immunosuppressive action of Treg cells. IL-10 is known to be an anti-inflammatory cytokine and is produced by multiple leukocyte populations. While inhibiting Th1/Th17 immune responses it stimulates type 2 immune responses such as the proliferation of B cells. Knockout studies in mice suggested that this cytokine is an essential immunoregulator in the intestinal tract. Due to its anti-inflammatory properties, IL-10 is crucial in mucosal immunotolerance. In the mammalian intestine, complex interactions between the microbiota and the mucosal immune system are controlled by Treg cells through the secretion of IL-10 and TGF-β (reviewed by Harrison and Powrie, 2013; Kole and Maloy, 2014). *IL-10* homologues have been cloned in a number of fish species. There are several studies in fish where environmental changes or nutritional disbalance leads to alterations in the expression of *IL-10* in the fish gastro-intestinal tract. For example, chronic inflammation was observed in the proximal intestine of Atlantic salmon after long-term hypoxic conditions. In parallel, increased levels of *IL-10* expression were recorded probably as a compensatory anti-inflammatory reaction (Niklasson et al., 2011). In grass carp, a phenylalanine-imbalanced diet results in decreased *IL-10* levels in the intestine thus promoting proinflammatory processes (Feng et al., 2015). Similar observations have been made in carp (Uran et al., 2008) and Atlantic salmon (Lilleeng et al., 2009) that developed enteritis along with decreased *IL-10* and *TGF-β* expression after being fed with a soybean meal diet.

5. Role of T cells in mucosal tolerance

Mucosal tolerance is a phylogenetically acquired feature avoiding inadequate immune responses to harmless food-, air- or waterborne antigens and to parasites, since inappropriate immune reactions to those antigens may result in allergic reactions. Recent studies have shown that peripheral non-responsiveness to orally administered antigens is preceded by a transient T-cell activation and is primarily due to the induction of functional T cell anergy (Sun et al., 1999). Additionally, the microenvironment of the GALT plays a central role in oral tolerance by supporting the growth of Treg cells that maintain intestinal homeostasis regardless of constant antigenic challenge. Furthermore, a recent study has also provided *in vivo* evidence for a major role of γδ IELs in the modulation of oral tolerance during food allergy (Frossard et al., 2015). With regard to tolerance, it is also interesting to note that mammalian M cells, indirectly contribute to oral tolerance, especially against bacteria (reviewed by Pabst and Mowat, 2012). In mammals, M cells are important antigen sampling cells in the gut. In human intestine, they form pockets at their proximal ends that are filled with B and T (mainly $CD4^+$) cells (Farstad et al., 1994). The pockets are thought to be the place where leukocytes primarily encounter antigens taken up by M cells. Interestingly, M-like cells were detected in the gut of salmonids where they were found to be associated with IELs (Fuglem et al., 2010).

In fish, mucosal tolerance is thought to be one of the reasons why bath or oral immunization is difficult to establish, especially when a Th1 response is crucial in combating the infection. Oral tolerance was reported in fish after repeated administration of antigens in Atlantic salmon (Piganelli et al., 1994; Udey and Fryer, 1978), rainbow trout (Davidson et al., 1994) and common carp (Joosten et al., 1997; Rombout et al., 1989). However, the mechanisms underlying these observations have not been clarified. In this context, it was recorded that repeated anal and oral intubation of rainbow trout with allogeneic cells induces allospecific cell-

mediated cytotoxic tolerance in recipients (Sato and Okamoto, 2007; Sato et al., 2005). Similarly, one bath administration of either plasmid DNA, β-glucan or lactoferrin to naïve fish induced the transcription of several cytokines such as TNF-α or IL-1β, while these differences were no longer observed after four bath treatments (Zhang et al., 2009). Thus, repeated booster vaccinations by mucosal routes (oral, spray or bath) may lead to tolerance against the corresponding pathogen and this is something that should be considered in vaccination strategies in fish. However in mammals, oral antigens tending to provoke an undesirable tolerance can still induce an effective adaptive immune response when administered along with adjuvants that trigger a pro-inflammatory state (Nagler-Anderson, 2000).

The immune-tolerant environment of the mammalian intestine is largely controlled by Treg cells (reviewed by Harrison and Powrie, 2013; Kole and Maloy, 2014). A Treg-like phenotype ($CD4^-2^+CD25^+Foxp3^+$) has been described in pufferfish and depletion of this subset resulted in an enhanced mixed lymphocyte reaction (MLR) and NCC activity *in vitro*, and inflammation of the intestine *in vivo* (Wen et al., 2011). It is extremely important to undertake additional studies of Treg responses associated to tolerance in fish, as this is a key issue to be taken into consideration when designing mucosal vaccines.

6. Conclusions

There are still many aspects of T cell function in teleosts that remain to be clarified, however, a great effort has been made in the past years by identifying orthologs of mammalian genes implicated in T cell function and by producing antibodies against T cell markers that allow the isolation and further characterization of these cells at a functional level. Besides, fish intestinal immunology constitutes one of the main research fields in which future fish immunology should focus, as the results obtained in this area will be extremely relevant for the development of oral vaccines where unstable vaccine antigens and oral tolerance may hamper vaccine efficacy. Intensified adjuvant research may, however, solve the issue of oral tolerance. Furthermore, studies on intestinal immune responses would also be of significance to researchers dealing with fish nutrition. One of the major challenges in this field is to overcome the need of fish meal and fish oil for saving natural resources, while the inclusion of plant proteins and oils in fish feed can induce gastroenteritis in fish. Since intestinal T lymphocytes are not only known to be implicated in triggering local and systemic immune responses but also in tolerance induction, whatever we can learn from their function within the teleost digestive tract will surely be important in the understanding of the whole immunity network in fish.

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Identification of Teleost Skin CD8α$^+$ Dendritic-like Cells, Representing a Potential Common Ancestor for Mammalian Cross-Presenting Dendritic Cells

Aitor G. Granja, Esther Leal, Jaime Pignatelli, Rosario Castro, Beatriz Abós, Goshi Kato, Uwe Fischer and Carolina Tafalla

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Identification of Teleost Skin CD8α$^+$ Dendritic-like Cells, Representing a Potential Common Ancestor for Mammalian Cross-Presenting Dendritic Cells

Aitor G. Granja,* Esther Leal,* Jaime Pignatelli,* Rosario Castro,* Beatriz Abós,* Goshi Kato,† Uwe Fischer,† and Carolina Tafalla*

Although fish constitute the most ancient animal group in which an acquired immune system is present, the presence of dendritic cells (DCs) in teleosts has been addressed only briefly, and the identification of a specific DC subset in teleosts remained elusive because of the lack of specific Abs. In mice, DCs expressing CD8α$^+$ in lymphoid tissues have the capacity to cross-present extracellular Ags to T cells through MHC I, similarly to tissue-derived CD103$^+$ DCs and the human CD141$^+$ DC population. In the current study, we identified a large and highly complex subpopulation of leukocytes coexpressing MHC class II and CD8α. This CD8α$^+$ MHC II$^+$ DC-like subpopulation constituted ∼1.2% of the total leukocyte population in the skin, showing phenotypical and functional characteristics of semimature DCs that seem to locally regulate mucosal immunity and tolerance in a species lacking lymph nodes. Furthermore, we identified trout homologs for CD141 and CD103 and demonstrated that, in trout, this skin CD8$^+$ DC-like subpopulation expresses both markers. To our knowledge, these results provide the first evidence of a specific DC-like subtype in nonimmune tissue in teleosts and support the hypothesis of a common origin for all mammalian cross-presenting DCs. ***The Journal of Immunology*****, 2015, 195: 1825–1837.**

Dendritic cells (DCs) belong to the family of professional APCs, together with macrophages and B lymphocytes. DCs have a superior capacity to present Ags through MHC class I and II to T lymphocytes and induce their activation and proliferation, consequently triggering CD4$^+$ and CD8$^+$ T cell responses. Together with their role in inducing specific immunity against invading pathogens, mammalian DCs maintain tolerance to self- or innocuous Ags (1).

Although teleost fish constitute the first animal group in which an adaptive immune system is present, a phenotypically distinct DC subtype has not been identified. A few previous studies showed indirect evidence that suggested the presence of DCs in fish. In salmonids, the description of cells containing Birbeck granules (2), cells with DC-like morphology (3), or cells expressing transcripts of DC markers, such as CD208/LAMP3 (4), suggested the presence of DCs. In rainbow trout (*Oncorhynchus mykiss*), a mammalian protocol was adapted to obtain hematopoietic cultures enriched in cells that showed a greater capacity than B cells or macrophages to stimulate proliferation in an MLR (5). These cells had many characteristic features of mammalian DCs, such as phagocytic capacity, responsiveness to TLR ligands, the expression of DC marker genes (CD83, CD209) or molecules (MHC class II), and migratory capacities. In zebrafish (*Danio rerio*), a leukocyte subpopulation enriched from lymphoid tissues by affinity to the lectin peanut agglutinin was shown to possess morphological and functional features of mammalian DCs (6). In this same species, enrichment of lymphoid cultures by CD209/DC-SIGN expression defined a cell type that coexpressed MHC class II, CD80/86, and CD83 (7). Despite these previous studies, the identification of a specific DC subset in teleosts remained because of the lack of specific Abs.

In mammals, DC subsets differ in morphology, anatomical location, surface markers, use of transcription factors, and functionality; consequently, this cellular divergence has greatly complicated a complete understanding of their role in the initiation of the adaptive immune response. DCs can be divided into two major subtypes: nonlymphoid tissue-migratory and tissue-resident DCs, sometimes categorized as conventional DCs (cDCs) and plasmacytoid DCs. cDCs are implicated in maintaining tolerance and triggering adaptive immune responses against invading pathogens (1, 8, 9), whereas plasmacytoid DCs are primarily focused on secreting type I IFN in response to viral infections (10). Among cDCs, murine lymphoid tissue–resident DCs include two major subsets defined as CD4$^+$CD8α$^-$CD11b$^+$ and CD8α$^+$CD4$^-$CD11b$^-$ DCs, respectively. Recently, an additional CD4$^-$CD8α$^-$CD11b$^+$ DC subtype was described (9). In the same species, CD8α$^+$ DCs congregate in T cell areas of secondary lymphoid tissues, whereas they are absent from nonlymphoid tissues (11) and are considered a nonmigratory DC population derived from blood-borne DC precursors (12) that is specialized in MHC class I presentation and cross-presentation of cell-associated Ags (13). In contrast, different tissue DC subsets coexist in environmental surfaces, such as the skin. Langerhans cells, the first DC subpopulation described (14), express high levels of langerin, a C-type lectin receptor,

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The online version of this article contains supplemental material.

Abbreviations used in this article: cDC, conventional DC; DC, dendritic cell; EST, expressed sequence tag; FSC, forward scatter; IRF, IFN regulatory protein; poly I:C, polyinosinic-polycytidylic acid; SSC, side scatter; TNP–KLH, TNP hapten conjugated to the keyhole limpet hemocyanin carrier protein; VHSV, viral hemorrhagic septicemia virus.

and CD1a (9). Langerin is also expressed in lymphoid CD8α$^+$ DCs and on a subpopulation of tissue DCs that coexpress high levels of CD103$^+$ (15). This CD103$^+$ subpopulation has a high migratory capacity and is able to efficiently cross-present Ags to CD8$^+$ T lymphocytes (16). Recent data suggest that, in mice, both of these cross-presenting DCs, lymphoid CD8α$^+$ DCs and tissue-derived CD103$^+$ DCs, form a common DC lineage. The functionality of these two DC subtypes is dependent on similar transcription factors (17), and they are derived exclusively from pre-DCs under the control of Flt3 ligand, IFN regulatory protein (IRF)8 (18, 19), and Batf3 (20, 21). Additionally, both populations use the CLEC9A lectin to recognize necrotic cells (22) and express the chemokine receptor XCR1 (23). They also express higher levels of TLR3 than do other DC subtypes and, consequently, are responsive to virus-derived intracellular dsRNAs (24). In humans, a discrete CD141$^+$ (BDCA-3 or thrombomodulin) DC population in blood also exhibits a cross-presenting function (25). This cell population also expresses CLEC9A (26), XCR1 (23), TLR3 and IRF8 (27), suggesting them as an equivalent of mice cross-presenting cells. In light of these results, some investigators predicted that a CD11c^{hi}CD1a$^+$CD141^{hi}CLEC9A$^+$ TLR3^{hi} DC subtype should exist in nonlymphoid human tissues (28). In 2012, Haniffa et al. (29) succeeded in identifying this population in human skin, skin-draining lymph nodes, liver, and lung. Through an extensive and detailed phenotypic identification of diverse DC subsets, the investigators demonstrated that human skin CD141^{hi} DCs are closely related to human blood CD141$^+$ DCs and to mice cross-presenting cells (lymphoid CD8α$^+$ DCs and CD103$^+$ migratory DCs) (29). Such a cross-presenting DC subtype in human epithelia was identified as one of the main targets for clinical studies aimed at inducing efficient CD8$^+$ T cell responses against intracellular pathogens and tumors (30).

In the current study, we identified for the first time, to our knowledge, a specific DC-like subpopulation in teleosts that is characterized by surface expression of MHC class II and CD8α. These CD8α$^+$MHC II$^+$ cells with DC-like morphology were identified in a number of tissues but were found to be most abundant in the skin, providing the first evidence of a functional dendritic-like cell in a nonlymphoid tissue. These cells did not express transcripts of T cell markers (CD3, TCRα, TCRβ, CD8β), but they showed high transcription levels of characteristic DC markers, such as DC-SIGN or LAMP3. Additionally, trout skin CD8α$^+$MHC II$^+$ cells were phagocytic for both polystyrene beads and apoptotic cells and were responsive to TLR ligands and to in vivo stimulation, regardless of presenting a semimature profile with high levels of CCR7 surface expression and intermediate levels of CD83 and costimulatory molecules (1). The data presented in the current study lay the foundation for future studies aimed at identifying additional DC markers for the differentiation of other DC subsets in teleosts that would permit functional studies to understand how Ag presentation is regulated in lower vertebrates. Importantly, the identification of CD141 and CD103 expression in this tissue-resident CD8α$^+$ subpopulation that expresses common unique markers of cross-presenting DCs, such as TLR3, Batf3, and IRF8, strongly supports the hypothesis of a common ancestor for vertebrate cross-presenting DCs.

Materials and Methods

Generation and proteomic characterization of an anti-trout MHC class II mAb

Massive immunizations of 6–8-wk-old BALBc mice were performed using large amounts of Ags from rainbow trout head kidney. Animals were immunized i.v. at days 0, 15, 30, and 45. Mice were sacrificed 3 d after the last immunization, and splenocytes were isolated. Generation of hybridomas by fusion of mouse splenocytes with SP2 myeloma cells, isolation of clones, and purification of specific anti-trout mAbs were performed as previously described (31). Rainbow trout head kidney protein lysates were used to test the specificity of specific Abs. The clone 1A1-5C10 mAb recognized a protein ~30 kDa in Western blotting (Supplemental Fig. 1A) and immunoprecipitation assays (Supplemental Fig. 1B), which were performed as previously described (32). For the identification of such Ag, a similar immunoprecipitation was performed, and the 30-kDa band from silver-stained gels was excised manually, deposited in 96-well plates, and processed automatically in a PROTEINEER DP (Bruker Daltonics). The digestion protocol used was described previously (33). Digested peptides of each sample were subjected to one-dimensional nano-liquid chromatography–electrospray ionization/multistage mass spectrometry analysis using a nano-liquid chromatography system (Eksigent Technologies, AB Sciex) coupled with a high-speed TripleTOF 5600 mass spectrometer (AB Sciex) with a DuoSpray Ion Source. The analytical column used was a silica-based reversed-phase column C18 ChromXP 75 μm × 15 cm, with 3 μm particle size and 120 Å pore size (AB Sciex), which was switched on-line with a C18 ChromXP trap column. Mass spectrometry and multistage mass spectrometry data were acquired using information-dependent acquisition mode with Analyst TF 1.5.1 Software (AB Sciex). For information-dependent acquisition parameters, a 0.25-s MS survey scan in the mass range of 350–1250 Da was followed by 35 MS/MS scans of 100 ms in the mass range of 100–1800 (total cycle time: 3.8 s). Raw data file-conversion tools generated mgf files that also were searched against the National Center for Biotechnology Information database with "*Oncorhynchus mykiss* + *Salmo salar* + *Mus musculus*" taxonomy restriction, containing 530,414 protein-coding genes, using Mascot Server version 2.5 (Matrix Science). Search parameters were set as follows: carbamidomethyl (C) as fixed modification and oxidation (M) as variable modification. Peptide mass tolerance was set to 25 ppm and 0.05 Da for fragment masses, and one missed cleavage was allowed. Only the peptides with an individual molecular weight search score ≥ 20 were considered correctly identified.

Experimental fish

Female rainbow trout (*O. mykiss*) ~ 50 g were obtained from Centro de Acuicultura El Molino (Madrid, Spain) and maintained at the animal facilities of the Centro de Investigación en Sanidad Animal in an aerated recirculating water system at 16°C, with a 12:12-h light/dark photoperiod. Fish were fed twice a day with commercial food (Skretting) and were acclimatized to laboratory conditions for ≥2 wk prior to any experimental procedures. All of the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and were approved by the Instituto Nacional de Investigación Agraria y Alimentaria Ethics Committee.

Tissue sampling

Rainbow trout were killed by MS-222 (Sigma) overdose, and blood was extracted with a heparinized needle from the caudal vein and diluted 10 times with Leibovitz medium (L-15; Invitrogen) supplemented with 100 IU/ml penicillin together with 100 μg/ml streptomycin (Life Technologies), 10 U/ml heparin (Sigma), and 5% FCS (Life Technologies). Spleen, head kidney, thymus, intestine, gills, and skin were collected. Single-cell suspensions from spleen, head kidney, gills, and thymus were obtained using 100-μm nylon cell strainers (BD Biosciences). The intestines were opened lengthwise, washed in PBS, and cut into small pieces. The skin was cut off the fish with a scalpel, muscle tissue was removed, and the skin was cleaned with ice-cold PBS and cut into small pieces. For both tissues, the cell-extraction procedure started with 30 min agitation at 4°C in L-15 medium with penicillin and streptomycin and 5% FCS, followed by agitation in PBS with 1 mM EDTA and 1 mM DTT for 30 min. Finally, the tissues were digested with 0.15 mg/ml collagenase (Sigma) in L-15 for 1.5 h at 20°C. All cell suspensions were placed onto 30/51% discontinuous Percoll (GE Healthcare) density gradients and centrifuged at 500 × *g* for 30 min at 4°C. The interface cells were collected and washed twice in L-15 containing 5% FCS. When required, cells were treated with LPS (100 μg/ml), polyinosinic-polycytidylic acid (poly I:C; 50 μg/ml), or TNP hapten conjugated to the keyhole limpet hemocyanin carrier protein (TNP–KLH; 5 μg/ml) for 16 h.

Flow cytometry

The anti-trout CD8α (mAb rat IgG; 7 μg/ml) and the anti-trout CCR7 (pAb rabbit IgG; 2 μg/ml) used in this study were characterized previously (34, 35). The anti-trout MHC class II β-chain (mAb mouse IgG1; 2 μg/ml) was characterized above. Primary cells were incubated for 30 min with primary Abs, washed twice with staining buffer (PBS containing 1% FCS and 0.5% sodium azide), and stained for 20 min with secondary Abs for anti-CD8α

[R-PE F(ab′)$_2$ fragment of goat anti-rat IgG (H+L); Life Technologies] and anti-CCR7 [Alexa Fluor 488 F(ab′)$_2$ fragment of goat anti-rabbit IgG (H+L); Life Technologies], when needed. An Alexa Fluor 647 fluorescently-labeled version of MHC class II Ab was used for flow cytometry (previously conjugated using the Alexa Fluor 647 Protein Labeling Kit; Life Technologies). After incubation, cells were washed three times with staining buffer and analyzed on a FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuest Pro software. Cell populations were sorted by flow cytometry on a BD FACSAria III (BD Biosciences), using their forward scatter (FSC)/side scatter (SSC) and fluorescence characteristics. In all cases, isotype controls for mouse mAbs, rat mAb, and rabbit polyclonal Ab (BD Biosciences) were tested in parallel to discard unspecific binding of the Abs. Flow cytometry analysis was performed with FlowJo 10 (TreeStar).

Transcriptional studies

Total cellular RNA was isolated from trout spleen- and skin-sorted populations or the RTS11 (rainbow trout macrophages) cell line (36) using the Power SYBR Green Cells-to-CT Kit (Invitrogen), following the manufacturer's instructions. RNA was treated with DNase during the process to remove genomic DNA that might interfere with the PCR reactions. Reverse transcription also was performed using the Power SYBR Green Cells-to-CT Kit (Invitrogen), following the manufacturer's instructions. To evaluate the levels of transcription of the different genes, real-time PCR was performed with a LightCycler 96 System (Roche) using SYBR Green PCR Core Reagents (Applied Biosystems) and specific primers (Supplemental Fig. 2). Each sample was measured in duplicate under the following conditions: 10 min at 95°C, followed by 40 amplification cycles (15 s at 95°C and 1 min at 60°C). A melting curve for each PCR was determined by reading fluorescence at every degree between 60 and 95°C to ensure that only a single product had been amplified. The expression of individual genes was normalized to the relative expression of trout housekeeping gene EF-1α elongation factor, and the expression levels were calculated using the $2^{-\Delta Ct}$ method, where ΔCt is determined by subtracting the EF-1α value from the target Ct. No template negative controls were included in all of the experiments.

Phagocytic activity

For the analysis of bead phagocytosis, skin leukocytes were seeded in 24-well plates (Nunc) at a cell density of 1×10^6 cells/well and incubated for 16 h at 20°C with fluorescent beads (FluoSpheres Microspheres, 1.0 μm, Crimson Red Fluorescent 625/645, 2% solids; Life Technologies) at a cell/bead ratio of 1:10 or without beads as negative controls. Cells were harvested using a standard cell scraper (Corning). Noningested beads were removed by centrifugation (100 × *g* for 10 min at 4°C) over a cushion of 3% (w/v) BSA (Fraction V; Fisher Scientific) in PBS supplemented with 4.5% (w/v) D-glucose (Sigma). Cells were resuspended in staining buffer, labeled with the flow cytometry Abs specified for each assay, and analyzed on a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences).

For the analysis of phagocytosis of apoptotic/dead cells, single-cell suspensions from spleen were obtained as described above, and the resulting splenocytes were labeled with CellTrace Far Red stain (Life Technologies) at a final concentration of 1 μM, following the manufacturer's instructions. Labeled cells were incubated for 16 h at 20°C in the presence of 1 μM A23187 calcium ionophore to induce apoptosis, as verified by >90% Annexin-V positive staining of splenocytes. Then, fluorescently labeled apoptotic splenocytes were added to skin leukocytes or RTS11 macrophages disposed into 24-well plates (Nunc) at a density of 1×10^6 cells/well and a effector/apoptotic cell ratio of 1:10. Plates were incubated for 16 h at 2 or 20°C. Cells were harvested using a standard cell scraper (Corning) and resuspended in staining buffer. For flow cytometric analysis, cells were stained for CD8α, and the percentages of CellTrace Far Red$^+$ CD8$^+$ leukocytes and CellTrace Far Red$^+$ RTS11 macrophages were calculated by subtracting the frequency of positive events at 2°C (binding) from the frequency at 20°C (uptake).

In vivo stimulations

Rainbow trout were nonlethally challenged with viral hemorrhagic septicemia virus (VHSV) through bath infection. Briefly, groups of three fish ~ 50 g were transferred to 2 l viral solution containing 5×10^5 $TCID_{50}$/ml VHSV, strain 0771. After 1 h of viral adsorption at 16°C, fish were transferred to their original tanks (20 l) and maintained for 16 h. In other experiments, groups of three rainbow trout were maintained in 10-l tanks and exposed to zymosan particles in the water at a final concentration of 0.01% (w/v) for 16 h. After treatment, fish were sacrificed by overexposure to MS-222. Skin was sampled and processed for flow cytometry analysis and for extraction of RNA for subsequent gene expression analysis. Mock-treated groups maintained under the same experimental conditions were included in the VHSV and zymosan experiments.

Immunofluorescence assay

Skin leukocyte suspensions obtained from rainbow trout of clone C25 were used for the immunofluorescence analysis of CD8α$^+$MHC II$^+$ cells. The separated leukocytes were seeded on a poly-L-lysine–coated slide and incubated at 16°C for 30 min. After gently washing with the culture medium, the slides were dried and fixed in ice-cold acetone for 5 min. Fixed cell slides were incubated with mAbs against trout CD8α and MHC class II diluted with the culture medium for 1 h at 4°C. Slides were washed with the culture medium and incubated with anti-rat IgG (H+L) Alexa Fluor 488 conjugate and anti-mouse IgG1 Alexa Fluor 568 (both from Life Technologies) for 30 min at 4°C. Slides were counterstained with Hoechst 33342 (Life Technologies) and examined with an ECLIPSE Ti-S Inverted Research Microscope (Nikon, Tokyo, Japan). Digital images were captured using NIS-Elements BR 3.2 software (Nikon).

Immunohistochemistry and confocal microscopy

Rainbow trout skin from anesthetized and exsanguinated rainbow trout was embedded in PolyFreeze cryostat mounting medium (Sigma), immediately snap-frozen in liquid nitrogen, and stored at −80°C until used. Cryostat sections with a thickness of 20 μm were prepared using a Leica CM3050 microtome and placed on SuperFrost glass slides (Menzel-Gläser). Dry sections were fixed in acetone for 10 min, air-dried, encircled with a hydrophobic compound (ImmunoPen; Calbiochem), incubated for 1 h at room temperature with a blocking solution (TBS buffer [pH 7.5], containing 0.01% BSA, 0.02% Tween-20, and 10% rabbit and goat serum), and stained with Abs against trout MHC class II β-chain (Alexa Fluor 647 fluorescently-labeled version of MHC class II Ab) and trout CD8α (mAb rat IgG). Samples were washed and incubated with the CD8α secondary Ab Alexa Fluor 488 F(ab′)$_2$ fragment of goat anti-rat IgG (H+L) (Life Technologies). Samples were counterstained with 1 μg/ml DAPI (Sigma). Three-dimensional image stacks (12 μm thickness) were acquired with an inverted Zeiss Axiovert LSM 510 META microscope. Images were analyzed with Volocity (Improvision), Imaris (Bitplane), and ImageJ (National Institutes of Health); maximum-intensity projections of the three-dimensional stacks are shown.

Scanning electron microscopy

Sorted skin CD8$^+$MHC II$^+$ cells from the myeloid gate were plated on poly-L-lysine–coated cover glasses before being fixed with 2.5% (v/v) paraformaldehyde in PBS for 10 min at room temperature. After three washes in PBS, cells were subjected to sequential dehydration with gradient ethanol (50, 70, 90, and three times at 100%; 5 min for each procedure), followed by isoamyl acetate treatment (three times, 5 min each). Critical-point drying with CO_2 was performed, samples were attached to stub holders and coated with gold, and a scanning electron microscope (JEOL JSM-7600F) was used for observation.

MLR

Skin CD8$^+$ dendritic-like cells were isolated by cell sorting, as described above. The cells were cultured for 12 h in L-15 medium supplemented with 5% FCS in the absence or the presence of the T cell–dependent Ag TNP–KLH. Because there are no Abs against extracellular pan-T cell markers available for rainbow trout, we used T cell–enriched cultures as responder cells. These T cell–enriched cultures from isogeneic and allogeneic splenocytes were obtained by depletion of splenocytes labeled with anti-IgM, anti-MHC class II mAbs and mAbs specific for thrombocytes (37) and myeloid cells (38), using flow sorting. The resulting negative population, representing ~10% of splenocytes, was labeled with CellTrace Far Red (Life Technologies). The enrichment in T cells was assessed by intracellular staining with an anti-CD3 Ab (39), which showed >90% of CD3$^+$ cells in the cultures. DCs were cocultured with isogeneic (auto-MLR) or allogeneic (allo-MLR) T cell–enriched splenocytes at a 1:50 DC/splenocyte ratio. After 24 h, cells were harvested, and total RNA was isolated for gene expression analysis, as described above. In parallel, 4 d after the MLRs were set up, cocultured samples were analyzed by flow cytometry to measure cell proliferation through the degree of dilution of the CellTrace Far Red vital marker.

Identification of the CD141 gene in rainbow trout

The human CD141 protein sequence was used as a query against expressed sequence tag (EST) databases from rainbow trout (*O. mykiss*) in the

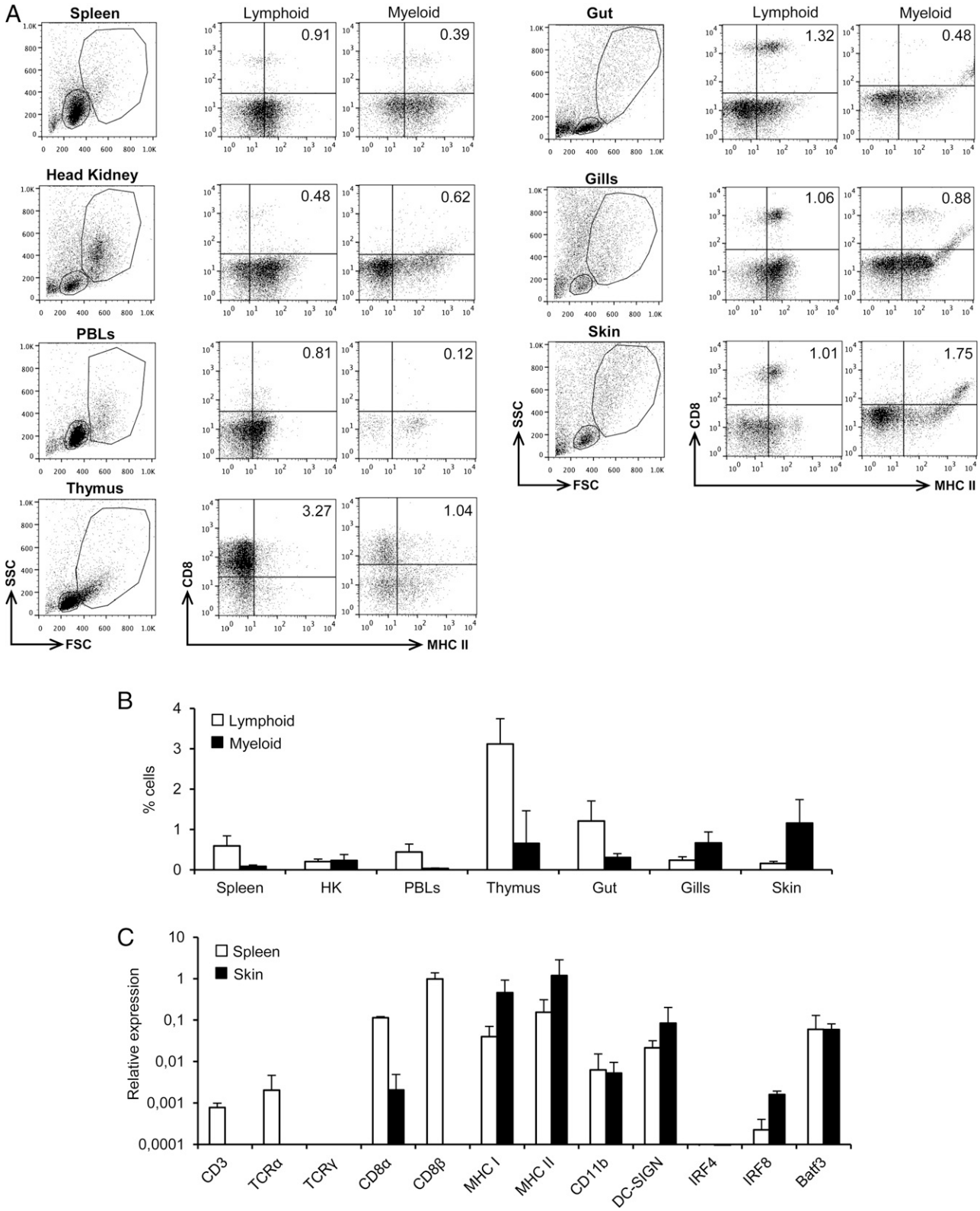

FIGURE 1. Distribution of CD8α+MHC II+ cells among leukocytes from different organs. (**A**) Flow cytometry analysis of rainbow trout leukocytes isolated from trout tissues (spleen, head kidney, PBLs, thymus, gut, gills, and skin) and stained with anti-CD8α and anti–MHC class II β-chain mAbs. For each individual tissue, FSC/SSC profiles are shown (*left panels*) and gates for lymphoid and myeloid cells were defined. Two-color CD8/MHC class II dot plots of gated cells are also shown (*right panels*). Percentage of CD8α+MHC II+ cells among the total number of cells is shown in the upper right corner. (**B**) Mean (± SD) percentages of CD8α+MHC II+ cells from three independent experiments (n = 9 fish). (**C**) The levels of transcription of several immune markers known in rainbow trout were analyzed by real-time PCR. Myeloid CD8α+MHC II+ cells from spleen and skin were isolated by cell sorting, and RNA was obtained. The relative expression of the indicated genes in relation to the endogenous control EF-1α was calculated for each sample, and the mean values (± SD) from three independent experiments, containing three animals/experiment, were calculated.

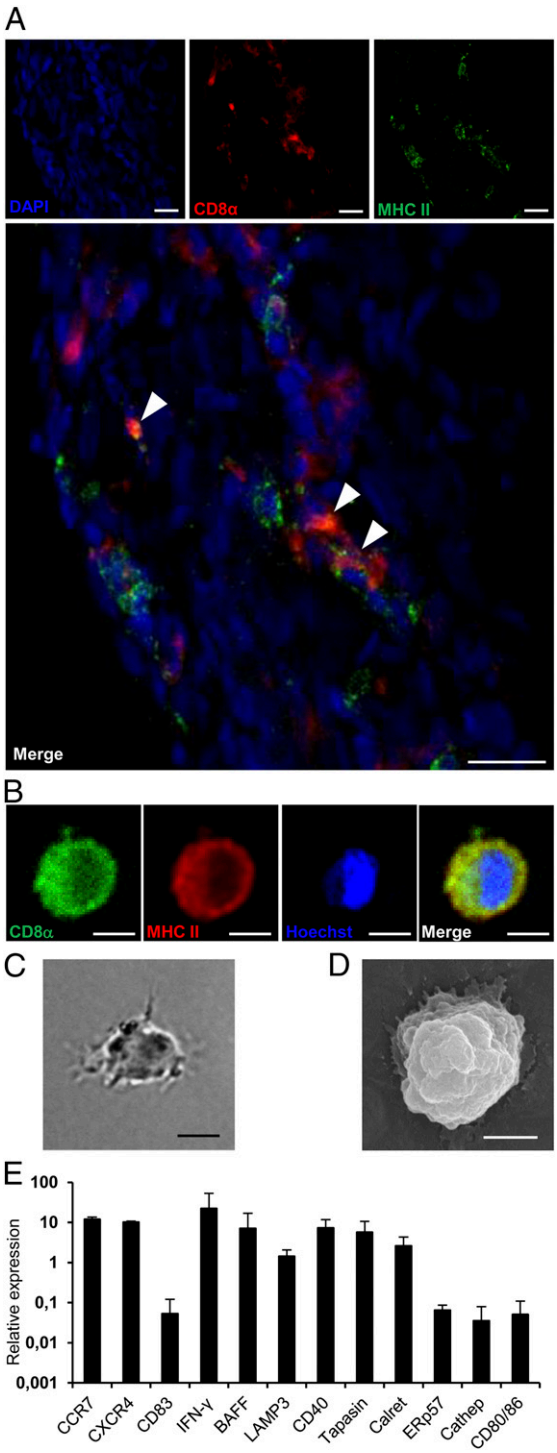

FIGURE 2. Location, morphology and gene expression of skin CD8α$^+$ MHC II$^+$ cells. (**A**) For immunofluorescence analysis, cryostat sections with a thickness of 20 μm were prepared from rainbow trout skin, fixed, and labeled with anti-CD8α (red) and anti–MHC class II (green) Abs, counterstained with Hoechst (blue), and analyzed by confocal microscopy. Arrowheads show double positive cells. Scale bars, 20 μm. (**B**) For identification of CD8α$^+$MHC II$^+$ cells, total leukocytes from skin were fixed and labeled with anti-CD8α (green) and anti–MHC class II (red) Abs, counterstained with Hoechst (blue), and analyzed by fluorescence microscopy. Scale bars, 5 μm. (**C**) CD8α$^+$MHC II$^+$ cells from skin were isolated by cell sorting and then incubated onto poly-L-lysine–treated glass slides, fixed, mounted, and analyzed by light microscopy. Scale bar, 5 μm. (**D**) Myeloid CD8α$^+$MHC II$^+$ cells from skin also were analyzed using a scanning electron microscope. Scale bar, 5 μm. (**E**) Sorted myeloid skin CD8α$^+$MHC II$^+$ cells were analyzed by real time-PCR. Relative expression of the indicated genes to the endogenous control EF-1α was calculated for each sample, and mean (± SD) values from three independent experiments, including three fish per experiment, were obtained.

National Center for Biotechnology Information's database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using tBLASTn searches. A rainbow trout EST that encoded a CD141-like sequence was identified (accession number CA377463). The sequence lacked a stop codon; therefore, 3′ RACE was performed to obtain the complete sequence using cDNA from PBLs, a 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen), and primers CD141-3′ RACE (5′-GGTGATGGTGGTGAAGGATGCGCTATGGC-3′) and CD141-3′ RACE nested (5′-GGGGCTACACATGCTCGTGTAAAGAAGG-3′). An overlapping fragment that contained the final segment of the CD141 coding sequence and the 3′ untranslated region was amplified. Primers were then designed to amplify the full coding sequence, which was again sequenced. The complete CD141 nucleotide sequence was analyzed within the ExPASy Molecular Biology server (http://us.expasy.org) and deposited in GenBank under accession number KP203844 (http://www.ncbi.nlm.nih.gov/nuccore/KP203844).

Phylogenetic analysis

Phylogenetic analyses of the CD141 and CD103 protein sequences were performed using MegAlign Software (DNASTAR, Madison, WI) and the Clustal V algorithm. Statistical parameters of phylogenetic trees were determined by bootstrap analysis using 1000 replicates.

Statistical analysis

Statistical analyses were performed using a two-tailed Student *t* test with the Welch correction when the *F* test indicated that the variances of both groups differed significantly. The differences between the mean values were considered significant on different degrees, where $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.005$ (GraphPad Prism 4 software).

Results

CD8α$^+$ cells that express MHC class II are present in all rainbow trout tissues

Through the combined use of an anti–MHC class II β-chain and an anti-CD8α Ab in flow cytometry, we observed that a considerable percentage of CD8α$^+$ cells expressed MHC class II on the cell membrane (Fig. 1A). These double-positive CD8α$^+$MHC II$^+$ cells were present in all tissues examined, including spleen, head kidney (main hematopoietic organ), thymus, gut, gills, and skin, whereas only a few were detected in blood. Because CD8α$^+$ cytotoxic T cells are not expected to express MHC class II on the cell membrane (40), and, in contrast, certain mammalian DC subsets are double positive for CD8α and MHC class II (11), our results suggested the possibility of these cells being DCs. Cells were analyzed according to their FSC/SSC profile (Fig. 1A), including cells within the lymphoid gate (FSC^{low}SSC^{low}) representing lymphocytes and thrombocytes (the latter being nucleated in teleosts) or within the myeloid gate (FSC^{high}SSC$^{low/high}$), in which larger cells, such as macrophages and neutrophils, are commonly found (41). Because of their size and morphology, DCs would be expected to appear in the myeloid gate. However, two types of CD8α$^+$MHC II$^+$ cells were found in all tissues examined: one population with lymphoid morphology and another one with myeloid morphology. Although the thymus was the organ in which the highest percentages of lymphoid CD8α$^+$MHC II$^+$ cells were identified (3.11% of total leukocytes), CD8α$^+$MHC II$^+$ cells within the myeloid gate were most abundant in skin (1.16%), followed by the gills (0.67%), the thymus (0.65%), and the gut (0.3%) (Fig. 1B). Strikingly, secondary lymphoid tissues, such as spleen and head kidney, harbor <0.1% and 0.25%, respectively, CD8α$^+$MHC II$^+$ cells within the myeloid gate, suggesting that these double-positive cells are preferentially located in peripheral mucosal tissues in teleosts.

Transcriptional profile of spleen and skin CD8α+MHC II+ cells

Seeking further evidence that CD8α+MHC II+ cells were DCs, we sorted spleen and skin CD8α+MHC II+ cells within the myeloid gate and skin CD8α+MHC II+ cells from the lymphoid gate following the gating strategy described in Supplemental Fig. 3A and 3B. Sorted cells were analyzed on their expression of different signature genes through real-time PCR. Skin myeloid CD8α+ MHC II+ cells showed no CD3, TCR-α, TCR-γ, or CD8β transcription, ruling out the possibility of them being cytotoxic T cells (Fig. 1C). In concordance with their staining patterns, skin myeloid CD8α+MHC II+ cells expressed CD8α and MHC class II transcripts. Furthermore, these cells also transcribed DC-SIGN and CD11b (42). Interestingly, skin myeloid CD8α+MHC II+ cells also transcribed IRF8 and Batf3, which are essential for the development of mammalian cross-presenting DCs (18–21), but not IRF4, which is implicated in the development of other CD8− DC subtypes in mammals (43). Compared with skin CD8α+MHC II+ cells, a different transcription profile was recorded for splenic myeloid CD8α+MHC II+ cells, showing significant CD8β, CD3, and TCR-α mRNA expression levels, suggesting the presence of mixed cell populations (Fig. 1C). Although no TCR transcripts were detected in skin CD8α+MHC II+ cells from the lymphoid gate, CD3 transcripts were found (Supplemental Fig. 3C). Consequently, further research needs to be undertaken to clarify the nature of these populations. When the transcriptional profile of skin CD8α+MHC II+ cells from the myeloid gate was compared with that of CD8α+ cytotoxic T cells from spleen (CD8α+ MHC II− cells from the lymphoid gate), we verified that skin CD8α+ MHC II+ cells had a distinct DC-like profile (Supplemental Fig. 4). CD8α+ cytotoxic T cells expressed typical signature transcripts (i.e., CD3, TCR-α, TCR-γ, CD8α, CD8β, and MHC class I), whereas they were unable to transcribe other DC markers, such as MHC class II or DC-SIGN. In contrast, to rule out the possibility of these cells being macrophages, their transcriptional profile was compared with that of rainbow trout macrophages from the RTS11 cell line. Although macrophages transcribed MHC class I, MHC class II, and DC-SIGN as expected, they did not transcribe CD8α (Supplemental Fig. 4), in agreement with the fact that they do not express CD8α protein on the cell surface (data not shown) or Batf3. Overall, our transcriptional results strongly support that skin CD8α+MHC II+ cells within the myeloid gate correspond to a subset of teleost DCs, and our subsequent experiments focused exclusively on this DC-like subpopulation.

The location of CD8α+MHC II+ cells was analyzed by immunofluorescence of trout skin sections. We observed disperse myeloid CD8α+MHC II+ cells located primarily in the epidermal layer (Fig. 2A). Moreover, when we analyzed skin leukocytes by fluorescence microscopy, we observed round globular CD8α+ MHC II+ cells (Fig. 2B). To further characterize the morphology of these cells, they were sorted by flow cytometry and analyzed by

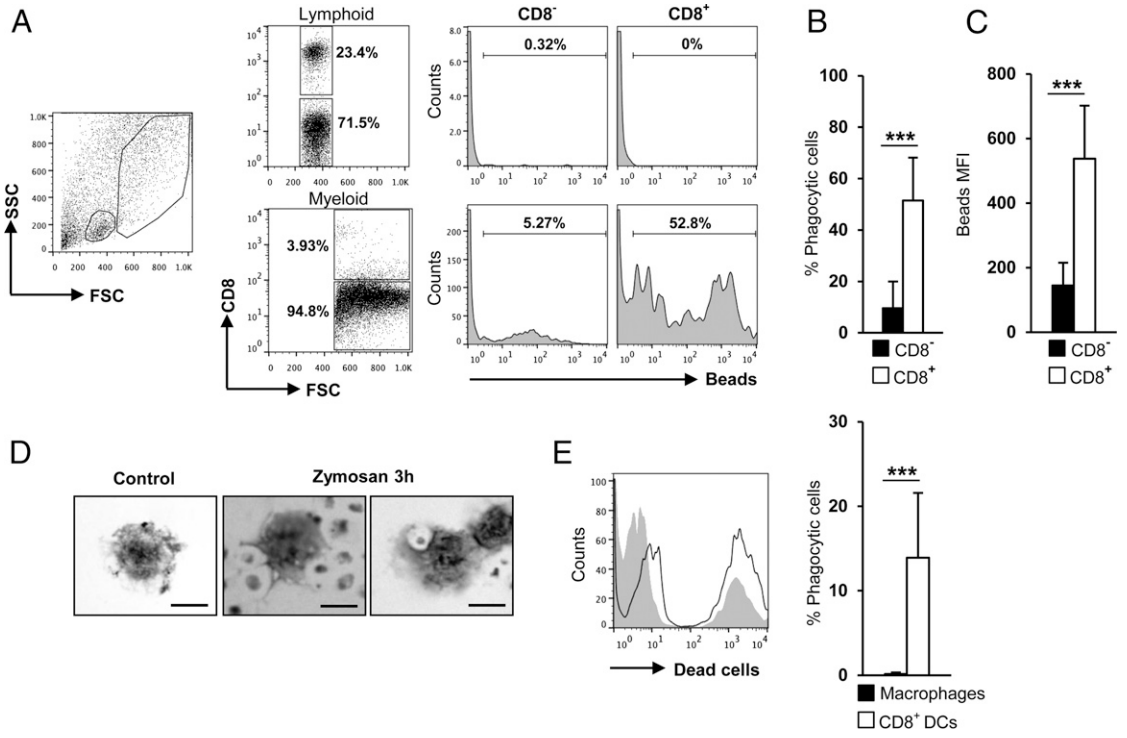

FIGURE 3. Phagocytic capacities of skin CD8+ cells. Leukocytes from trout skin were incubated with Crimson Red fluorescent polystyrene beads (1 μm diameter) at a ratio of 1:10 (cell/beads) for 16 h and then centrifuged through a 3% BSA + 4.5% glucose gradient to remove adhered beads. (**A**) Cells were stained with mAb to CD8α and analyzed by flow cytometry. Lymphoid cells (*upper panels*) and myeloid cells (*lower panels*) were gated, and CD8− and CD8+ cells were further selected to analyze and measure the fluorescence of internalized beads (line graphs). The percentages of cells containing beads were determined using control samples without beads to establish the phagocytosis gate shown in the line graphs. Average percentage of phagocytic CD8− and CD8+ cells (**B**) and median fluorescence intensity of the beads (**C**) were used to determine the phagocytic capacity of each cell type. Data are representative of nine individual fish from three independent experiments. (**D**) Giemsa staining of CD8+MHC II+ skin cells after incubation with zymosan particles for 3 h (zymosan) compared with the same cell type after incubation with control medium. Scale bar, 5 μm. (**E**) Skin leukocytes or RTS11 trout macrophages were incubated with labeled apoptotic splenocytes at a 1:1 ratio (effector/apoptotic cells), as described in *Materials and Methods*. Uptake of apoptotic cells by skin CD8+ DCs was analyzed by flow cytometry to determine the percentage of CellTrace Far Red+ CD8+ cells after subtracting the frequency of positive events at 2˚C (binding; filled graph) from the frequency at 20˚C (uptake; open graph) (*left panel*). Uptake of apoptotic cells by RTS11 macrophages was analyzed in parallel. A representative bar graph shows uptake of apoptotic cells by skin CD8+ cells and the percentage of phagocytic cells obtained in skin leukocytes and macrophages (n = 9 fish, mean ± SD). ***$p \leq 0.005$.

light microscopy (Fig. 2C) and scanning electron microscopy (Fig. 2D). Cells showed a round irregular morphology with small membrane projections, as described previously for the corresponding subpopulation of DCs in mammals (44). Interestingly, further transcriptional analysis of skin myeloid CD8α$^+$MHC II$^+$ cells revealed that they showed an activated phenotype with extremely high CCR7, CXCR4, IFN-γ, BAFF, LAMP-3, and CD40 transcription levels and intermediate levels of CD83 (Fig. 2E). Additionally, skin CD8α$^+$MHC II$^+$ cells transcribed MHC class I–associated molecules, showing high levels of tapasin and calreticulin and intermediate levels of ERp57 (Fig. 2E). Moreover, skin CD8α$^+$MHC II$^+$ cells transcribed cathepsin Z and CD80/86, known to be associated with MHC class II–dependent Ag presentation in mammals. This last molecule, with similar homologies to mammalian CD80 and CD86, previously was designated CD80/86 (45). These results reveal that skin CD8α$^+$MHC II$^+$ cells have the essential machinery for both MHC class I— and MHC class II–mediated Ag presentation. The transcriptional profile, together with the morphological characterization, led us to conclude that these skin CD8α$^+$MHC II$^+$ cells are, in fact, a teleost DC-like subpopulation.

Phagocytic capacity of teleost skin CD8$^+$MHC II$^+$ cells

Although it was widely assumed that activated DCs lose their ability to phagocytose, Platt et al. (46) demonstrated that mature DCs retain their capacity to capture, process, and present Ags internalized via endocytic receptors, whereas only their capacity to execute micropinocytosis is downregulated through maturation. Trout skin CD8$^+$MHC II$^+$ cells showed a high phagocytic capacity, in contrast to lymphoid CD8α$^+$ cells that were not phagocytic at all (Fig. 3A, 3B). After 16 h of incubation with 1 μm Crimson red–labeled polystyrene beads, ~53% of the CD8α$^+$MHC II$^+$ cells had internalized beads, whereas only ~5% of CD8α$^-$ cells within the myeloid gate had internalized beads (Fig. 3A, 3B). Additionally, the median fluorescence intensity in CD8α$^+$MHC II$^+$ cells was significantly higher than that observed in CD8α$^-$ cells within the myeloid gate (Fig. 3C), indicating that the average number of particles internalized per cell was higher for CD8α$^+$ MHC II$^+$ cells than for CD8α$^-$MHC II$^+$ cells. Sorted CD8$^+$MHC II$^+$ cells were also capable of internalizing different types of particulate Ags, such as zymosan (Fig. 3D). These results strengthen our findings that trout skin CD8α$^+$MHC II$^+$ cells within the myeloid gate are, in fact, phagocytic CD8$^+$ DCs.

A distinctive feature of mammalian cross-presenting DCs is their superior capacity to uptake apoptotic and/or dead cells (22); thus, we also compared the capacity of trout skin CD8$^+$MHC II$^+$ cells and trout RTS11 macrophages to phagocytose apoptotic cells. As shown in Fig. 3E, skin dendritic-like cells were capable of uptaking apoptotic splenocytes, whereas macrophages were not.

Response through TLRs

The pattern of TLR expression is commonly used in mammals to characterize DC subtypes. Thus, we examined the transcription levels of TLRs in skin CD8$^+$MHC II$^+$ cells, including all known trout TLR genes (i.e., TLR1, TLR2, TLR3, TLR5, TLR7, TLR8a and TLR9), together with TLR22, a fish-specific cell surface sensor of dsRNA (47). Interestingly, trout skin CD8$^+$MHC II$^+$ cells transcribed high levels of TLR1, TLR5, TLR7, TLR8a, TLR9, and TLR22, intermediate levels of TLR3, and very low levels of TLR2 (Fig. 4A). Although murine CD8$^+$ DCs do not express TLR7 (48), TLR3 is considered a signature marker for cross-presenting DCs, murine CD103$^+$ tissue and CD8$^+$ lymphoid DCs, and the human CD141$^+$ DC lineage (29). Consequently, we analyzed whether trout skin sorted CD8$^+$ dendritic-like cells responded to stimulation with poly I:C, a known ligand for teleost TLR3 and TLR22 (49), or bacterial LPS, as an irrelevant TLR3 ligand. We found that poly I:C provoked a significant upregulation of CCR7, BAFF, IFN-γ, MHC class I, MHC class II, and CD83 in the skin CD8$^+$ DC-like subpopulation, whereas no significant changes in gene expression were found after incubation with LPS (Fig. 4B). These results demonstrate that, similarly to mammalian cross-presenting DCs, trout skin CD8$^+$ dendritic-like cells are responsive to TLR3 ligands.

Teleost skin CD8α$^+$MHC II$^+$ cells express CD141 (BDCA-3/thrombomodulin) and CD103 (integrin α E)

Multiple lines of evidence point to a common cross-presenting DC lineage for mice lymphoid CD8$^+$ DCs and tissue-migratory CD103$^+$ DCs (17–19, 22–24). In humans, blood CD141$^+$ cells also cross-present Ags and exhibit markers in common with mice CD8$^+$ DCs (23, 25–27), suggesting a common origin for all mammalian cross-presenting cells (29). To investigate whether such an ancestor was present in an ancient vertebrate, such as rainbow trout, we searched the databases for trout CD141 (also designated as BCDA3 or thrombomodulin) and CD103 (also designated as integrin α E) sequences to determine whether trout skin CD8α$^+$MHC II$^+$ cells express these markers.

Using the human CD141 protein sequence, we searched within trout EST databases and identified a sequence with close homology to CD141 (GenBank accession number CA377463.1). This sequence lacked the 3′ end; thus, we conducted 3′ RACE to obtain the full coding sequence. Posterior phylogenetic analysis through neighbor joining of the predicted protein with different mamma-

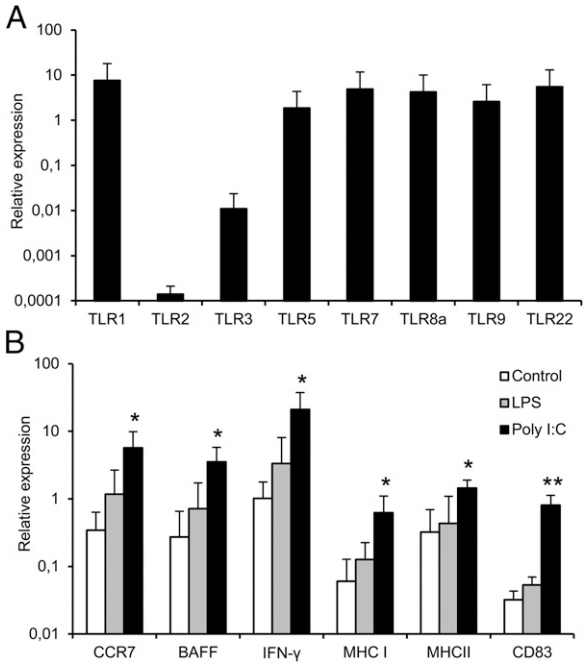

FIGURE 4. Analysis of TLR expression and responses of skin CD8$^+$ dendritic-like cells to TLR ligands. Skin CD8$^+$MHC II$^+$ cells were isolated by cell sorting, and RNA was extracted. (**A**) The expression of each indicated TLR gene relative to the endogenous control EF-1α was calculated for each sample, and mean (± SD) values from three independent experiments, containing three animals/experiment, were calculated. (**B**) Trout leukocytes from skin were purified and cultured in L-15 medium supplemented with 5% FCS for 16 h in the presence of 100 μg/ml LPS, 50 μg/ml poly I:C, or control medium. Then, CD8$^+$ dendritic-like cells were isolated by cell sorting, and RNA was obtained. The expression of the indicated genes relative to the endogenous control EF-1α was calculated for each sample, and mean (± SD) values from three independent experiments, containing three animals/experiment, were calculated. *$p \leq 0.05$, **$p \leq 0.01$.

lian and fish thrombomodulin sequences confirmed this identification (Fig. 5A). CD141 transcription was detected at extremely high levels in trout skin sorted CD8$^+$ dendritic-like cells, whereas it was not expressed in CD8α$^+$MHC II$^+$ cells from the lymphoid gate or in spleen cytotoxic T cells identified as lymphoid CD8α$^+$ MHC II$^-$ cells (Fig. 5B).

Similarly, we searched the rainbow trout EST databases for a CD103 homolog using the human CD103 sequence as a template. In this case, we found a sequence with high homology to human CD103 that contained a full coding region (GenBank accession number CDQ67442.1). Further phylogenetic analysis confirmed that this sequence was, in fact, a rainbow trout CD103 homolog (Fig. 5C). Interestingly, trout skin sorted CD8α$^+$MHC II$^+$ cells also transcribed CD103 (Fig. 5D). CD8α$^+$MHC II$^+$ cells from the lymphoid gate and splenic cytotoxic T cells also transcribed CD103, but with lower expression levels. The fact that skin DCs from an ancient vertebrate, such as the rainbow trout, coexpress CD8α, CD141, and CD103 strongly supports the existence of a common origin for all mammalian cross-presenting DCs.

Teleost skin CD8α$^+$MHC II$^+$ cells prime T cells

One of the main defining features of DCs is their capacity to stimulate T cells. To demonstrate their T cell–activating potential, we performed an MLR. For this, we sorted skin CD8α$^+$MHC II$^+$ cells and incubated them overnight in the presence of TNP–KLH (a T cell–dependent Ag) or left them unstimulated. This CD8$^+$ DC-like subpopulation was coincubated with isogeneic (auto-MLR) or allogeneic (allo-MLR) splenocyte cultures enriched in T cells from which all APCs had been depleted. As expected, the addition of CD8$^+$ dendritic-like cells to the T cell–enriched splenocyte cultures significantly induced cell proliferation in isogeneic and allogeneic cultures (Fig. 6A, 6B). The fact that dendritic-like cells were previously incubated with TNP–KLH had no effect on their T cell–activating capacities (Fig. 6A, 6B), pointing to an activated state of skin-resident CD8$^+$ dendritic-like cells. When transcriptional analyses were performed, we observed that T-bet mRNA levels were increased significantly in auto-MLRs, whereas Eomes mRNA levels were significantly higher in auto-MLRs in which dendritic-like cells were pretreated with TNP–KLH and in allo-MLRs performed with unstimulated dendritic-like cells (Fig. 6C). Additionally, FoxP3 mRNA levels were higher in both auto-MLR and allo-MLR cultures in which dendritic-like cells were not stimulated with TNP–KLH. Finally, neither perforin nor CD40L mRNA levels were induced by CD8$^+$ dendritic-like cells. Altogether, these results demonstrate a capacity for the teleost skin CD8$^+$ DC-like subpopulation to activate T lymphocytes exhibiting both stimulatory and regulatory phenotypes.

Skin CD8α$^+$MHC II$^+$ cells express high levels of CCR7 on their cell surface

In mammals, CCR7 governs DC migration from the skin to the lymphatic vessels in steady-state conditions, playing an important

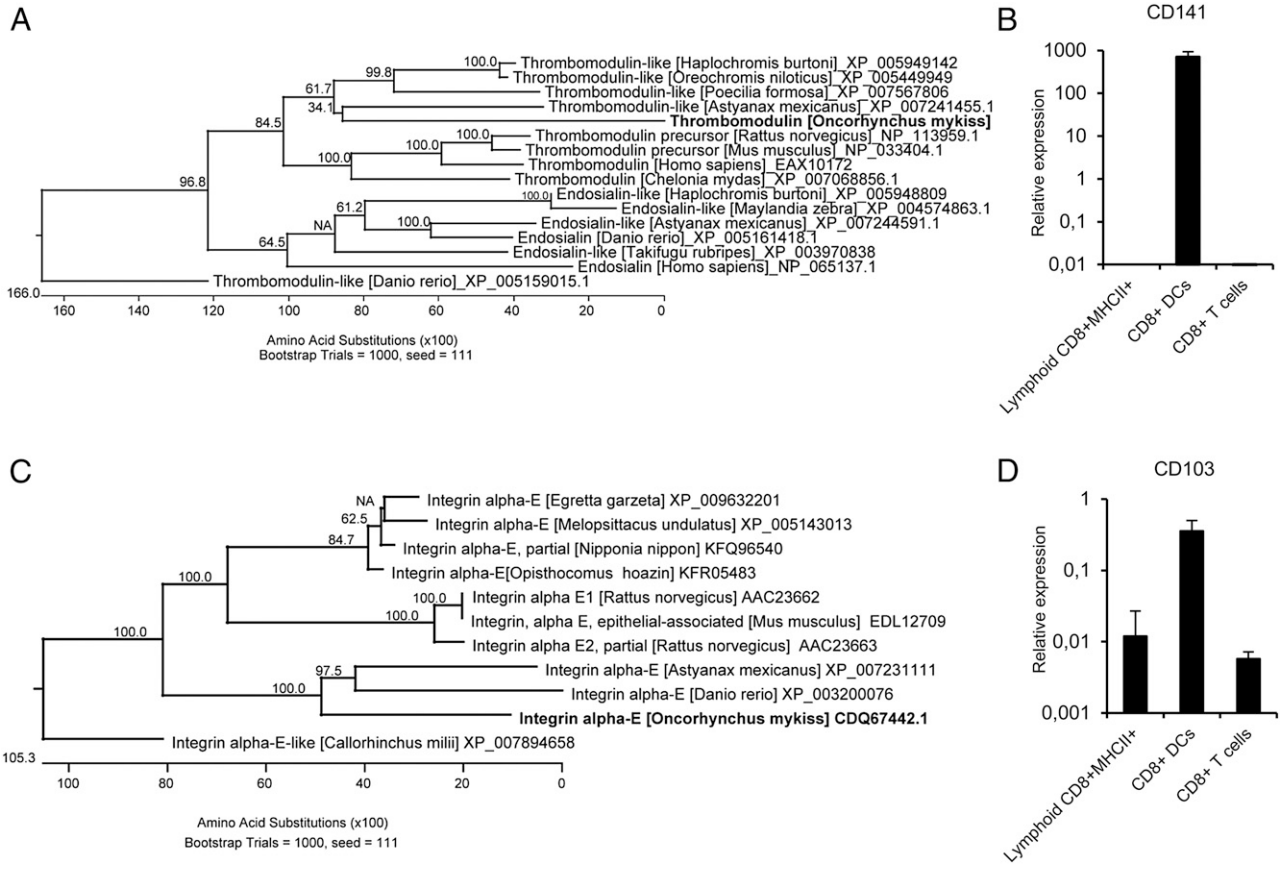

FIGURE 5. CD141 (BDCA-3) and CD103 (integrin α E) are present in rainbow trout and expressed in the CD8$^+$ DC-like subpopulation. Phylogenetic analyses of the CD141 (**A**) and CD103 (**C**) protein sequences were performed using MegAlign Software by comparison using the Clustal V algorithm and the neighbor-joining method. Statistical parameters of phylogenetic trees were determined by bootstrap analysis using 1000 replicates. Bootstrap values are shown above the branches. Skin CD8$^+$MHC II$^+$ cells from the lymphoid gate and skin CD8$^+$MHC II$^+$ cells (myeloid gate), as well as splenic CD8$^+$ T cells (CD8$^+$MHC II$^-$ events from the spleen lymphoid gate) were isolated by cell sorting, and RNA was obtained. The relative expression of CD141 (**B**) and CD103 (**D**) to the endogenous control EF-1α was calculated for each sample, and mean (± SD) values from three independent experiments, involving three animals/experiment, were calculated.

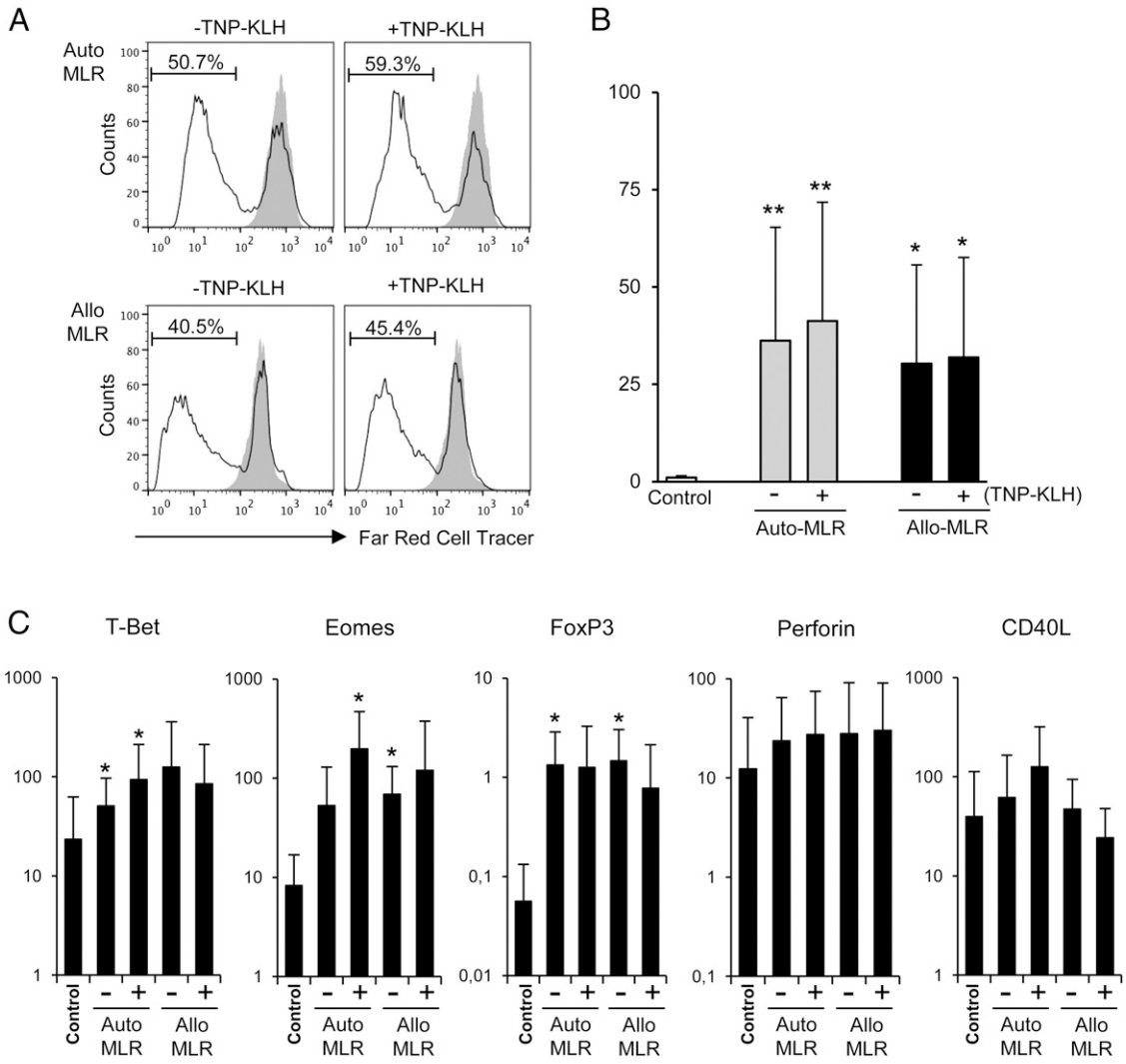

FIGURE 6. Analysis of T cell activation by CD8$^+$ dendritic-like cells. Trout skin CD8α$^+$MHC II$^+$ cells were isolated by cell sorting. Cells were cultured for 12 h in L-15 medium supplemented with 5% FCS in the absence or presence of the T cell–dependent Ag TNP–KLH. Then, DCs were cocultured with isogeneic (auto-MLR) or allogeneic (allo-MLR) T cell–enriched cultures of splenocytes, which were labeled with the division marker CellTrace Far Red, as described in *Materials and Methods*. Cocultures were set at a ratio of 1:50 DC/T cell–enriched splenocytes, incubated for 4 d, and analyzed by flow cytometry to measure the level of CellTrace Far Red dilution. (**A**) A representative example from seven individuals is shown; the percentage of proliferating cells is indicated. (**B**) Average percentages of proliferating cells were calculated to determine the proliferative capacity under each condition analyzed ($n = 7$ fish, mean ± SD). (**C**) In parallel, 24 h after the cocultures were set, RNA was isolated from the cocultured cells. The relative expression of each indicated gene to the endogenous control EF-1α was calculated for each sample, and (mean ± SD) values from seven individuals were calculated. *$p \leq 0.05$, **$p \leq 0.01$.

role in the maintenance of peripheral tolerance (50). Using a recently described anti-trout CCR7 polyclonal Ab (35), we evaluated the levels of CCR7 surface expression in skin CD8α$^+$MHC II$^+$ cells. Because CCR7 is known to be highly expressed in thymocytes (Fig. 7A, 7B) (51), skin expression of CCR7 was compared with the corresponding levels in thymic CD8α$^+$MHC II$^+$ cells (Fig. 7C). In trout skin, the percentages of myeloid cells with membrane-bound CCR7 were very high no matter whether they were CD8α$^+$MHC II$^+$ cells or not (Fig. 7A, 7B). However, a significantly higher CCR7 median fluorescence in the CD8$^+$ DC-like subpopulation indicates that the density of CCR7 molecules on the cell surface is highest in skin CD8α$^+$MHC II$^+$ cells (Fig. 7B). Similar cell surface expression patterns were recorded in thymocytes (Fig. 7D).

In vivo exposure to zymosan activates the skin CD8α$^+$ DC-like subpopulation

To understand whether skin CD8α$^+$MHC II$^+$ cells were responsive to in vivo stimulation, we bath exposed fish to zymosan or VHSV and analyzed its effect on the number of CD8$^+$ dendritic-like cells in the skin and in the transcriptional regulation of different DC activation markers. A significant increase in the percentage of skin CD8$^+$ dendritic-like cells was observed in fish exposed to zymosan (Fig. 8A) or infected with VHSV (Fig. 8B) in comparison with nonstimulated fish. This increase in the number of CD8α$^+$MHC II$^+$ cells in skin was accompanied by a significant increase in the transcription levels of BAFF, MHC class II, CD83, DC-SIGN, tapasin, and calreticulin in CD8$^+$ dendritic-like cells from both zymosan-treated (Fig. 8C) and virus-infected fish (Fig. 8D). In contrast, MHC class I and CD40 expression was significantly upregulated only after virus infection (Fig. 8D) and not by zymosan treatment (Fig. 8C). In contrast, ERp57 transcription was only upregulated by zymosan treatment (Fig. 8C). Additionally, transcription levels of IFN-γ were augmented in CD8$^+$ dendritic-like cells from both zymosan (Fig. 8C) and VHSV-treated (Fig. 8D) animals, although the increase was considerably higher in virus-infected fish, indicating antiviral responses of fish DCs.

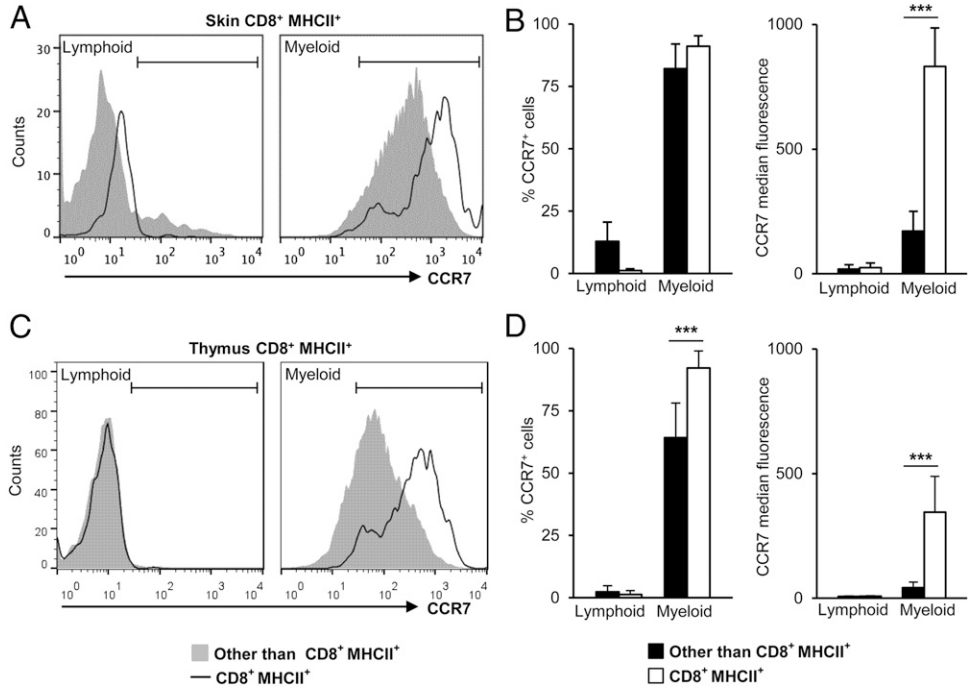

FIGURE 7. The trout skin CD8$^+$ DC-like subpopulation expresses high levels of CCR7. Trout leukocytes from skin (**A**) and thymus (**C**) were purified, stained with specific Abs to CD8α, MHC class II, and CCR7, and analyzed by multicolor flow cytometry. Cells were gated as lymphoid and myeloid cell on the basis of FSC and SSC. Then, CD8$^+$MHC II$^+$ cells were gated on those populations, and CCR7 fluorescence was determined in CD8$^+$ DCs (open line graphs) and compared against the general CCR7 level in each gate (filled line graphs). Data represent the CCR7$^+$ cell population after exclusion of negative cells of the conjugate-only controls (data not shown). Average percentages of CCR7$^+$ cells (*left panels*) and the corresponding median fluorescence intensities (*right panels*) in lymphoid and myeloid cells of skin (**B**) and thymus (**D**). Data are representative of nine individual fish from three independent experiments. $***p \leq 0.005$.

Discussion

Jawed fish are the first evolutionary group in which adaptive immune responses are present. This is not a general rule for fish species, because no MHC class II and CD4 genes were found in the Atlantic cod genome (52). However, most fish species investigated possess these molecules; consequently, it would be expected that all of the elements implicated in Ag processing and presentation also exist in this animal group. Although there is evidence for the existence of DCs in teleosts, the lack of adequate immunological tools has hampered a full phenotypic and functional characterization of DC lineages, and only a few studies have analyzed the properties of DC-like populations following enrichment protocols (5–7). In the current work, we identified, in trout, a large and highly complex subpopulation of leukocytes, of irregular shape and coexpressing MHC class II and CD8α, which resemble mammalian CD8$^+$ DCs. In the skin, which is all mucosal in fish, the myeloid CD8α$^+$MHC II$^+$ subpopulation, residing in the epidermis, transcribes DC marker genes, such as CD11b, DC-SIGN, CD80/86, CD83, LAMP3, or CD40, but no T cell markers. Moreover, the expression of both MHC class I–associated molecules (tapasin, calreticulin, and ERp57) and MHC class II–associated molecules (cathepsin Z and CD80/86) confirms that these cells contain the machinery needed for professional Ag presentation (53). Supporting our phenotypic characterization, trout skin CD8α$^+$ MHC II$^+$ cells also exhibited functional features similar to mammalian DC subsets, such as a strong phagocytic capacity and the ability to activate T cell proliferation and differentiation, which is a characteristic property of DCs. Skin CD8α$^+$MHC II$^+$ myeloid cells induced lymphocyte proliferation using allogeneic and isogeneic splenocyte targets, and this capacity was not affected by preincubation with a T cell–dependent Ag. This suggests a certain degree of maturation of trout skin CD8α$^+$MHC-II$^+$ myeloid cells, because only mature human DCs show T cell–activating properties in an auto-MLR (54). A further transcriptional analysis confirmed that trout skin CD8α$^+$MHC II$^+$ myeloid cells were in a rather mature state, expressing high CCR7, CXCR4, IFN-γ, BAFF, LAMP-3, and CD40 mRNA levels, along with intermediate levels of CD80/86 and CD83; this last molecule is only expressed upon DC maturation in mammals (55). Surprisingly, no IL-12 mRNA was detected in these cells, even after stimulation (data not shown). Although cross-presentation in mice takes place in a Th1 environment with IL-12 production, as seen in mice CD8$^+$ DCs (56) and human CD141$^+$ DCs (25), human CD141$^+$ skin DCs do not produce IL-12 either (29). This suggests that the potential of cross-presenting DCs to produce IL-12 is influenced by the tissue environment.

In concordance with this semiactivated transcriptional profile, trout skin CD8$^+$ dendritic-like cells expressed high CCR7 levels in steady-state conditions. It is well known that DCs reprogram their expression of chemokine receptors during maturation; CCR7 is one of the chemokine receptors expressed on mature DCs that is absent in immature DCs (57). In mammals, upon stimulation, CCR7 guides skin DCs to enter the skin-draining lymph nodes and encounter lymphocytes that are also recruited to the lymph nodes by CCR7 ligands (58). Because fish lack lymph nodes, CCR7 may be maintaining DCs in the mucosa, which is the location in fish where innate and adaptive immune responses are initially orchestrated. Interestingly, in mammals, CCR7 deficiency reduces the numbers of DCs in the peripheral lymph nodes, but this reduction does not affect all DC subpopulations equally; although CD4$^-$CD8$^-$ DC numbers were minimally affected, CD4$^+$ and CD8$^+$ DCs were strongly reduced by the lack of CCR7 (50). Further evidence demonstrated that CCR7 is also responsible for

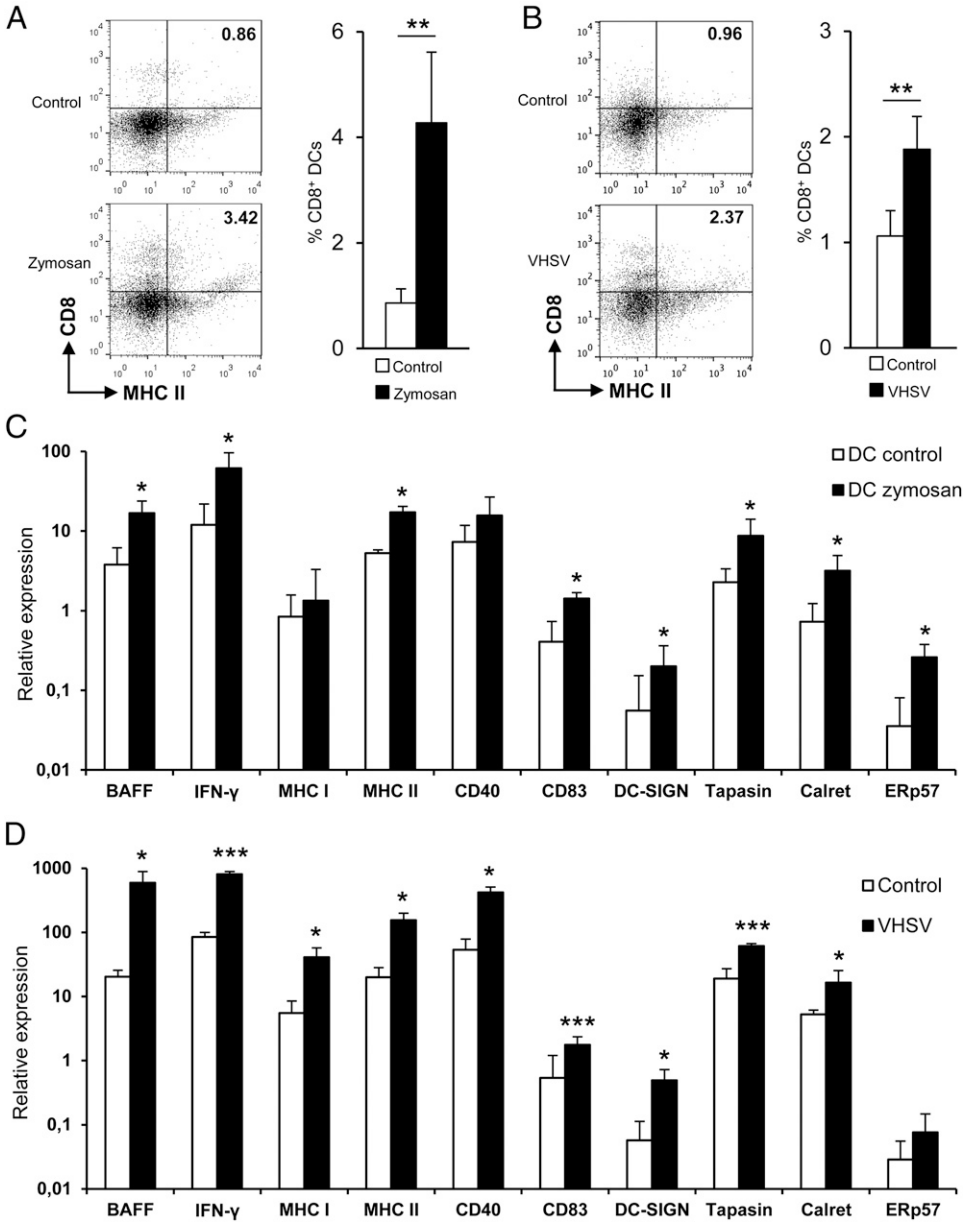

FIGURE 8. Effect of zymosan particles and VHSV on skin CD8$^+$ dendritic-like cells in vivo. Trout were treated by immersion in water containing 0.01% (w/v) zymosan particles or VHSV (5×10^5 $TCID_{50}$/ml) or control water for 16 h. Then, leukocytes from the skin were isolated, costained with Abs specific to CD8α and MHC class II, and analyzed by flow cytometry. CD8 and MHC class II levels on skin leukocytes from zymosan-treated (**A**) or VHSV-treated (**B**) trout were analyzed and compared with control animals. Mean percentage (± SD) of CD8$^+$MHC II$^+$ cells among the total number of skin cells was determined in nine animals analyzed from three independent experiments (*right panels*). In parallel, RNA from cells sorted from the skin CD8$^+$ DC-like subpopulation was obtained for the zymosan-treated fish, VHSV-treated fish, or control fish. The relative expression of each indicated gene to the endogenous control EF-1α was calculated for zymosan-treated (**C**) and VHSV-treated (**D**) animals. Data are mean ± SD and are representative of nine animals analyzed from three independent experiments. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.005$.

a continuous turnover of DCs from the skin to the draining lymph nodes in a process called steady-state migration; this is corroborated by the fact that some skin DCs in the epidermis already express CCR7 (50). In fact, these skin DCs found in the draining lymph nodes, without having received inflammatory signals, are in a semimature state, showing high MHC class II levels and intermediate expression of costimulatory molecules, such as CD80 or CD86 (50). This characteristic DC expression profile has been cataloged as a "semimature" state and is believed to induce tolerance (59), because DC maturity or activation levels at the time of Ag encounter determine the promotion of tolerance instead of immunity (1, 8). Trout skin CD8$^+$ dendritic-like cells also showed high CCR7 and MHC class II expression levels and had levels of costimulatory molecules that could be upregulated by virus infection, zymosan, or poly I:C, suggesting that they are also in this semimature state that regulates peripheral tolerance. Because fish lack lymph nodes, the identification of semimature CD8$^+$ DCs in trout skin suggests that, in teleosts, both tolerance and presentation of foreign Ags crossing epithelial barriers are immediately triggered in the skin. We show in this study that the trout skin CD8$^+$ DC-like subset induced the regulation of FoxP3 in both isogeneic and allogeneic splenocytes in the absence of additional stimuli. In contrast, skin CD8$^+$ DC-like cells previously stimulated with TNP–KLH induced a cytotoxic T cell profile in isogeneic splenocytes, as concluded from upregulated levels of T-bet and Eomes. These observations suggest the potential of skin CD8$^+$ DC-like cells to induce tolerance and immunity in rainbow trout. In agreement with this hypothesis, previous studies showed that fish mucosal surfaces, including the skin, are rich in CD3$^+$ T cells (39), suggesting that the establishment of tolerance or activation of T cells in response to water-borne Ags is a local process. Consequently, higher percentages of myeloid CD8α$^+$MHC II$^+$ cells were identified in other mucosal tissues, such as gills or gut, in comparison with the lower percentages observed in lymphoid tissues, such as the spleen or head kidney. Further characterization of these cells in other tissues remains to be done; we focused on skin DCs in this study because the percentage of CD8α$^+$MHC II$^+$ cells was higher in the skin, and because it was in the skin where human DCs with functional homology to the CD8$^+$ DC lineage had been fully characterized (29).

Unexpectedly, spleen CD8α$^+$MHC II$^+$ cells of the myeloid gate transcribed several T cell markers, such as CD3 and TCRα, along with the transcription of DC marker genes. Skin CD8α$^+$MHC II$^+$ cells from the lymphoid gate also transcribed CD3 but did not contain any TCR transcripts. Because we are lacking specific Abs against trout surface CD3 or TCRα, we cannot unequivocally state whether these two populations correspond to a heterogeneous cell population or whether they correspond to a true DC subpopulation. In fact, enriched rat CD45$^+$CD2$^-$ thymocytes that are triple negative for CD8, CD4, and CD3 contained rearranged TCRβ-chain and pre–Tα-chain transcripts, defining a common DC/T bipotential intermediate cell in rat adult and fetal thymus (60); therefore, it might be possible that these trout CD8α$^+$MHC II$^+$ cells are DC precursors containing T cell transcripts. Future studies by our group will try to resolve this question.

Because CD8α is only expressed in cross-presenting lymphoid DCs in mice, whereas tissue cross-presenting DCs are defined by surface expression of CD103 (in mice) or CD141 (in human), we searched trout EST databases for these two molecules. We found trout homologs to both CD141 and CD103, which allowed us to establish that trout skin CD8$^+$ DCs were coexpressing these two markers. These findings support the hypothesis that mammalian cross-presenting DCs, including murine lymphoid CD8$^+$ DCs, murine tissue CD103$^+$ DCs, and the human CD141 lineage, share a common ancestor that has been conserved through evolution in teleost fish. Previous evidence that led other investigators to formulate the hypothesis includes the expression of common transcription factors, such as Batf3 (20, 21) and IRF8 (18, 19), the sensitivity to TLR3 ligands, and the use of CLEC9A and XCR1 (18, 19, 22–24, 29). We were able to identify CLEC9A and XCR1 homologs in trout EST databases, but we showed that the skin CD8$^+$ DC-like subpopulation expressed both Batf3 and IRF8, distinctive transcription factors essential in the development of cross-presenting DCs. Additionally, trout skin CD8$^+$ DCs expressed TLR3 and responded to poly I:C, also an established TLR3 ligand in teleosts (49), by upregulating CCR7, BAFF, IFN-γ, MHC class II, and CD83 transcription, leading the cells to a further activation level. Likewise, trout skin CD8$^+$ dendritic-like cells also transcribed TLR22, a fish-specific TLR known to sense dsRNA in the cell surface, in contrast to TLR3, which primarily detects intracellular dsRNA (47), suggesting that teleost skin CD8$^+$ DCs are well equipped to sense viral infections and, consequently, activate CD8$^+$ T cells through cross-presentation. In fact, skin CD8$^+$ dendritic-like cells significantly enhanced the transcription of BAFF, IFN-γ, MHC class I, MHC class II, CD40, CD83, DC-SIGN, tapasin, and calreticulin after water-borne infection with VHSV, an RNA virus. Moreover, the transcriptional profiles observed in the MLRs performed in this study are consistent with a capacity of skin CD8$^+$ DCs to activate CD8$^+$ T cells, because a significant augmentation of T-bet and Eomes expression was observed when splenocytes were primed with purified isogeneic CD8$^+$ DCs stimulated earlier with TNP–KLH. Finally, the fact that skin CD8$^+$ dendritic-like cells upregulate MHC class I–associated molecules, such as tapasin, calreticulin, and ERp57 upon stimulation with the extracellular Ag zymosan, suggests cross-presenting capacities.

In conclusion, we achieved for the first time, to our knowledge, an extensive characterization of a tissue-resident DC subset in teleosts defined by surface expression of MHC class II and CD8α. Our results strongly support that skin epidermal CD8$^+$ DCs in trout are a phenotypical and functional equivalent of mammalian DCs, laying the groundwork for further studies dealing with Ag presentation in fish. The presence of semiactivated DCs in fish mucosa reveals that, in the absence of lymph nodes, tolerance and Ag presentation are organized locally. Finally, the fact that these cells coexpressed distinctive mammalian markers of different cross-presenting DC subsets strongly supports the ascription of cross-presenting cells in vertebrates to a common DC lineage that arose ≥450 million years ago with the appearance of adaptive immunity.

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Disclosures

The authors have no financial conflicts of interest.

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MARCH5 gene is duplicated in rainbow trout, but only fish-specific gene copy is up-regulated after VHSV infection

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ABSTRACT

Ubiquitination regulates the activity, stability, and localization of a wide variety of proteins. Several mammalian MARCH ubiquitin E3 ligase proteins have been suggested to control cell surface immunoreceptors. The mitochondrial protein MARCH5 is a positive regulator of Toll-like receptor 7-mediated NF-κB activation in mammals. In the present study, duplicated MARCH5-like cDNA sequences were isolated from rainbow trout (*Oncorhynchus mykiss*) comprising open reading frames of 882 bp (MARCH5A) and 885 bp (MARCH5B), respectively. Trout MARCH5A and MARCH5B-encoding sequences share only 65% sequence identity. Phylogenetic analyses including an additionally isolated MARCH5-like sequence from whitefish (*Coregonus maraena*) suggest that teleosts possess an additional MARCH5 gene copy resulting from a fish-specific whole genome duplication. Coding sequences of MARCH5A and MARCH5B genes from trout are distributed over six exons. Hypothetical MARCH5 proteins from trout comprise four transmembrane helices and a single motif similar to a RING variant domain (RINGv) including eight highly conserved cysteine and histidine residues. A 'reverse-northern blot' analysis revealed furthermore a MARCH5B Δexon5 transcript variant. Both MARCH5 genes from trout show a strain-, tissue- and cell-specific expression profile indicating different functional roles. Fish-specific MARCH5A gene for instance might be involved in defense mechanisms, since *in vivo*-challenge with the viral pathogen VHSV caused a significant 1.7-fold elevated copy number of the respective gene in gills four days after infection, whereas MARCH5B transcript level did not increase.

1. Introduction

Ubiquitination is the posttranslational attachment of the polypeptide ubiquitin to target proteins, reviewed in [1]. This multi-enzyme process is carried out by a cascade of concerted reactions involving ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin-protein ligase E3, reviewed in [2]. Unlike to E1 and E2, E3 ubiquitin ligases display substrate specificity. Eleven members of the MARCH (membrane-associated RING-CH) ubiquitin E3 ligase family have been identified in human so far [3]. The majority shares an N-terminal C_4HC_3-type RING (really interesting new gene) finger [4]. Furthermore, two transmembrane spans are characteristic for MARCH proteins, although MARCH5 bears 4 and MARCH6 even 13 transmembrane domains, whereas MARCH7 and MARCH10 lack the respective domain.

The precise physiological function of the MARCH family remains as yet unknown, but there is evidence for an association with immune defense [5]. Therefore, MARCH family members have also been termed as MIR (modulators of immune recognition). It has been hypothesized that MARCH-dependent ubiquitination allows internalization, recycling or lysosomal degradation of cell surface immunoreceptors, reviewed in [6]. Human MARCH4 and MARCH9 were suggested to influence surface expression of MHC-I (major histocompatibility complex, class I) molecules [4]. Similarly, human MARCH1 and MARCH8 proteins have been identified as potent regulators of MHC-II surface expression [7]. Furthermore, human MARCH8 is reported to influence the expression of the co-stimulatory molecule CD86 (B7-2) [8]. Both MHC and CD86 molecules are essential for antigen presentation and/or the subsequent

Abbreviations: aa, amino acid; EST, expressed sequence tag; mAb, monoclonal antibody; MARCH, membrane-associated RING-CH; MIR, modulator of immune recognition; MHC, major histocompatibility complex; ORF, open reading frame; qRT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; RACE, rapid amplification of cDNA ends; RING, really interesting new gene; RINGv, RING-variant; TLR, Toll-like receptor; VHSV, viral hemorrhagic septicaemia virus.

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activation of effector immune cells. MARCH9 has been suggested to control expression of the intercellular adhesion molecule ICAM1, which is important for the onset and manifestation of inflammatory responses [9]. However, TLR (Toll-like receptor)-signaling and other dendritic cell maturation signals are known to counteract ubiquitination of immunoreceptors [5]. It might be noteworthy that the MARCH-homolog MIR proteins have been initially identified in double-stranded DNA virus KSHV (Kaposi's sarcoma-associated herpes virus) [10] as down-modulators of MHC-I.

Human MARCH5 is reported to participate in the regulation of mitochondrial networks [11]. It has been shown that MARCH5 acts as a critical regulator of mitochondrial division and interconnection in mammals [12]. Most likely the MARCH5-dependent ubiquitination and subsequent degradation of the dynamin 1-like protein and further members of the mitochondrial scission complex plays a central role. Furthermore, it has been hypothesized that MARCH5 also interacts with the membrane protein mitofusin 2 promoting mitochondrial fusion. The balanced regulation of mitochondrial fission and fusion rates contributes to the cellular fitness concerning for example essential mechanisms like Ca^{2+} buffering [13]. Recently, it has been shown that human MARCH5 protein positively regulates TLR7 signaling by ubiquitination of TANK (TRAF family member-associated NF-κB activator) [14]. TANK is known as an inhibitor of TLR-dependent NF-κB activation by suppressing the autoubiquitination of the downstream factor TRAF6 (TNF receptor-associated factor 6).

In this manuscript, MARCH5-like genes from the salmonid fishes rainbow trout and maraena whitefish are characterized including bioinformatic analyses and expression profiles in healthy trout and after infection with the single-stranded RNA rhabdovirus VHSV (viral hemorrhagic septicaemia virus), causing severe hemorrhages in different organs and tissues. VHSV is a serious threat for salmonid aquaculture industry with high mortality rates predominantly among rainbow trout and Atlantic salmon, reviewed in [15].

2. Materials and methods

2.1. Sampling and nucleic acid extraction

Two-year old farmed rainbow trout (*Oncorhynchus mykiss*) of the imported strain TCO steelhead II-WA or of the local selection steelhead strain Born (BORN), and one-year old maraena whitefish (*Coregonus maraena*) were purchased from Binnenfischerei Mecklenburg GmbH Schwerin (Frauenmark, Germany).

In order to isolate MARCH5 cDNA and genomic DNA sequences as well as to investigate its tissue-specific expression, tissues (adipose tissue, gills, head kidney, heart, intestine, liver, muscle, and spleen) from six healthy imported trout and six healthy BORN trout as well as liver tissue from three healthy maraena whitefish were collected and immediately frozen in liquid nitrogen. For RNA isolation, flash-frozen tissue samples were homogenized individually in 1 ml TRIzol Reagent (Invitrogen, Karlsruhe, Germany). RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with in-column DNase treatment for 30 min. DNA was isolated from flash-frozen liver tissue using QIAamp DNA Micro Kit (Qiagen). DNA and RNA quantity was assessed at the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). The integrity of RNA was determined by agarose gel electrophoresis.

2.2. Isolation of MARCH5 sequences from rainbow trout and maraena whitefish

Subsequent to BLAST searches, we designed oligonucleotides (Sigma–Aldrich, Taufkirchen, Germany) specific for MARCH5A utilizing two ESTs with the GenBank accession numbers CB490059 and CX722962 as well as for MARCH5B utilizing three overlapping ESTs with the accession codes CA343783, CR376203, and BX085190 (Table 1).

RNA was extracted from livers of steelhead rainbow trout and maraena whitefish. 5 μg of total RNA were reverse-transcribed using Superscript II™ (Invitrogen) to generate a cDNA template for PCR amplification of MARCH5A and 5B fragments. In order to obtain full-length trout MARCH5A cDNA sequence, 5′- and 3′-RACE experiments were conducted using the Gene Racer Super Script™II RT Module (Invitrogen) according to a touchdown-PCR protocol. This included a 5-min pre-incubation at 95 °C, a denaturing step at 94 °C for 30 s, an annealing step at temperatures decreasing from 68 to 59 °C during the first 10 cycles (temperature decrement of 1 °C per cycle) for 30 s, and an elongation step at 72 °C for 2 min, followed by 30 cycles with 30 s at 94 °C, 30 s at 60 °C, 2 min at 72 °C, and final elongation at 72 °C for 7 min. A total of 41 cycles was performed.

Coding MARCH5 mRNA sequence of maraena whitefish was generated using primer pairs CM_MARCH_f1, -r1; and CM_MARCH_f2, -r2 (Table 1) according to a 30-cycle PCR protocol.

Introns of both MARCH5 genes were generated in touchdown-PCRs using genomic trout DNA as template and the oligonucleotides listed in Table 1. In order to amplify intron 1, a genomic walking library from rainbow trout DNA was established using the BD GenomeWalker™ Universal Kit (BD Biosciences, Erembodegem, Belgium).

Each nucleotide position of both cDNA and genomic DNA fragments was sequenced at least four times.

2.3. Southern blot analysis on RACE PCR fragments

3′-RACE PCR products from spleen and gill tissue amplified with OM_MARCH5B_f2 gene-specific oligonucleotide (Table 1) were run together with a PCR-generated MARCH5B fragment as a positive control on 2% agarose gel and washed twice in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and twice in neutralization solution (0.5 M Tris–HCl [pH 7.5], 1.5 M NaCl). DNA was transferred on a positively charged nylon membrane (Roche, Mannheim, Germany) by overnight capillary blotting in 20× SSC and eventually UV-cross-linked. In parallel, a 169-bp digoxigenin-labelled probe for hybridization reaction, which corresponds to exon 4 of trout MARCH5 was synthesized using the oligonucleotides OM_MARCH5-SB_f and -r (Table 1) and the PCR DIG Probe Synthesis Kit (Roche). Prehybridization and overnight hybridization were carried out at 43 °C in DIG Easy Hyb solution (Roche). Membranes were washed twice in low stringency buffer (2× SSC, 0.1% SDS) at room temperature for 5 min each and then twice in high stringency buffer (0.1× SSC, 0.1% SDS) at 43 °C for 20 min each. The blots were visualized according to the DIG Nucleic Acid Detection Kit (Roche).

2.4. Flow-sorting of trout lymphocytes

Leukocytes from head kidney, spleen, gills, and intestine were prepared and incubated with anti-CD8α monoclonal antibodies (mAb) in mixed medium (MM), i.e. Iscove's DMEM/Ham's F-12 (Sigma–Aldrich, Steinheim, Germany) at a ratio of 1:1, supplemented with 10% fetal bovine serum (FBS) and 0.1% sodium azide for 30 min. The cells were washed twice with MM and incubated with FITC-conjugated goat anti-rat IgG (H + L) (Jackson ImmunoResearch, Newmarket, England) for 20 min. Flow-sorting was performed with a MoFlo™ high speed cell sorter (Dako, Eching, Germany). The lymphocytes were sorted into two populations, anti-CD8α mAb-positive and anti-CD8α mAb-negative cells. After

Table 1
Primers used in this study.

Primer Name	Sequence (5′–3′)	Positions within cDNA/gene/EST (GenBank accession code)
amplification of MARCH5A 3′-end from rainbow trout:		
OM_MARCH5A-3R_f	CGACACAGAGGACAGCGGAAA	177–197 (CB490059)
OM_MARCH5A-3Rn_f	TTTAGGAGGTGCATTACATTTCCA	295–318 (CB490059)
amplification of MARCH5A 5′-end from rainbow trout:		
OM_MARCH5A-5R_r	ACCACACCGGCAGCAGTGAAT	64–44 (CX722962)
OM_MARCH5A-5Rn_r	CACTGTCCATCTGCGGAGGAA	24–4 (CX722962)
amplification of MARCH5A coding sequence from maraena whitefish:		
CM_MARCH_f1	TCCATTCACTGCTGCCGGTGT	303–323 (FN550946)
CM_MARCH_f2	ACCGCCACATCCTCAACTACC	800–820 (FN550946)
CM_MARCH_r1	TGGGCTCTACAGTGTACACACA	1252–1231 (FN550946)
CM_MARCH_r2	TCTATCTCCCCTCTACTTCTCC	958–937 (FN550946)
amplification of MARCH5B coding sequence from rainbow trout:		
OM_MARCH5B_f1	TTCAACAAGGCCAAGCTACC	225–244 (CA343783)
OM_MARCH5B_f2	ATCATGGTGGGCTCCATTTA	270–289 (CR376203)
OM_MARCH5B_r1	GTAGGTGACAGCGGTCCAGT	308–289 (CR376203)
OM_MARCH5B_r2	GCTGCTCAGAGGGGTACAAG	506–487 (BX085190)
amplification of MARCH5A intron sequences from rainbow trout (together with Genome Walker primer AP1 and AP2)		
OM_MARCH5AIntr1_r1	ACCACACCGGCAGCAGTGAAT	428–408 (FN400889)/574–554 (FN400890)
OM_MARCH5AIntr2_f1	GGCTCGATGAGAAGCAGAAA	263–282 (FN400889)/254–273 (FN400890)
OM_MARCH5AIntr2_r1	ATAGACTGTCCCCACCACCA	444–425 (FN400889)/590–571 (FN400890)
OM_MARCH5AIntr3_f1	TGGTGGTGGGGACAGTCTAT	425–444 (FN400889)/571–590 (FN400890)
OM_MARCH5AIntr3_r1	TCTGCCACAGCCTCAGTATG	625–606 (FN400889)/965–946 (FN400890)
OM_MARCH5AIntr4_f1	CATACTGAGGCTGTGGCAGA	606–625 (FN400889)/946–965 (FN400890)
OM_MARCH5AIntr4_r1	CCACCAATTGTGCAAGTTCT	1247–1228 (FN400890)
OM_MARCH5AIntr5_f1	TCTCCTCATTTTGTCTGTCATAGT	1152–1175 (FN400890)
OM_MARCH5AIntr5_r1	TACACCTTCAACACTCCCTTCAT	1764–1742 (FN400890)
probe synthesis for Southern Blotting:		
OM_MARCH5-SB_f	GTGGGCCATAAGAAGGGCTTG	487–507 (FN400889)
OM_MARCH5-SB_r	CAATAGCAGCTGTAGTTTAGAGG	654–632 (FN400889)
quantification of MARCH5A/MARCH5B/ACTB/EEF1A1 from rainbow trout:		
OM_MARCH5A_LC_f	ATTTCCAAACAGAGAACTTGTCCA	1110–1133 (FN400889)
OM_MARCH5A_LC_r	TGGGCTCTACAGTGTACAGACA	1264–1243 (FN400889)
OM_MARCH5B_LC_f	CCATCCTGGGTGGGATTGCG	828–847 (FN677805)
OM_MARCH5B_LC_r	GCTCCACCCACTCCCCCTTT	996–977 (FN677805)
OM_ACTB_LC_1	CCCTCCACCATGAAGATCAAGA	1015–1036 (AF157514)
OM_ACTB_LC_r1	GGGATGGGTACAGTCTGTTTAG	1197–1176 (AF157514)
OM_EEF1A1_LC_f1	TGATCTACAAGTGCGGAGGCA	136–156 (NM_001124339)
OM_EEF1A1_LC_r1	CAGCACCCAGGCATACTTGAA	236–216 (NM_001124339)

sorting, total RNA was extracted from 30,000 cells of each cell population using RNeasy Micro Kit (Qiagen). The production and characterization of mAbs against rainbow trout CD8α has been described in [16].

2.5. VHSV infection

VHSV strain 861 was grown on EP/F cells (FLI cell culture collection) and titrated according to the method of Reed and Muench. One year old steelhead rainbow trout were kept in a semicircular water system in 300 L tanks at 15 °C and fed commercial dry pellets. Two groups of 25 fish each were intraperitoneally infected with the VHSV strain 861 at a dosage of 100 $TCID_{50}$/100 μl/fish, while a control group of the same size was injected with the same amount of cell culture medium. Fish were anesthetized with benzocaine (Sigma–Aldrich) and organ samples (spleen, head kidney and gills) were collected at day 0, 2, 4, and 7 after injection from both infected and control fish. Organ pieces of about 100 mg were subsequently transferred into RNAlater (Qiagen), stored overnight at 4 °C and transferred to −20 °C until RNA extraction. RNA was robot-extracted (Tecan, Männedorf, Switzerland) from organ pieces of 10–15 mg using the NucleoSpin II Kit (Macherey-Nagel, Düren, Germany).

2.6. Quantitative real-time RT-PCR

Quantitative Real-Time RT-PCR (qRT-PCR) was carried out using the LightCycler® 480 Instrument and the LightCycler® 480 SYBR Green I Master Kit (Roche). First strand cDNA was synthesized from 1.5 or 0.9 μg of total organ RNA from healthy or VHSV-infected trout by utilizing the Super Script™II Kit (Invitrogen). Products were purified using High Pure PCR Product Purification Kit (Roche). The trout MARCH5A- or MARCH5B-specific oligonucleotides OM_MARCH5A_LC_f and -r or OM_MARCH5B_LC_f and -r (Table 1) were used to quantify a 157-bp or a 169-bp fragment, respectively. For MARCH5 mRNA quantification in healthy or VHSV-infected trout, a 183-bp β-actin (ACTB) or a 101-bp eukaryotic elongation factor-1 (EEF1A1) fragment was amplified in parallel to serve as a control for both RNA integrity and qRT-PCR success utilizing the primers OM_ACTB_LC_f1 and -r1 or OM_EEF1A1_LC_f1 and -r1 [17], respectively (Table 1).

Quantification was performed in repeated runs with an initial denaturation step of 10 min at 95 °C, and then 40 cycles as follows: 15 s of denaturation at 95 °C, 10 s of annealing at 60 °C, 20 s of elongation at 72 °C, and ultimately 5 s quantification at 78 °C (MARCH5A), 79 °C (EEF1A1), 83 °C (MARCH5B), and 84 °C (ACTB), respectively. Copy numbers were calculated relative to dilutions of PCR-generated MARCH5 fragments as external standards (10^3–10^6 copies). PCR products were separated in 3% agarose gels to assess product size and quality. The housekeeping gene copy number was in all cases non-significantly different, except for quantification in flow-sorted lymphocytes. For this reason, we decided to normalize MARCH5 transcript number to EEF1A1.

Statistical significance of expression data was assessed with SPSS software (SPSS Inc., Version 15.0) using parametric *t*-test, non-parametric Mann and Whitney *U*-test or one-way ANOVA applying

the Bonferroni method. In all tests, a two-tailed *P*-value of 0.05 or less was considered significant.

2.7. Computational analyses

NCBI and Ensembl BLAST searches were conducted for sequence comparisons. Sequence alignments were carried out by ClustAlW multiple alignment [18]. Basic physical and chemical properties were analyzed by the ProtParam tool at the Expasy Proteomics Server [19]. Transmembrane helices of the MARCH5 protein were predicted with TMHmm v2.0 included in the HUSAR v3.0 package (DKFZ-Heidelberg, Germany).

Phylogenetic analysis of amino acid sequences was conducted using the Molecular Evolutionary Genetics Analysis package (MEGA4) [20]. The dendrogram was reconstructed with the Neighbor-Joining method based on Poisson-corrected distances and optimized manually. Node robustness was evaluated on a bootstrap analysis based on 10,000 iterations.

3. Results

3.1. Rainbow trout encodes a second MARCH5 gene producing transcript variants

In the present study, we isolated a full-length MARCH5-like cDNA sequence from rainbow trout (*O. mykiss*) with RACE technique based on two ESTs (GenBank accessions: CB490059, CX722962). The complete cDNA sequence of trout MARCH5 gene comprises 1318 bp (FN400889). The ORF of 882 bp is flanked by 120 and 316 bp of 5′- and 3′-UTR, respectively. In addition, a homologous sequence (FN550946) was isolated from maraena whitefish (*C. maraena*), a close relative to rainbow trout. Both fishes are classified as Salmoniformes. The ORF of maraena whitefish MARCH5-encoding cDNA is shorter by 3 bp due to an anticipated TAG stop codon. However, 3′-untranslated regions of maraena whitefish and rainbow trout MARCH5-encoding sequences share 91% identity and a canonical poly(A) signal AATAAA separated by 14 nucleotides from the poly(A) tail.

MARCH5 nucleotide sequences from rainbow trout and maraena whitefish share highest identity with each other (97%) and with a partial MARCH5 sequence of another salmonid fish, Atlantic salmon (*Salmo salar*, BT072360) representing about $^{3}/_{4}$ of the putative ORF. Database searches revealed that salmon possesses a second MARCH5 gene with lower sequence homology (NM_001140034). Moreover, two MARCH5 variants are also present in zebrafish (*Danio rerio*). This finding motivated us to search in the GRASP database for ESTs encoding a second rainbow trout MARCH5 variant. Three overlapping ESTs (CA343783, CR376203, BX085190) were identified, the assembled sequence was subsequently experimentally proven and unknown positions were corrected (FN677805, in the following termed as MARCH5B). The 885-bp ORF of MARCH5B is 3 bp or 6 bp longer than the firstly identified "MARCH5A" sequences from rainbow trout or maraena whitefish, respectively, and shares only 65% or 66% sequence identity with both MARCH5A sequences. In contrast, MARCH5B sequences from rainbow trout and its full-length counterpart in salmon show 94% identity.

The genomic structures of both trout MARCH5 genes (MARCH5A: FN400890; MARCH5B: FR749991) were determined by comparing cDNA and genomic sequences. The coding sequences of both, trout MARCH5A and MARCH5B gene includes six exons, although we detected additionally a MARCH5B Δexon5 splice variant *via* a 'reverse-northern blot' utilizing trout cDNA from spleen and gill tissue as template (Fig. 1A).

Fig. 1B depicts the exon/intron composition of both trout MARCH5 genes corresponding generally with its homologs from zebrafish as well as human. The lengths of the exon 2, 3, and 5 are throughout conserved. It might be noteworthy that the introns of MARCH5A gene from trout are not longer than 464 bp, whereas three introns within the paralogues MARCH5B gene comprise more than 1000 bp. Corresponding introns share accordingly no sequence homology. However, MARCH5A does not feature generally short introns, since zebrafish includes four-digit introns in both MARCH5 genes. The mammalian counterparts also contain extremely long introns (up to 29,335 bp in human). Remarkably, MARCH5 gene from three-spined stickleback *Gasterosteus aculeatus* contains an additional intron within the first exon.

As special features, trout MARCH5A gene contains a $(GT)_{39}$ dinucleotide microsatellite within intron 1, 35 bp downstream of the start codon and 9 bp downstream of exon 1. MARCH5B includes a $(TG)_9$ dinucleotide microsatellite located in intron 4, 67 bp downstream of exon 4.

3.2. MARCH5 proteins from trout and maraena contain characteristic domains

The ORF of MARCH5A gene from rainbow trout encodes a putative protein of 293 aa (CAZ64332), whereas its counterpart from maraena whitefish (CBE70290) is one amino acid residue shorter. Both proteins have a hypothetical molecular weight of 32 kDa and a theoretical pI of 8.5 as predicted by the ProtParam tool at the Expasy Proteomics Server. The conceptually translated trout MARCH5B protein includes an additional amino acid residue compared to its paralog. The theoretical pI of MARCH5B is 9.0.

Four transmembrane helices have been predicted for MARCH5A proteins from rainbow trout and maraena whitefish as well as for

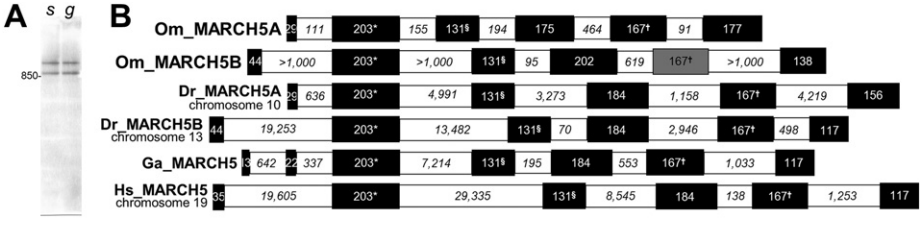

Fig. 1. A) Detection of a MARCH5B transcript variant by southern blotting on RACE PCR fragments. 3′-RACE fragments were generated using total oligo-(dT) cDNA from spleen (s) and gills (g), immobilized and hybridized with a MARCH5 probe specific for exon 4. The lower band represents the MARCH5B Δexon5 splice variant, which is shorther in length by ~170 bp corresponding to exon 5; a characteristic marker band is indicated in bp on the left. To validate the Δexon5 splice variant, 3′-RACE fragments from splenic and branchial cDNA were cloned and sequenced. Procedure was repeated three times with different cDNA samples, a representative blot is shown here. B) Comparative illustration of MARCH5 gene structures from different vertebrates. The figure compares the lengths of exons (scaled black boxes) and introns (graduated white boxes) from duplicated MARCH genes in trout with those in zebrafish (Dr_MARCH5A, Ensembl accession: ENSDART00000039187; Dr_MARCH5B, ENSDART00000043069), as well as single copy MARCH genes in stickleback (Ga_MARCH5, ENSGACT00000003872), and human (Hs_MARCH5, ENST00000358935). Lengths are given in bp. The gray box represents a spliceable exon. Conserved exon lengths in ortholog and paralog MARCH5 genes are marked with a symbol (asterisk: trout MARCH5 exon 2; paragraph: exon 3; cross: exon 5).

MARCH5B variant from trout spanning from position (i) 100 to 122 or 105 to 127, respectively; (ii) 137 to 159 or 142 to 164; (iii) 205 to 224 or 219 to 238; and (iv) 234 to 251 or 248 to 265 (Fig. 2) corresponding to the respective positions in the human MARCH5 protein [12]. Hence, MARCH5B Δexon5 splice variant lacks one transmembrane helix. In contrast to full-length MARCH5B protein, both ends of the truncated MARCH5B variant are most likely situated at different sides of the membrane comparable to (human) MARCH6 ligase containing also an odd number of transmembrane domains.

A single motif similar to a RING variant domain (RINGv) was found in both hypothetical MARCH5A protein sequences from residue 12 to 66 and in MARCH5B protein from residue 17 to 71 corresponding to the consensus pattern C-X_2-C-$X_{10–45}$ (15 in trout and whitefish)-C-X-C-X_7-H-X_2-C-$X_{11–25}$ (18 in trout and whitefish)-C-X_2-C (in short C_4HC_3). RING domain sequences characteristically comprise a cluster of cysteine and histidine residues that coordinate zinc ions [21]. MARCH5 amino acid sequences from rainbow trout and maraena whitefish contain one histidine residue at position 41 (variant A) or 46 (variant B; Fig. 2), as well as seven cysteine residues from position 12 to 66 (variant A) or 17 to 71 (variant B) that might constitute cysteine bridges, which are typical for E3 ubiquitin ligases. As expected, the respective amino acid residues are well conserved among MARCH5 proteins from vertebrates as illustrated by the amino acid sequence alignment in Fig. 2. Moreover, the comparison visualizes the presence of fish-specific MARCH5 proteins, namely MARCH5A, whereas piscine MARCH5B proteins resemble tetrapod MARCH5 proteins represented by MARCH5 from chicken *Gallus gallus* and human. Fig. 2 accents a total of 56 amino acid residues specific for either MARCH5A or MARCH5B as indicated by arrows. It clearly reveals first that the N-termini of MARCH5A variants from trout, whitefish, zebrafish, and pike are five amino acid residues shorter than MARCH5B variants from trout, salmon, and zebrafish as well as from chicken. Second, the C-termini of the selected piscine MARCH5A sequences are significantly (six to 20 residues) longer than piscine MARCHB variants as well as tetrapod MARCH5. Furthermore, species-specific deletions or insertions are present in MARCH5 proteins, e.g. an additional stretch of six amino acid residues after position 188 within trout and salmon MARCH5B protein.

3.3. *MARCH5 genes are duplicated in several teleostean species*

The mammalian MARCH family consists of eleven members (MARCH1 to -11) showing a distinct tissue distribution [6]. Our searches at the Ensemble genome browser and in GenBank revealed that the teleostean fishes zebrafish *D. rerio*, three-spined stickleback *G. aculeatus*, Japanese rice fish *Oryzias latipes*, Japanese pufferfish *Takifugu rubripes*, and spotted green pufferfish *Tetraodon nigroviridis* encode presumably only eight MARCH genes, MARCH2, -4, -5, -6, -7, -8, -9, and -11. Zebrafish possesses a MARCH1 gene (NCBI protein accession code: CAX14354), at present uniquely among teleosts. Similarly, MARCH3 has been experimentally validated only in *Salmonidae* so far. Recently, we isolated a MARCH3 cDNA sequence from rainbow trout (NCBI: FR851411). In parallel, the Atlantic salmon's ortholog was published (NCBI: NP_001135117). MARCH10 is obviously absent in all piscine species. Although clawed frog's genome lacks also MARCH10, it does encode MARCH3. In brief words, the MARCH family in non-vertebrates seems to differ from the mammalian one. Moreover,

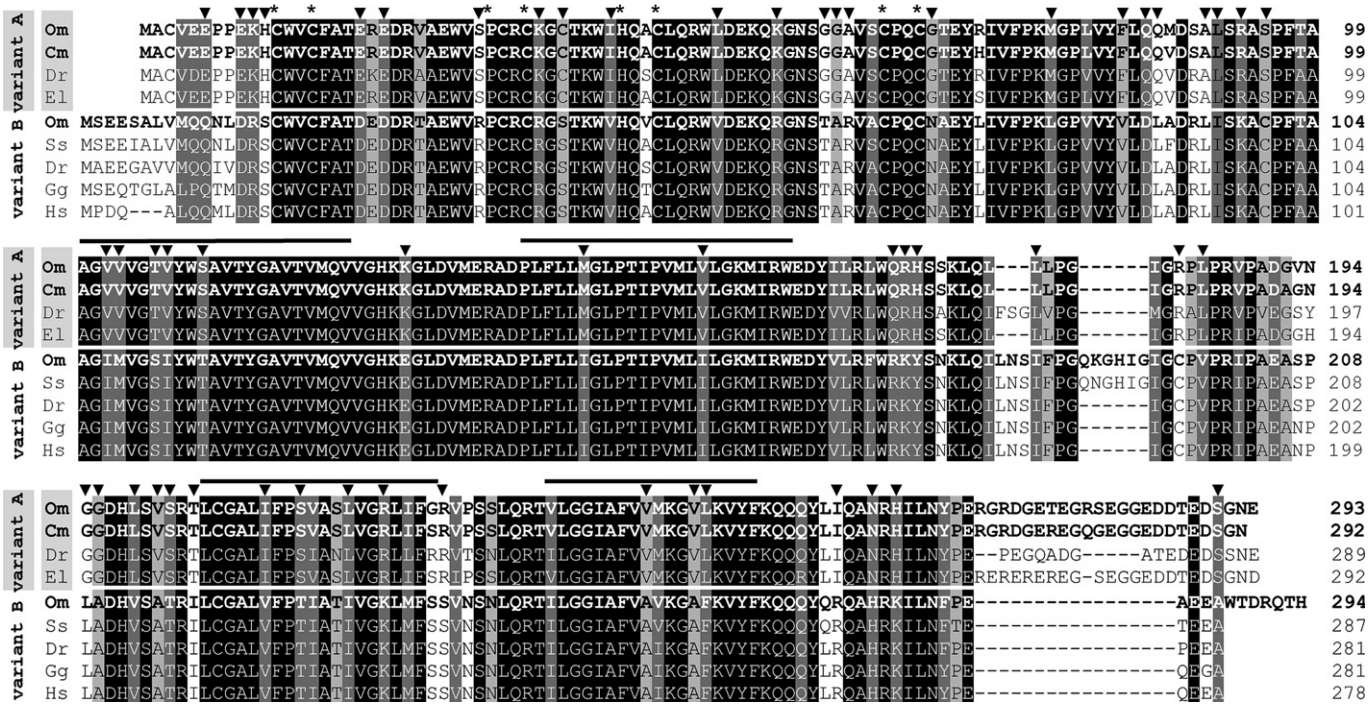

Fig. 2. Multiple alignment of MARCH5 amino acid sequences. The comparison includes MARCH5 variant A (highlighted in gray along the left margin) and variant B sequences from the teleostean species rainbow trout (Om, variant A, GenBank accession: CAZ64333; variant B, CBK39084), maraena whitefish (Cm, CBE70290), zebrafish (Dr, variant A, NP_956033; variant B, NP_001076296), northern pike (El, ACO13898), and Atlantic salmon (Ss, ACI33369), as well as chicken (Gg, NP_001012924), and human (Hs, NP_060294). Sequences that have been generated for the present study are printed in bold face letters. Amino acids are numbered along the right margin. Identical amino acids are highlighted in black; strongly similar amino acids are printed in white letters with dark gray underlay; weakly similar amino acids are printed in black letters with light gray underlay. Conserved residues of the RINGv domain, which is critical for ubiquitin transfer activity of several E3 ubiquitin ligases, are indicated with an asterisk above the respective alignment section. Arrows mark amino acid residues, which are specific for either MARCH5A or MARCH5B variants. Four predicted transmembrane domains of MARCH5 proteins from rainbow trout and maraena whitefish are indicated with a solid line.

it appears that some MARCH family members are duplicated in bony fish, i.e. MARCH6, -9, and MARCH5, described here.

MARCH5-like amino acid sequences from rainbow trout, maraena whitefish, and Atlantic salmon (ACN58677) share 95–97% sequence identity. In comparison with human MARCH family members, MARCH5A proteins from trout and whitefish show higher homology to MARCH5 (NP_060294; 61%–62%) than to human MARCH1 to -4 and -6 to -11 (≥14%). MARCH5B from trout shares even 94% sequence identity with human MARCH5 protein, but a similarly low identity with human MARCH5 paralogs (5%–19%). To support the assumption that both trout sequences described here are co-orthologs of the mammalian MARCH5 protein, a comprehensive phylogenetic dendrogram including representatives of all members of the MARCH E3 ubiquitin ligase family was reconstructed (Fig. 3). The dendrogram contains all human MARCH amino acid sequences and their respective piscine counterparts as well as MARCH5 proteins from insect *Culex quinquefasciatus*, frog *Xenopus tropicalis*, reptile *Anolis carolinensis* and bird *G. gallus*.

The dendrogram clearly separates the MARCH proteins into seven major families, (i) MARCH1/-8, (ii) MARCH2/-3, (iii) MARCH4/-9/-11 including two piscine MARCH9 gene variants, (iv) MARCH5 including piscine A and B gene variants, (v) MARCH6 including two piscine gene variants, (vi) MARCH7, and (vii) MARCH10 without piscine members. Among the MARCH5 proteins, the insect amino acid sequence is clearly separated (bootstrap confidence level: 100%) from vertebrate counterparts. MARCH5 protein from mosquito contains indeed a few additional sequence features that are absent in vertebrate MARCH5 proteins.

This phylogenetic analysis proves that MARCH5 sequences from rainbow trout and maraena whitefish are related to tetrapod MARCH5. Moreover, the dendrogram reveals that piscine MARCH5 proteins are separated into distinct clades with 77% bootstrap support. On the one hand, MARCH5A amino acid sequences from zebrafish and pike *Esox lucius* form a sister group to MARCH5A proteins from the salmonids trout, salmon and whitefish with 88% bootstrap confidence. On the other hand, MARCH5B clade comprises sequences from salmon, zebrafish, and trout which are grouped with MARCH5 from stickleback, rice fish, pufferfishes and even chicken and human.

3.4. MARCH5A gene is highly expressed in the immunorelevant tissues spleen, gills, and head kidney

We analyzed the expression of MARCH5A and MARCH5B gene in eight different tissues of six clinically healthy steelhead rainbow trout *via* qRT-PCR using oligonucleotides specific for either trout MARCH5A or -5B (Table 1). The highest concentration of MARCH5A copies has been found in immune-related tissues, i.e. spleen, gills, head kidney (Fig. 4A). MARCH5A mRNA abundance was significantly lower ($P = 0.0045$) in heart, intestine and liver. Hence, 18.8 ± 1.7-fold more MARCH5A copies were detected in splenic compared to hepatic tissue. These data are contrasted by MARCH5B gene, which is more equally expressed among the selected tissues (Fig. 4B). The highest MARCH5B mRNA concentration is present in heart, followed by head kidney, gills, and spleen. Liver shows again lowest mRNA copy number, but cardiac MARCH5B expression is only 1.6 ± 0.4-fold higher than hepatic expression. These tissue-specific expression profiles are furthermore reflected in a different MARCH5A/B transcript number. In head kidney, gills, spleen as well as muscle and adipose tissue, 0.4- to 1.2-fold more MARCH5A transcripts are present than MARCH5B copies. *Vice versa*, liver, gut, and heart contain 3.4- to 4.6-fold more MARCH5B than MARCH5A copies.

Assuming an importance of a pronounced MARCH5A expression in the immunocompetent tissues spleen, gills, and head kidney, we wanted to examine, to which extent MARCH5A and MARCH5B are expressed in leukocytes of the respective organs. However, Fig. 4C and D illustrates that more MARCH5B copies were detected in both, anti-CD8α mAb-positive and -negative leucocytes, namely branchial lymphocytes, >pronephrocytes, >splenocytes.

Besides the tissue- and cell-specific expression differences, we also found strain-specific differences. We are investigating the genomic potential of a local rainbow trout selection strain BORN, which has been bred since 1975 at the fishery institute in Born in brackish costal water of the southwestern Baltic Sea with varying temperature, between 2.5 and 6 practical salinity units (PSU), differently strong pollutant and pathogen pressure [22,23]. MARCH5A mRNA level is significantly lower in muscle tissue of the selection strain BORN in comparison to the imported steelhead strain (fold change of 0.47 ± 0.07, $P = 0.005$; Table 2). In the other seven tissues, the expression of MARCH5A in both trout strains was non-significantly different. In contrast, MARCH5B expression was significantly lower in all tissues examined (head kidney, spleen, gills, heart, intestine, adipose tissue, muscle), except for liver, with fold changes ranging from 0.40 ± 0.07 (muscle, $P = 0.002$) to 0.72 ± 0.05 (head kidney, $P = 4 \times 10^{-4}$) compared to imported steelheads (Table 2).

3.5. MARCH5A is up-regulated in gills after VHSV

We evaluated both, MARCH5A and MARCH5B mRNA expression levels in spleen, gills and head kidney after *in vivo* challenge with VHSV. The respective organs were chosen, since they showed highest MARCH5A mRNA abundance. Samples were taken at day 0, 2, 4, and 7 and quantified *via* qRT-PCR analysis. Fig. 5A illustrates that on the one hand, expression of MARCH5A gene remained on a similar level in spleen and head kidney during evaluation period. On the other hand, MARCH5A expression slightly increased in gill tissue (1.61-fold change, $P = 0.34$) two days after VHSV infection, peaked significantly at day 4 post-challenge (2.68 ± 0.83-fold change, $P = 0.03$) and almost returned to control levels (1.13 ± 0.31-fold change, $P = 0.67$) one week after infection. In contrast, expression of MARCH5B in gills is not elevated, but eventually slightly depressed at day 7 post-challenge (0.61 ± 0.14-fold change) with a marginal statistical significance ($P = 0.06$). Similarly, MARCH5B copy number is significantly down-regulated in spleen four days (0.65 ± 0.06-fold change, $P = 0.01$) and seven days (0.53 ± 0.15-fold change, $P = 0.04$) after challenge. In head kidney, significant changes of the MARCH5B mRNA level were not apparent.

4. Discussion

4.1. Teleosts possess a tetrapod-ortholog and a fish-specific MARCH5 variant

We isolated two MARCH5 DNA sequences from rainbow trout (*O. mykiss*) termed as variant A and B. Both MARCH5 variants are transcribed and encode MARCH5-characteristic amino acid residues and motifs. However, both genes contain specific intron sequences and the putative proteins comprise distinct amino acid positions that allow separation of variant A from variant B. Phylogenetic analyses suggest MARCH5 variant A as a fish-specific gene, whereas variant B seems truly ortholog to the respective gene in tetrapods. Variant A was not identified in tetrapods, but has been found in maraena whitefish (*C. maraena*) in the present study as well as in zebrafish [24], salmon, and northern pike [25] in the frame of cDNA sequencing projects. Zebrafish, as well as salmonids and the closely related pike evolved early among bony fishes. Large scale sequencing and analyses of gene loci in bony fish have

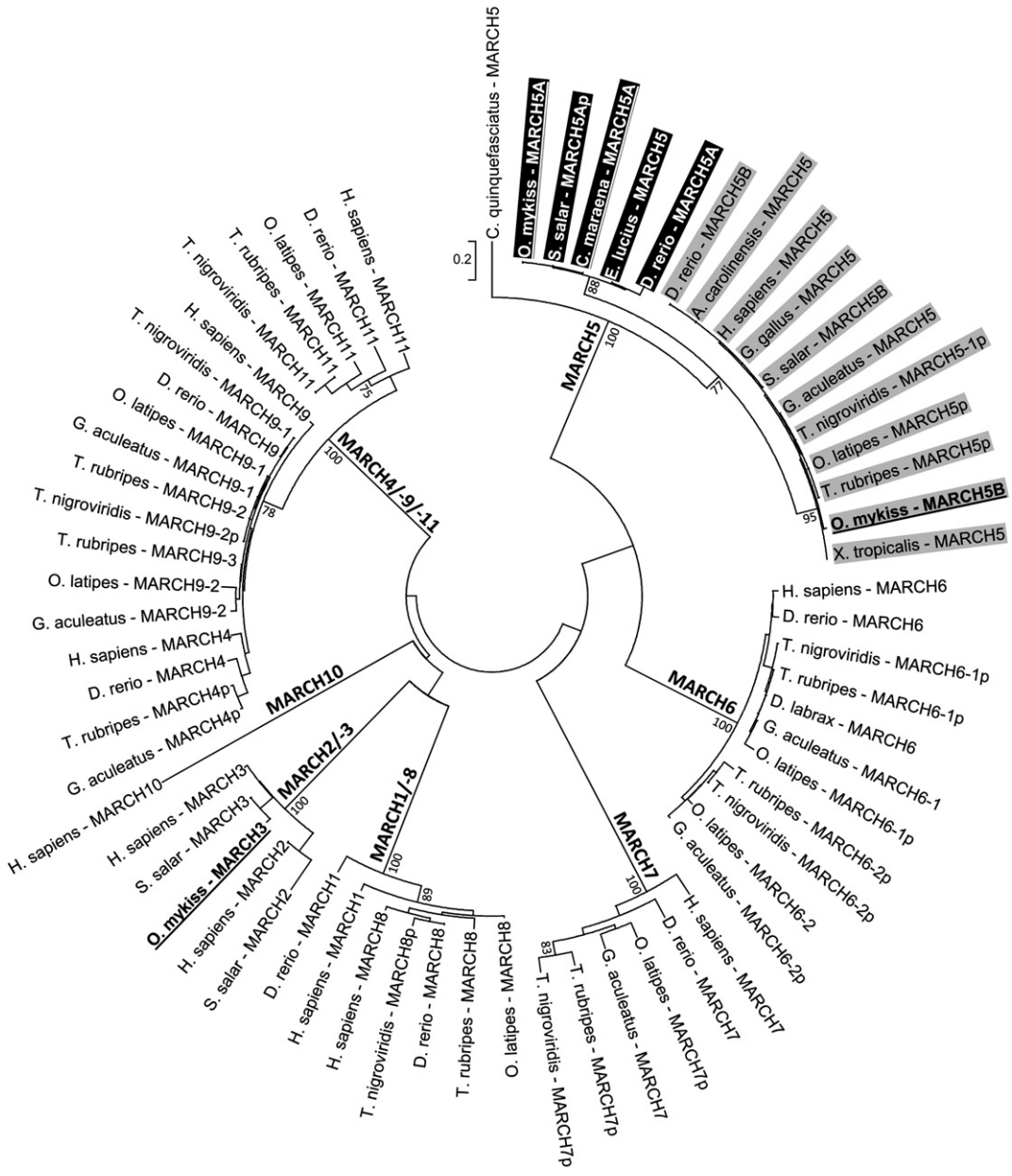

Fig. 3. Circular phylogenetic tree of MARCH amino acid sequences. The MEGA4 dendrogram was reconstructed with the Neighbor-Joining Method using the Poisson correction distance model. Numbers at the nodes indicate the percentage of bootstrapping after 10,000 replications. Only bootstrap values above 75% are indicated. The scale bar represents a genetic distance of 0.2 amino acid substitutions per site. The tree includes MARCH protein sequences from the following species (with corresponding GenBank [GB] and Ensembl [E] accession codes): *Anolis carolinensis* (MARCH5, GB: XP_003224783), *Coregonus maraena* (MARCH5A, GB: CBE70290), *Culex quinquefasciatus* (MARCH5, GB: XP_001862231), *Danio rerio* (MARCH1, GB: CAX14354; MARCH4, GB: NP_001038876; MARCH5A, GB: NP_956033; MARCH5B, GB: CAX13539; MARCH6, GB: XP_002660875; MARCH7, GB: NP_001108052; MARCH8, GB: NP_001154907; MARCH9, GB: XP_001339845; MARCH11, GB: XP_001338632), *Dicentrarchus labrax* (MARCH6, GB: CBN81886); *Esox lucius* (GB: ACO13898), *Gallus gallus* (MARCH5, GB: NP_001012924), *Gasterosteus aculeatus* (MARCH4, E: ENSGACP00000008045; MARCH5, E: ENSGACP00000003859; MARCH6-1, E: ENSGACP00000003448; MARCH6-2, E: ENSGACP00000019168; MARCH7, E: ENSGACP00000020033; MARCH9-1, E: ENSGACP00000015512; MARCH9-2, E: ENSGACP00000000754), *Oncorhynchus mykiss* (MARCH3, GB: CCA64454; MARCH5A, GB: CAZ64333; MARCH5B, GB: CBK39084), *Oryzias latipes* (MARCH5, E: ENSORLP00000000908; MARCH6-1, E: ENSORLP00000009594; MARCH6-2, E: ENSORLP00000016019; MARCH7, E: ENSORLP00000005266; MARCH8, E: ENSORLP00000009376; MARCH9-1, E: ENSORLP00000020760; MARCH9-2, E: ENSORLP00000019029; MARCH11, E: ENSORLP00000007969), *Salmo salar* (MARCH2, GB: NP_001135370; MARCH3, GB: NP_001135117; MARCH5A, GB: ACN58677; MARCH5B, GB: ACI33369), *Takifugu rubripes* (MARCH4, E: ENSTRUP00000027579; MARCH5, E: ENSTRUP00000010692; MARCH6-1, E: ENSTRUP00000022643; MARCH6-2, E: ENSTRUP00000013780; MARCH7, E: ENSTRUP00000043983; MARCH8, E: ENSTRUP00000008831; MARCH9-1, E: ENSTRUP00000006082; MARCH9-3, E: ENSTRUP00000047405; MARCH11, E: ENSTRUP00000003675), *Tetraodon nigroviridis* (MARCH5, E: ENSTNIP00000006615; MARCH6-1, E: ENSTNIP00000010989; MARCH6-2, E: ENSTNIP00000009099; MARCH7, E: ENSTNIP00000012345; MARCH8, E: ENSTNIP00000011522; MARCH9-1, E: ENSTNIP00000021407; MARCH9-2, E: ENSTNIP00000005476; MARCH11, E: ENSTNIP00000010823), and *Xenopus tropicalis* (MARCH5, GB: NP_001116950). Additionally the tree includes human amino acid sequences of MARCH1 (GB: NP_060393), -2 (GB: NP_001005415), -3 (GB: NP_848545), -4 (GB: NP_065865), -5 (GB: NP_060294), -6 (GB: NP_005876), -7 (GB: AAH65014), -8 (GB: AAH66988), -9 (GB: NP_612405), -10 (GB: AAH35021), and MARCH11 (GB: NP_001096032). MARCH5A and -5B sequences are highlighted with black and gray underlay, respectively. Sequences isolated in our laboratory are underlined. The letter '*p*' indicates partial sequences.

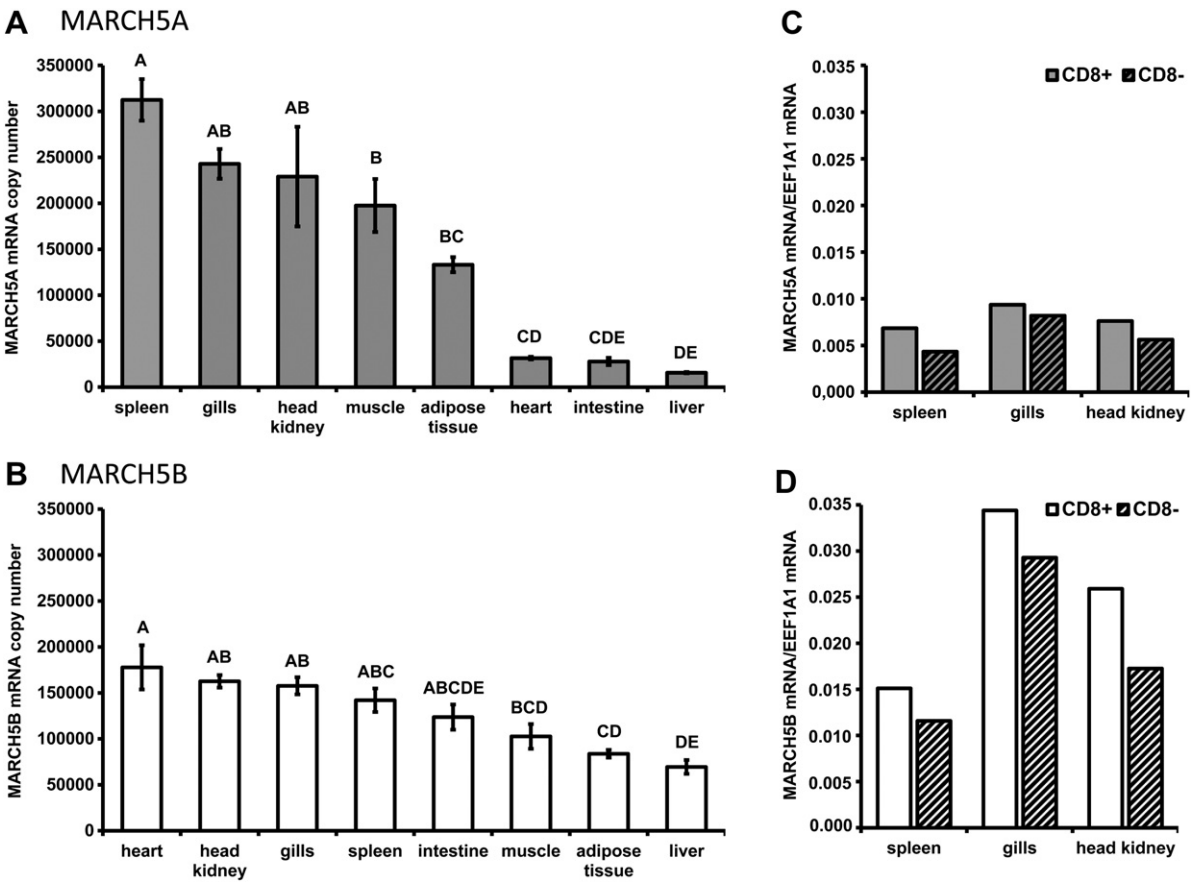

Fig. 4. (A) MARCH5A and (B) MARCH5B transcript number in 75 ng reverse-transcribed RNA isolated from selected tissues of six clinically healthy steelhead rainbow trout. Each bar represents the mean ± SEM. ACTB and EEF1A1 copy numbers were measured in parallel and showed no significant alteration. Different capital letters (A–E) above the bars indicate statistically significant ($p < 0.01$) differences in MARCH5A and -B expression between the different tissues detected by the post-hoc Bonferroni test for each gene. (C) MARCH5A and (D) MARCH5B copy number in anti-CD8α mAb-positive and -negative (open and hatched columns, respectively) leukocytes. Relative expression of MARCH5A and MARCH5B gene against EEF1A1 level in splenocytes, pronephrocytes and branchial lymphocytes is shown. The experiment was repeated with corresponding results.

indicated that teleosts contain duplicate copies of many single copy human genes [26]. Hence, MARCH5 duplicates might be the result of the third round of whole genome duplication (3R) in vertebrates [27]. This 3R duplication event occurred 226–316 million years ago near the origin of bony fishes [28]. Since teleosts are thought to be monophyletic [29], MARCH5A is expected to be present in all teleostean species. The question remains, why teleosts retained both MARCH5 gene copies. Neither variant A nor variant B has been silenced, since expression was confirmed for both. In the case of two parallely expressed duplicate genes, it is possible that both gene products fulfill different tasks (neofunctionalization) or partition the ancestral function (subfunctionalization) [30]. But indeed there are only sparse examples of neofunctionalization or subfunctionalization in fish like the antifreeze glycoprotein gene in Antarctic fishes that derived from a trypsinogen-like protease gene [31] or two closely related protocadherin 15 genes, pcdh15a and pcdh15b, that are required exclusively for function and morphology in the ear and eye, respectively [32]. Nevertheless, we cannot exclude a subfunctionalization phenomenon regarding the MARCH5 genes from trout since both proteins share a lower degree of conservation. Both genes show additionally different expression patterns.

Table 2
MARCH5A and MARCH5B expression ratio between imported and BORN trout.

Tissue	MARCH5A		MARCH5B	
	Expression difference BORN/Import	*P*-value	Expression difference BORN/Import	*P*-value
spleen	0.88	0.36	**0.71**	**0.01**
gills	0.97	0.74	**0.68**	**0.01**
head kidney	1.16	0.54	**0.72**	**0.0004**
white muscle	**0.47**	**0.005**	**0.40**	**0.002**
adipose tissue	0.89	0.37	**0.48**	**0.0006**
heart	1.04	0.73	**0.64**	**0.03**
intestine	1.36	0.12	**0.60**	**0.04**
liver	1.15	0.06	0.79	0.22

Values in bold are significant ($P < 0.05$).

4.2. Duplicated MARCH5 genes from rainbow trout show different expression patterns and might perform different physiological demands

Pronounced MARCH5A mRNA copy numbers have been detected in the immunocompetent organs spleen, head kidney, and gills from rainbow trout. Spleen and head kidney are the major lymphoid and hematopoietic organs of fish, reviewed in [33]. Recently, a gill lymphoid structure termed as “interbranchial lymphoid tissue” has been reported as a quantitatively very important site of T cell aggregation [34] representing an immune-competent barrier against the external. Although MARCH5B gene shows also highest expression values in those three organs together with heart, the MARCH5B copy number in the other organs is not clearly lower as seen in the MARCH5A expression profile. In liver for example, MARCH5A accounts for ~5% of the respective expression

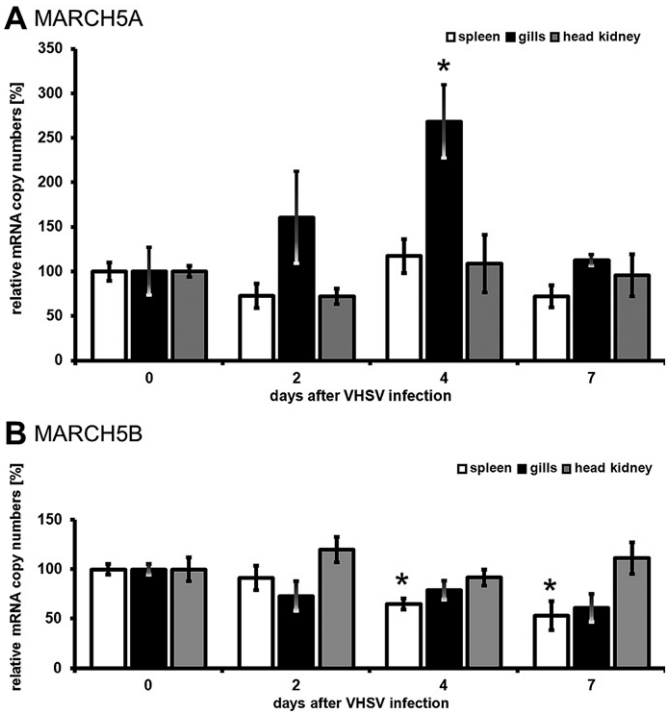

Fig. 5. Relative MARCH5A (A) and MARCH5B (B) copy number after infection of steelhead rainbow trout with VHSV. Spleen (white), gills (black), and head kidney (gray) from three trout at each time point were examined. Each bar represents the mean ± SEM; mean values of MARCH5A and MARCH5B mRNA number from not infected trout were set as 100%. Asterisks indicate significantly different MARCH5 expression levels with $P < 0.05$ compared to the respective tissue of healthy trout.

in spleen, while MARCH5B amounts to ~49%. Expression of both MARCH5 genes in leukocytes from spleen, head kidney, and gills of healthy fish has been proven, although a prevailing MARCH5A copy number compared to the MARCH5B transcript level could not be found. This indicates that MARCH5A expression is elevated in another cell type. It might be conceivable that MARCH5A expression is down-regulated in leukocytes to limit the production of proinflammatory mediators in healthy fish. In this case, TANK might be hardly ubiquitinylated by MARCH5A and is supposed to repress TLR-mediated NF-κB activation. Indeed, a significant increase of MARCH5A copy number by 1.7-fold was observed in gills four days after viral infection. Matsuki et al. suggested an alternative scenario hypothesizing that surface molecules are MARCH-dependently degraded and substituted with newly synthesized proteins to guarantee efficient defense mechanisms [35]. This immunomolecule metabolism might also explain MARCH5A up-regulation in gills of infected fish. Salmonid gills are equipped with extremely thin mucous membranes and may therefore depend on excellent defense mechanisms.

However, a more or less steady MARCH5A mRNA copy number in spleen and head kidney might doubt the involvement of MARCH5 in piscine immune mechanisms. Whether the elevated MARCH5A expression in gills supports the TLR-dependent NF-kB activation needs to be proven. However, our current transcriptome profiling study exploiting gill tissues from rainbow trout infected with *Aeromonas salmonicida* subsp. *salmonicida* shows a comparable up-regulation of MARCH5A gene (results not shown). In detail, a 60-mer sequence (derived from a *D. rerio* sequence with the TIGR accession TC101431) corresponding to trout MARCH5A indicated a significant up-regulation at day 7 after infection compared to healthy animals. This microarray result was verified in a qRT-PCR (2.40 ± 0.24-fold change, $P = 0.03$, $n = 4$). On the other hand, a sequence corresponding to MARCH5B (derived from an annotated *S. salar* EST sequence with the GenBank accession NM_001140034) indicated almost no alteration of expression level (−1.01 to 1.13 within an evaluation period of 21 days, verified by qRT-PCR).

As discussed before, several MARCH proteins including MARCH1, -3, and -10 appear to be absent in some teleostean species, whereas others including MARCH5, -6, and -9 are probably duplicated. The varying number of MARCH proteins among mammalian and non-mammalian vertebrates might indicate alternate functional spectra of the MARCH family members in different species. Regarding human MARCH5, two functions have been described. Firstly, MARCH5 regulates mitochondrial fission and fusion [11]. Secondly, MARCH5 affects TLR signalling [14]. It might be assumed that piscine MARCH5 proteins share the original functions of ancestral MARCH5 factor, i.e. MARCH5A controls immune mechanisms and MARCH5B modifies mitochondrial morphology according to the subfunctionalization theory [30].

Moreover, we found MARCH5A and -5B differentially expressed in several tissues of two trout strains, the economically important steelhead trout TCO and the local steelhead selection strain BORN. A decreased MARCH5B expression appears characteristic for BORN trout probably correlating with decreased ubiquitination in BORN trout tissues, which influences protein degradation/recycling processes. Unfortunately, the physiological role of MARCH5 and its paralogs in teleosts as well as in mammals is not completely understood. The structural characterization and expression analysis of two trout MARCH5 genes and the MARCH5B splice variant provides the basis for further research work.

5. Conclusions

In summary, the present manuscript discusses the differently composed MARCH gene family among vertebrates. It is likely, that some MARCH family members are absent in most fishes, whereas others are duplicated compared to mammals. We provide evidence for the existence of a duplicated MARCH5 gene in at least some teleostean species. In rainbow trout, both MARCH5 genes show characteristic expression profiles in two strains, different tissues and cell types suggesting a similar, but not overlapping activity. MARCH5A might be involved in immune regulation, but further research is required to clarify its definite physiological role in fish.

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The expression of CD8α discriminates distinct T cell subsets in teleost fish

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ABSTRACT

CD8, belonging to the TCR complex, is the main marker molecule of CTLs. Although *CD8* genes have been detected in many fish species, the analysis of teleost CD8$^+$ cells has been limited because of the lack of antibodies. Using newly established mAbs against rainbow trout CD8α, we found high ratios of CD8α$^+$ cells in trout thymus, gill and intestine, but relatively low abundance in pronephros, spleen and blood. Accordingly, tissue sections revealed many CD8α$^+$ cells in thymus, numerous intra- and subepithelial CD8α$^+$ cells in intestine and gill and few scattered CD8α$^+$ cells in spleen and pronephros. In secondary lymphoid tissues, CD8α$^+$ lymphocytes, which did not react with anti-thrombocyte or anti-IgM mAbs, expressed *CD8α*, *CD8β* and *TCRα*, while *Ig* and *CD4* transcripts were found in CD8α$^-$ lymphocytes. In contrast, considerable *CD4* expression in CD8α$^+$ thymocytes suggests the presence of double-positive early T cells. Highly expressed *TCRγ*, *LAG3* and *CTLA4* in CD8α$^+$ lymphocytes imply that they constitute a heterogeneous population different from found in non-mucosal tissues. PHA stimulation resulted in an up-regulation of CTL effector genes (*perforin*, *granulysin* and *IFN-γ*) in CD8α$^+$ pronephrocytes, while both Th1 (*IFN-γ*) and Th2 (*IL-4/13A*) cytokines were up-regulated in CD8α$^-$ pronephrocytes. Although the basic characteristics of CD8α$^+$ lymphocytes seem similar in teleost and mammals, features such as the low proportion of teleost CD8α$^+$ lymphocytes in blood and their high abundance in respiratory tissue reveal a unique dynamics and distribution.

1. Introduction

In higher vertebrates, the adaptive arm of the cellular immune system is mainly represented by helper T (Th) cells and cytotoxic T lymphocytes (CTLs). The TCR co-receptors CD4 and CD8 are the key distinctive markers of these two respective subsets. During T cell differentiation in the thymus, T cell precursors express both CD4 and CD8 molecules, while, after a maturation process, the lymphocytes leaving the thymus have been committed to a particular lineage/subset and have lost the expression of either CD4 or CD8. The CD4/TCR/CD3 and CD8/TCR/CD3 complexes can functionally interact with MHC molecules on antigen presenting cells, with CD4 and CD8 increasing the signaling capacity of T cells by intracellular recruitment of LCK kinase. LCK phosphorylates CD3 molecules, serving as an important trigger for T cell activation. The CD8α subunit of the CD8αβ heterodimer can bind MHC class I molecules on opposing cells by its ectodomain (Miceli and Parnes, 1991; Salter et al., 1989; Sun et al., 1995; Wang et al., 2009) and can intracellularly bind LCK by its cytoplasmic tail (Shaw et al., 1990; Turner et al., 1990). The CD8β molecule, which is an ancient duplication of CD8α, can also bind MHC class I molecules and is important for proper complex orientation (Wang et al., 2009) and for targeting the CD8αβ molecules to the lipid rafts where the TCR/CD3 molecules are located (Pang et al., 2007). There is no report on CD8β function outside of the CD8αβ complex, but enigmatic CD8αα homodimers are found in various cell types – in particular some NK, dendritic and gut intraepithelial T lymphocytes – and these may have an immune inhibitory rather than a stimulatory function (Cheroutre and Lambolez, 2008).

Teleosts possess a well-developed adaptive immune system based on polymorphic MHC antigens and somatically diversified antigen receptors (TCR and BCR) that are expressed on distinct lymphocyte populations (Litman et al., 1999; Flajnik and Kasahara, 2001). However, teleost T cell function is largely unknown mainly because of the lack of population-specific mAbs. The first suggestive evidence for CTL activity in fish derived from experiments showing a memory component in allograft-specific cell-mediated cytotoxicity. Later on, experiments using clonal fish and infected MHC class

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I-matched cell lines (syngeneic or with MHC class I identical to that of the fish) as targets extended these first evidences (Nakanishi et al., 2002; Fischer et al., 2006). Other lines of evidence for a T cell response in fish like catfish cytotoxic lymphocyte cell lines (Stuge et al., 2000) or TCRβ CDR3 spectratyping (Boudinot et al., 2001) also did not directly demonstrate the existence of a $CD8^+$ T cell subset responsible for Ag specific cell-mediated cytotoxicity. In 2000, the first teleost *CD8α* sequence for rainbow trout was reported by Hansen and Strassburger (2000), while its *CD8β* sequence was reported in 2005 by Moore et al. (2005). Since then it has been demonstrated that *TCR* and *CD8α* mRNA expressions in ginbuna crucian carp, rainbow trout and catfish can be up-regulated by virus infection or allogenic stimulation (Fischer et al., 2003; Somamoto et al., 2005, 2006, 2009; Stuge et al., 2000; Utke et al., 2007), suggesting the existence of piscine $CD8^+$ CTLs similar to those of higher vertebrates. At the molecular level, it was shown that the rainbow trout CD8α cytoplasmic tail has the capacity to bind LCK like in humans (Hayashi et al., 2010). Moreover, another TCR co-receptor gene CD4 has been cloned in a number of fish species. Intriguingly, two CD4 molecules, both of which possess a typical LCK binding motif in their cytoplasmic tails, have been identified in fish. One was designated CD4-1 (aliases CD4, CD4L-1) and contains four extracellular Ig domains in all fishes investigated to date (Dijkstra et al., 2006; Edholm et al., 2007; Laing et al., 2006; Nonaka et al., 2008; Suetake et al., 2004) like in higher vertebrates. The other molecule termed CD4-2 (aliases CD4L-2, CD4REL) (Dijkstra et al., 2006; Laing et al., 2006) shows significant similarities with other vertebrate CD4 sequences, but bears only two Ig domains in rainbow trout or three Ig domains in channel catfish (Edholm et al., 2007). The transcripts of these CD4s were predominantly expressed in IgM^- lymphocytes (Laing et al., 2006; Edholm et al., 2007), while their functions remain to be clarified.

In the recent past, a few antibodies against teleost TCR coreceptor molecules, especially CD8α, have been established (Table 1). Fugu $CD8\alpha^+$ PBL, comprising lymphocyte/thrombocyte and monocyte subpopulations were capable to respond to PHA, resulting in $CD8\alpha^+$ cell proliferation (Araki et al., 2008). In the inflammatory site of immune-stimulated fugu, considerable numbers of monocytes/macrophages and neutrophils that expressed *CD8α*, but not *CD8β* mRNA, were detected, which was reminiscent of mammalian $CD8\alpha\alpha^+$ cells. In ginbuna, alloantigen-specific cytotoxicity was found to be mediated by $CD8\alpha^+$ cells, but not by $CD8\alpha^-$ cells (Toda et al., 2009). Recently, Hetland et al. (2010) also produced mAbs against Atlantic salmon CD8α using recombinant *Escherichia coli* proteins for staining of paraffin-embedded tissue sections (they only investigated pronephros, spleen and gill) in virus-infected rainbow trout. However, they have neither shown flow cytometry data nor characterized flow-sorted cells using their mAbs.

In the present work, we report high numbers of $CD8\alpha^+$ cells in the thymus and mucosal tissues (intestine and gills), lower proportions in secondary lymphoid organs (spleen and pronephros; in terms of teleost T cell development and function, these are secondary lymphoid organs and referred to as such in this article), and a surprisingly low number (less than 1%) among PBL. Moreover, we demonstrate the existence of a $CD8\alpha^+$ lymphocyte subset which fairly expresses genes specific for CTLs (*CD8α* and *-β*, *TCRα*, *perforin*, *granulysin* and *IFN-γ*), as well as that of $CD8\alpha^-IgM^-$ lymphocytes, probably comprising Th cells, which express both Th1 cytokine *IFN-γ* and Th2 cytokine *IL-4/13A*. Whereas the expression of Th cell markers (*CD4-1* and *-2*) was barely detectable in extrathymic $CD8\alpha^+$ lymphocytes, the presence of CD4/CD8 double-positive T cells in thymus was suggested by the high expression of *CD4-1* and *-2* transcripts. In addition, expression profiles of various cell-specific markers suggest that CD8α is expressed not only on αβ T cell subpopulations, but also on γδ T cell and NK cell subpopulations, with pronounced differences in abundance of these cell types between tissues. The summarized data suggest that rainbow trout CD8α distribution contributes to compartmentalization of several lymphocyte lineages, similar as in mammals, whereas low numbers of $CD8\alpha^+$ lymphocytes in rainbow trout blood, pronephros and spleen and their high abundance in respiratory tissue are distinctly different from the situation in human and mouse.

2. Materials and methods

2.1. Fish

Homozygous isogenic rainbow trout (*Oncorhynchus mykiss*), clone C25, were derived from Nagano Prefectural Experimental Station of Fisheries, Japan. The clone was produced by gynogenesis over two generations with suppression of mitosis and meiosis in the first and second generations, respectively. The strain "Born" was obtained from the Landesforschungsanstalt für Landwirtschaft und Fischerei, Born, Germany. Fish were maintained in 400 l tanks at 15 °C in a partially recirculating water system and fed with commercial dry pellets. Fish were at the age of one to two years during sampling. For immunohistochemistry, thymi were sampled from fish up to one year of age.

2.2. Leukocyte preparation

Blood was drawn from the caudal vein using a heparinized syringe and immediately diluted in cold mixed medium (MM): Iscove's DMEM/Ham's F12 (Gibco) at a ratio of 1:1, supplemented with 10% fetal bovine serum (FBS) and 0.1% sodium azide. Fish organs (thymus, pronephros, spleen, gill and intestine) were removed and homogenized with a Potter-Elvehjem homogenizer to prepare a single cell suspension. The purged and opened hindgut was briefly but vigorously shaken in MM to discard the excess of mucus before homogenization. The diluted blood and cell suspensions were layered onto an isotonic Percoll (Biochrom AG) gradient ($r = 1.075$ g/ml) and centrifuged at $650 \times g$ for 40 min. Cells at the interphase were collected and washed twice using MM. The cell suspensions were kept on ice until further preparation.

2.3. Immunofluorescence staining and flow cytometry

Methods for making and characterization of mAbs reactive to rainbow trout CD8α are described in the supplementary methods and in supplementary Fig. 1. Cell suspensions from each organ were adjusted to a final concentration of 5×10^6 cells/ml in MM and immunostained with anti-CD8α mAbs and R-PE conjugated $F(ab')_2$ fragments of donkey anti-rat IgG (H + L) (Jackson ImmunoResearch) as described above. For double-staining of cell surface proteins on $CD8\alpha^+$ cells, anti-thrombocyte (mAb 42-1, mouse IgG1) (Köllner et al., 2004) and anti-B cell (mAb 4C10, mouse IgG1; and mAb N2, mouse IgG2b) (Fischer and Köllner, 1994; Thuvander et al., 1990) mAbs were used. Leukocytes from pronephros and spleen were incubated with anti-CD8α mAbs and either with mAb 42-1 (for thrombocyte staining) or with a combination of mAb 4C10 and N2 (for B cell staining). Cells were then labeled with the corresponding mouse isotype-specific FITC and TC (PE-Cy5) conjugates (Caltag). Controls were treated with secondary antibody conjugates only. Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson).

2.4. Immunohistochemistry

Rainbow trout organs (thymus, pronephros, spleen, gills, midgut and hindgut) sampled from anesthetized and exsanguinated rainbow trout were immediately snap-frozen in liquid nitrogen and stored at −20 °C until use. Cryostat sections with a thickness of

Table 1
Antibodies reactive to teleost T cell coreceptor molecules.

Marker	Fish species	Type of Ab	Suitable methods[a]	Reference
CD8α	Rainbow trout	Monoclonal Ab	FCM, CS, IHC, WB, IP	Present article
	Atlantic salmon	Monoclonal Ab	IHC	Hetland et al. (2010)
	Ginbuna crucian carp	Monoclonal Ab	FCM, CS, WB, IP	Toda et al. (2009)
	Torafugu	Polyclonal Ab	FCM, CS, WB, IP	Araki et al. (2008)
CD4-1	Ginbuna crucian carp	Monoclonal Ab	FCM, CS	Toda et al. (2011b)
	Green spotted puffer	Polyclonal Ab	WB	Wen et al. (2010)
CD4-2	Green spotted puffer	Polyclonal Ab	FCM, CS, IHC, WB	Wen et al. (2010)

[a] FCM, flow cytometry; CS, cell sorting (magnetic or fluorescence activated); IHC, immunohistochemistry; WB, western blot; IP, immunoprecipitation.

20 μm were prepared using a Leica CM3050 and placed on glass slides coated with poly-L-lysine (Sigma–Aldrich). Dry sections were acetone-fixed for 5 min, air dried and encircled with a hydrophobic compound (PAP Pen; Kisker).

For single labeling, sections were stained with anti-CD8α mAbs for 30 min. After washing, sections were post-fixed with 4% paraformaldehyde for 5 min and dyed with Alexa 488 conjugated goat-anti-rat-Ig (Molecular Probes). Controls were stained with secondary antibody conjugates only. Sections were mounted with an anti-bleaching counter staining buffer containing 10% glycerine, 2.5% DABCO (Sigma) and 0.0002% propidium iodide (PI) in PBS^{-}.

For double-labeling, frozen sections were first incubated with a mixture of anti-CD8α mAbs and with another population-specific mAb: anti-thrombocyte mAb 42-1 or anti-IgM mAb 4C10, washed, and then stained with secondary antibodies (Alexa 488 goat-anti-rat-Ig conjugate (Molecular Probes) and goat-anti-mouse IgG1-Cy3 (Caltag)). Controls were stained with secondary antibody conjugates only. Sections were then washed again twice and finally mounted with an anti-bleaching buffer containing 10% glycerine and 2.5% DABCO.

Sections from rainbow trout organs were examined by confocal laser scanning microscopy using a Zeiss LSM510 (Zeiss, Germany). For single-labeled sections, scanning was performed at an excitation laser line of 488 nm, and images from optical slices of approximately 1 μm thickness were generated. Filters and beam splitters were adjusted for the detection of green (FITC) and red (PI) emissions. For double-stained sections, two excitation laser lines (488 and 543 nm) were used, and filters and beam splitters were adjusted to detect green (FITC) and red (Cy3) emissions.

2.5. RT-PCR analysis of flow-sorted cells

Leukocytes from each organ were prepared as described above and incubated with anti-CD8α mAbs in MM for 30 min. The cells were washed twice with MM and incubated with FITC-conjugated goat anti-rat IgG (H + L) (Jackson ImmunoResearch) for 20 min. Flow-sorting was performed with a MoFlo (Dako) high speed cell sorter. The lymphocytes were sorted into two populations, $CD8\alpha^{+}$ and $CD8\alpha^{-}$ cells. After sorting, total RNA was extracted from 30,000 cells of each cell population using RNeasy Plus Micro Kit (Qiagen). One-step RT-PCR was carried out with gene-specific primers (Supplemental Table 1) using a OneStep RT-PCR Kit (Qiagen) according to the manufacturer's guidebook. The cycle numbers used for each gene are listed in the figures. PCR products were analyzed by standard agarose gel electrophoresis. Images of the PCR products were recorded with an FK-7512-IQ camera (PIEPER) and Phoretix Grabber software (Biostep).

2.6. Expression analysis of effector molecules in stimulated pronephrocytes

Leukocytes were isolated from pronephros as described above and incubated with anti-CD8α mAbs and a combination of mAb 4C10 and N2, followed by staining with FITC-conjugated goat anti-rat IgG (H + L) and R-PE-conjugated anti-mouse IgG subclass specific antibodies (Jackson ImmunoResearch). The stained leukocytes were separated into $CD8\alpha^{+}$, IgM^{+} and $CD8\alpha^{-}IgM^{-}$ cells with a MoFlo high speed cell sorter. The sorted cells were plated onto 96-well microplates and then incubated with or without 10 μg/ml of PHA-L (Biochrome AG) for 9 h. After stimulation, total RNA was extracted from each population, and the expression of the effector molecules, *perforin*, *granulysin*, *IFN-γ* and *IL-4/13A*, was examined by RT-PCR as described above. To measure the relative amounts of *IFN-γ* mRNA, real-time RT-PCR was performed using QuantiTect Probe RT-PCR Kit (Qiagen) and analyzed in an Mx3000P thermo cycler (Stratagene). Target quantity was determined using a relative standard curve method with total RNA from PHA-stimulated pronephrocytes. The expression level of mRNA from unstimulated $CD8\alpha^{+}$ cells was designated as the calibrator. Results were described as relative to the calibrator.

3. Results

3.1. Characterization of anti-rainbow trout CD8α mAbs

Established mAbs showed high reactivity with both live thymocytes and live epithelioid cell line Normal Rat Kidney clone 52E (NRK-52E; ATCC, CRL-1571) expressing trout CD8α in flow cytometry, while being negative with parental NRK cells (Supplementary Fig. 1A). To further verify the specificity of the mAbs, western blot analysis was performed to compare the binding of anti-CD8α and anti-HA mAbs to lysates of NRK control cells and of NRK cells expressing HA-tagged trout CD8α (Supplementary Fig. 1B). Under reducing conditions the anti-HA mAbs detected proteins at the expected size of ~32 kDa. Under non-reducing conditions the anti-HA mAbs bound to proteins running at less than 49 kDa and approximately 70 and 74 kDa, while those largest bands were also recognized by anti-CD8α mAbs. Moreover, proteins precipitated by anti-CD8α and anti-HA mAbs were detected by anti-HA mAbs and appeared at the same positions (Supplementary Fig. 1C). These results indicate that the established anti-CD8α mAbs recognize rainbow trout CD8α protein.

3.2. Rainbow trout $CD8\alpha^{+}$ cells are lymphocytes distinct from B cells and thrombocytes

To characterize the rainbow trout leukocyte subpopulation displaying CD8α molecules, pronephrocytes and splenocytes were stained with anti-CD8α mAbs and examined by immunofluorescence microscopy and flow cytometry. In confocal laser scanning microscopy, $CD8\alpha^{+}$ cells appeared as lymphocytes (Fig. 1A). Flow cytometry revealed that a relatively small proportion (approximately 2%) of whole pronephrocytes (Fig. 1B, left) and splenocytes (data not shown) expressed CD8α molecules on their cell surface. Positive cells represented a distinct, strongly labeled leukocyte subpopulation with scatter characteristics of small, non-granulated cells (FSC^{low}/SSC^{low}, respectively) (Fig. 1B, upper right), which is typical for both fish lymphocytes and thrombocytes (nucleated

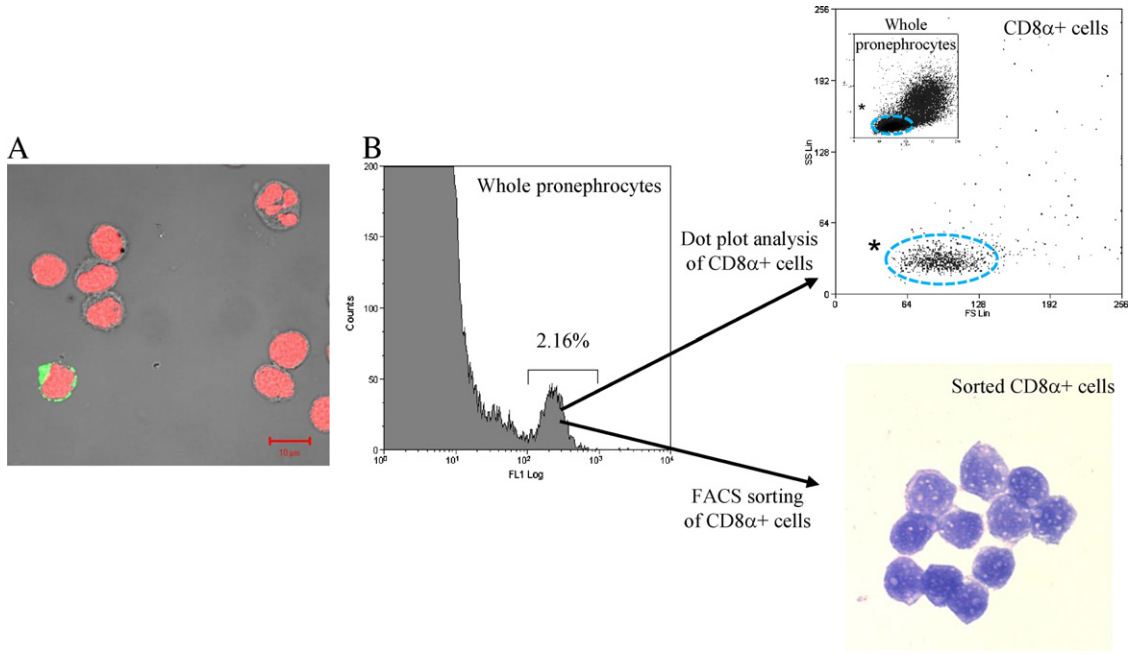

Fig. 1. Morphological features of CD8α⁺ pronephrocytes. Cells from trout pronephros were stained with anti-CD8α mAbs and FITC-conjugated goat anti-rat IgG. (A) Confocal laser scanning micrograph of unsorted pronephrocytes (green = anti-CD8α mAb; red = PI; grey = differential interference contrast, DIC). (B) Flow cytometry of rainbow trout pronephrocytes stained with anti-CD8α mAbs. (Left) histogram of fluorescence intensity (*X*-axis) versus cell number (*Y*-axis) of mAb-labeled whole pronephrocytes. 2.16% of the whole population is CD8α⁺. (Upper right) FSC versus SSC dot plot profile of CD8α⁺ pronephrocytes. The majority of positive cells originate from the FSC^{low}/SSC^{low} population. Inset in upper-right dot plot represents forward (FSC, *X*-axis) versus side (SSC, *Y*-axis) scatter dot plot profile of whole pronephrocytes. Trout pronephrocytes comprise three populations: lymphocytes (FSC^{low}/SSC^{low}), macrophages/blast cells (FSC^{high}/SSC^{low}) and granulated cells (FSC^{high}/SSC^{high}). Lymphocyte population is circled in blue dotted line. (Lower right) the morphology of sorted CD8α⁺ pronephrocytes. Cytospin preparation of sorted CD8α⁺ pronephrocytes was stained by Giemsa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

cells in non-mammalian vertebrates). The lymphocyte morphology of CD8α⁺ cells was also confirmed by microscopic analysis following cell sorting (Fig. 1B, lower right). Moreover, two-color flow cytometry with pronephrocytes (Fig. 2A) and splenocytes (Fig. 2B) using anti-thrombocyte mAb 42-1 or anti-IgM mAbs 4C10 and N2 indicated that the CD8α⁺ cells are neither thrombocytes nor IgM⁺ B cells.

3.3. *Fish CD8α⁺ cells are present in all lymphoid tissues and constitute follicle-like structures in the spleen*

To investigate the proportion and distribution of rainbow trout CD8α⁺ cells, flow cytometry with leukocytes from blood, lymphoid organs (thymus, pronephros and spleen) and mucosal tissues (gill and intestine) was performed (Fig. 3A). The proportion of CD8α⁺ cells in lymphoid cells from blood and lymphoid tissues is indicated in Fig. 3B. Flow cytometry showed a high percentage of CD8α⁺ cells among thymic (approximately 70%), intestinal (approximately 54%) and gill (approximately 25%) lymphocytes, whereas only low percentages of CD8α⁺ cells were recorded in splenocytes (approximately 2%) and pronephrocytes (approximately 4%). The percentage of CD8α⁺ cells among PBL was remarkably low (approximately 0.3%). Most of the CD8α⁺ cells in the blood and secondary lymphoid tissues were strongly labeled, while thymocytes showed varying staining intensities, indicating different amounts of surface CD8α molecules per cell (Fig. 3A).

To study the distribution of CD8α⁺ cells within the lymphoid tissues, cryostat sections of lymphoid organs and mucosal tissues were prepared and stained with anti-CD8α mAbs. In the thymus, CD8α⁺ cells were less abundant in the external (sub-epithelial) zones (Fig. 4A) of the organ whereas the internal (basal) zones harbored high numbers of CD8α⁺ cells (Fig. 4B and C). Some sections of the CD8α-rich compartments were highly vascularized (Fig. 4C). In the spleen (Fig. 4D–G), a rather low number of scattered CD8α⁺ cells were observed (Fig. 4D), with very few dense follicle-like clusters of positive cells (2–3 clusters per whole spleen section; Fig. 4E). Those clusters were frequently observed in viral hemorrhagic septicemia virus (VHSV) infected rainbow trout (data not shown). Neither solitary positive cells nor clustered cells were associated with melanomacrophage centers. No staining of IgM⁺ cells (Fig. 4F) or thrombocytes (Fig. 4G) with anti-CD8α mAbs was observed, confirming the specificity of the labeling. In the pronephros (Fig. 4H), the density of CD8α⁺ cells was higher than in the spleen, while the mesonephros (Fig. 4I) harbored less CD8α⁺ cells. No follicle-like structures, but some small clusters of up to five CD8α⁺ cells were observed, suggesting that the role of spleen and pronephros may be different in the fish CD8⁺ T cell responses. As in the spleen, pronephros CD8α⁺ cells were not linked to any morphological entity (e.g. to melanomacrophage centers). The primary and secondary lamellae of the gill contained numerous CD8α⁺ intra-epithelial cells (Fig. 4J). Another focus of positive cells was observed in a lymphoid structure of the junction between primary lamellae (Fig. 4K, dashed circle) that was recently described in salmonids (Haugarvoll et al., 2008) and that we now call interbranchial lymphoid tissue (ILT) (Koppang et al., 2010). Numerous clusters of CD8α⁺ cells were also seen in the epithelial tissue covering the gill arches (Fig. 4L). Like in the gill, the presence of intestinal CD8α⁺ cells was a frequent observation, with lower abundance in the midgut (Fig. 4N and O) than in the hindgut (Fig. 4P and Q). Although no follicle-like structures were found in the gut of rainbow trout, the lamina propria below the crypt epithelium (Fig. 4P) and the mucosal folds (Fig. 4Q) harbored clusters of lymphoid cells partially consisting of CD8α⁺ lymphocytes. Like in the spleen no staining of IgM⁺ cells with anti-CD8α mAbs was observed (Fig. 4O).

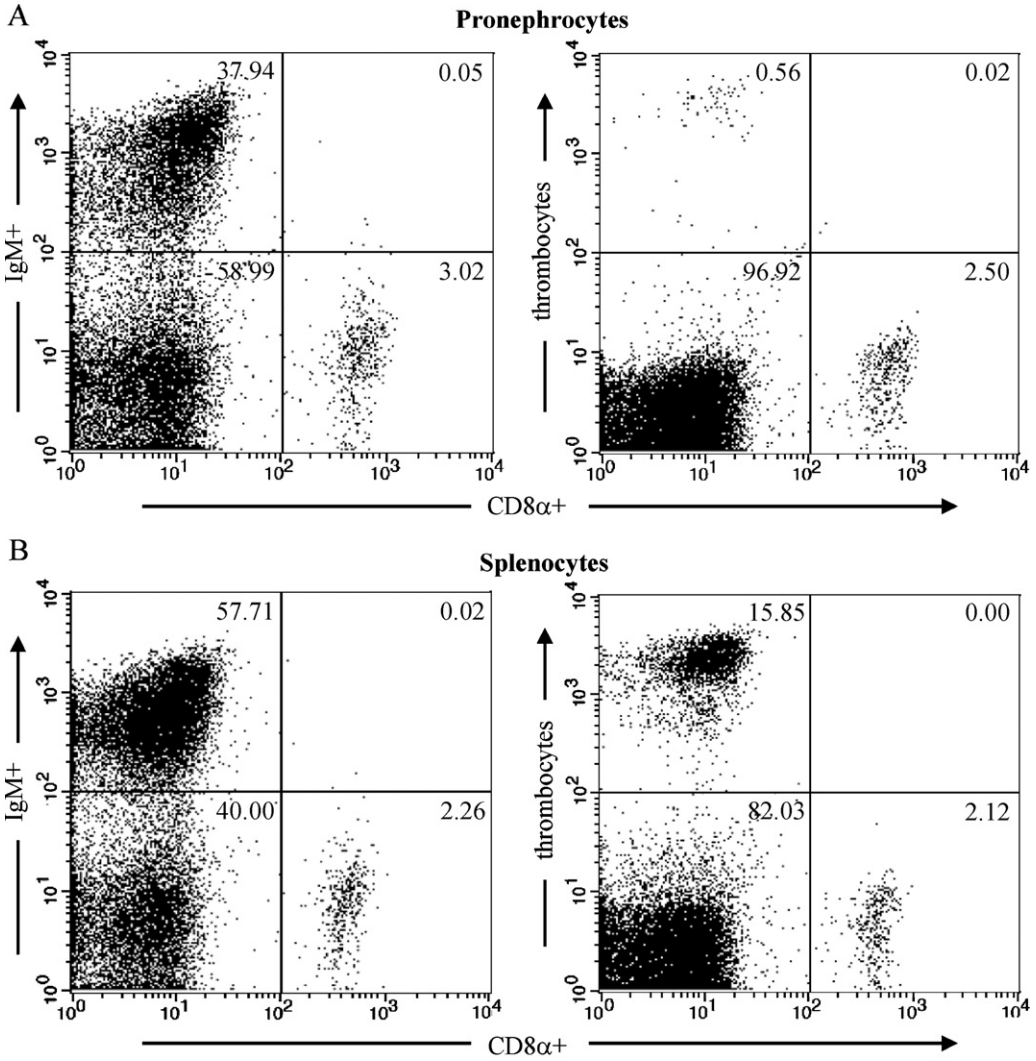

Fig. 2. Double-labeling of pronephrocytes and splenocytes with anti-CD8α and anti-IgM or anti-thrombocyte mAbs. Leukocytes from pronephros (A) and spleen (B) were stained with anti-CD8α mAbs and anti-IgM or anti-thrombocyte mAbs. FSC^{low}/SSC^{low} lymphocytes were gated and their fluorescence intensity was plotted. The *X*-axis depicts staining with the anti-CD8α mAb while the *Y*-axis represents IgM and thrombocyte staining. All $CD8\alpha^+$ cells are depicted in the lower right quadrants while the IgM^+ cells and thrombocytes appear in the upper left quadrants, respectively. The number of cells in the upper right quadrant representing double-stained cells was negligible. Data are from a single representative of two independent experiments. In each experiment, leukocytes from three individuals were pooled.

3.4. CD8α⁺ lymphocytes from thymus, blood, secondary lymphoid organs and mucosal tissues show distinct gene expression profiles

To further characterize the phenotype of rainbow trout $CD8\alpha^+$ cells, $CD8\alpha^+$ and $CD8\alpha^-$ lymphocytes from thymus, blood, secondary lymphoid organs and mucosal tissues were flow-sorted, followed by gene expression analysis of cell-specific marker (Fig. 5). Sorted $CD8\alpha^+$ and $CD8\alpha^-$ lymphocytes had a purity of more than 95% (data not shown). RT-PCR using total RNA from flow-sorted extrathymic lymphocytes showed that *CD8α* and *-β* were exclusively expressed in $CD8\alpha^+$ lymphocytes, and no such transcripts were detected in $CD8\alpha^-$ lymphocytes. In thymus, *CD8α* and *-β* were highly expressed in $CD8\alpha^+$ lymphocytes, while low levels of both transcripts were detected in $CD8\alpha^-$ lymphocytes. *CD4-1* and *-2* were readily detected in both $CD8\alpha^+$ and $CD8\alpha^-$ thymocytes, while in extrathymic lymphoid tissues only $CD8\alpha^-$ lymphocytes expressed substantial amounts of *CD4-1* and *-2*. However, some *CD4-1* and *-2* expressions were also recorded for $CD8\alpha^+$ lymphocytes. The expression of *IgM* and *IgT* was exclusively detected in $CD8\alpha^-$ lymphocytes, while *TCRα* was expressed in all investigated lymphocyte populations. These results strongly suggest that fish possess distinct T cell subsets with quasi-exclusive expression of CD8 and CD4-1/2.

In addition, we also investigated the expression of NK and γδ T cell markers because a subpopulation of these cells expresses CD8αα homodimers in mammals (Cheroutre and Lambolez, 2008). For this, we have cloned the rainbow trout *DAP12* (Supplemental Fig. 2) to analyze its expression together with another potential NK cell marker *FcRγ*. Overall, both rainbow trout *FcRγ* and *DAP12* transcripts were predominantly found in extrathymic $CD8\alpha^-$ lymphocytes, while $CD8\alpha^+$ lymphocytes from secondary lymphoid organs (pronephros and spleen) also expressed considerable amounts of these genes. The expression of *TCRγ* was detected at higher levels in thymic and mucosal lymphocytes when compared to other lymphocytes (see also Supplemental Fig. 3), suggesting the prevalence of fish γδ T cells in thymus and mucosal tissues as in mammals. Importantly, *TCRγ* expression by $CD8\alpha^+$ and $CD8\alpha^-$ lymphocytes suggests the existence of γδ T cells in either cell population, as is known for mammals. The costimulatory receptor gene *CD28*, which in mammals is constitutively expressed by naive T cells, showed an expression pattern similar to *TCRα*. Two other receptor genes, *CTLA4* and *LAG3*, were predominantly expressed in

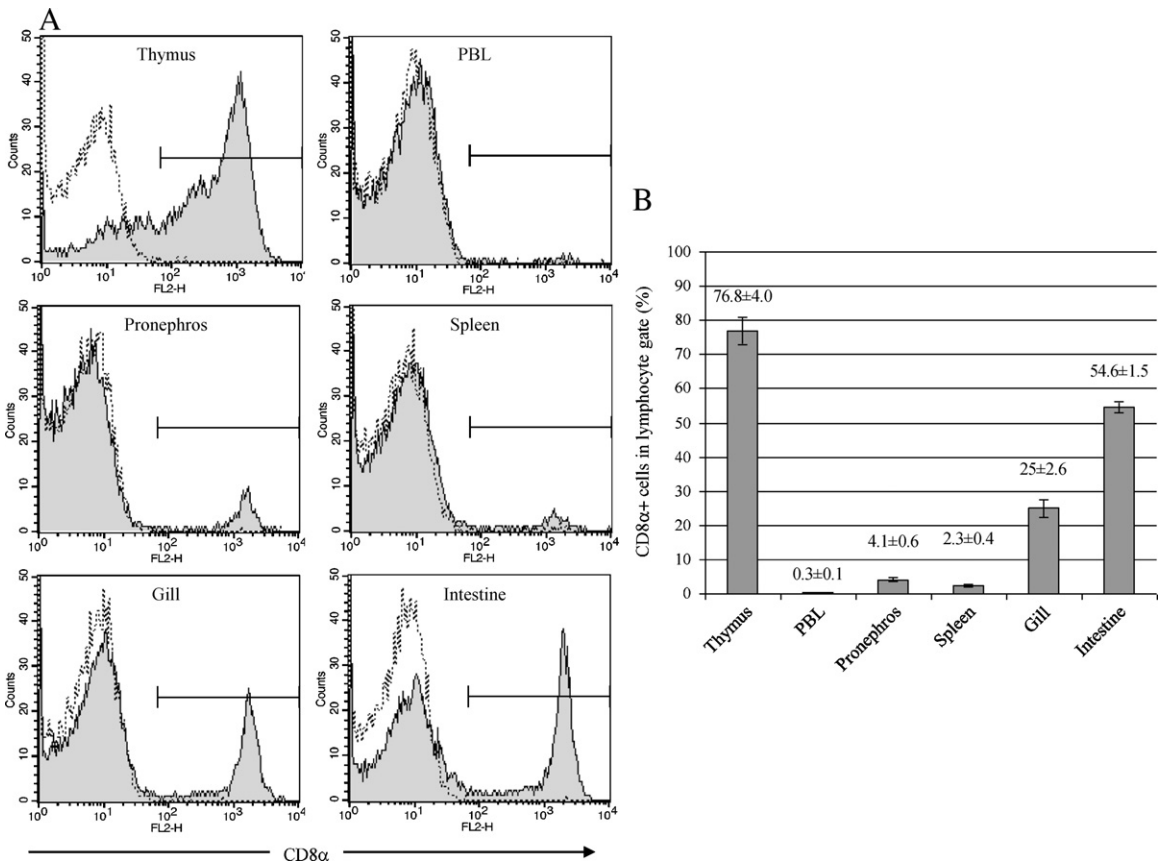

Fig. 3. Flow cytometry of CD8α$^+$ cells in rainbow trout lymphatic tissues. Cell suspensions from various lymphatic tissues (peripheral blood, pronephros, spleen, thymus, gill and intestine) were stained with anti-CD8α mAb and R-PE-conjugated goat anti-rat IgG. (A) Histograms from FSC^{low}/SSC^{low} cells were recorded. Shaded histograms depict mAb staining and the dashed lines represent control staining with the secondary antibodies alone. The histograms are representative of four individuals. (B) Percentages of CD8α$^+$ lymphocytes in various tissues of rainbow trout. The numbers indicate percentages (mean ± SE, $n = 4$) of positive cells.

both mucosal CD8α$^+$ and CD8α$^-$ lymphocytes. Mammalian CTLA4 and LAG3 are expressed on activated lymphocytes and are constitutively expressed on subsets of regulatory T cells (Corthay, 2009). In mammals, integrin β7 (ITGB7) forms heterodimers with ITGA4 and integrin αE (ITGAE, CD103), which are expressed on T cells to mediate homing to and retention in mucosal tissues, respectively (Gorfu et al., 2009). Expression of these adhesion molecules in rainbow trout is reminiscent of the situation in mammals. Rainbow trout *ITGA4* showed highest expression levels in CD8α$^+$ PBL, while *ITGAE* and *ITGB7* were abundantly expressed in thymic and mucosal CD8α$^+$ lymphocytes. To our knowledge the present study is the first to analyze expression of these integrin genes in fish.

3.5. Rainbow trout CD8α$^+$ cells express CTL effector molecules

In mammals, CD8$^+$ αβ T cells typically express a number of effector molecules such as perforin, granulysin and IFN-γ, that are released in large amounts upon activation and differentiation and that are involved in target cell lysis and viral clearance. To determine whether rainbow trout CD8α$^+$ cells harbor the same repertoire of effectors molecules, the mRNA expression of *perforin*, *granulysin* and *IFN-γ* was quantified in CD8α$^+$ and CD8α$^-$ lymphocytes sorted from pronephros and spleen in naive fish. *Perforin* and *granulysin* were predominantly, but not exclusively, expressed in CD8α$^+$ lymphocytes, while *IFN-γ* was expressed in similar amounts by both CD8α$^+$ and CD8α$^-$ lymphocytes (Fig. 6A). As shown in Fig. 6B, PHA stimulation of rainbow trout whole pronephrocytes enhanced the expression of *perforin*, *granulysin* and *IFN-γ* over time. To further dissect the capacity of rainbow trout lymphocyte subpopulations to express *perforin*, *granulysin* and *IFN-γ*, we compared the responses of sorted CD8α$^+$, IgM$^+$ and CD8α$^-$IgM$^-$ lymphocytes from pronephros to PHA stimulation. PHA treatment strongly enhanced expression of the three CTL effector genes in CD8α$^+$ lymphocytes (Fig. 6C) while only a modest enhancement of *IFN-γ* was detected in CD8α$^-$IgM$^-$ cells; the *IFN-γ* expressing CD8α$^-$IgM$^-$ pronephrocytes may comprise CD4$^+$ Th cells. No significant increase in the expression of the three CTL effector molecules was detected for the IgM$^+$ population. Furthermore, we confirmed the *IFN-γ* expression level by using real-time PCR, and PHA stimulation caused a 702-fold increase in *IFN-γ* expression in CD8α$^+$ lymphocytes and a 5.4-fold increase in CD8α$^-$IgM$^-$ cells (Fig. 6D). Interestingly, *IL-4/13A*, a teleost homolog of Th2 cytokines IL-4 and IL-13 in higher vertebrates (Ohtani et al., 2008), was only abundant in PHA-stimulated CD8α$^-$IgM$^-$ pronephrocytes (Fig. 6B and C), and not in non-stimulated cells or other investigated pronephrocyte subpopulations. This is very suggestive for the existence of CD8α$^-$ Th cells secreting Th2 cytokines as known in mammals.

4. Discussion

In the present paper, we describe the first comprehensive analysis of teleost CD8α$^+$ lymphocytes by using newly established mAbs against rainbow trout CD8α. The study comprised immunocytochemistry, immunohistochemistry, gene expression profiling, and immune stimulation experiments. Our results strongly suggest that CD8α is an ancient marker for distinct compartments of αβ T cells, γδ T cells and NK cells. Gene expression profiles were con-

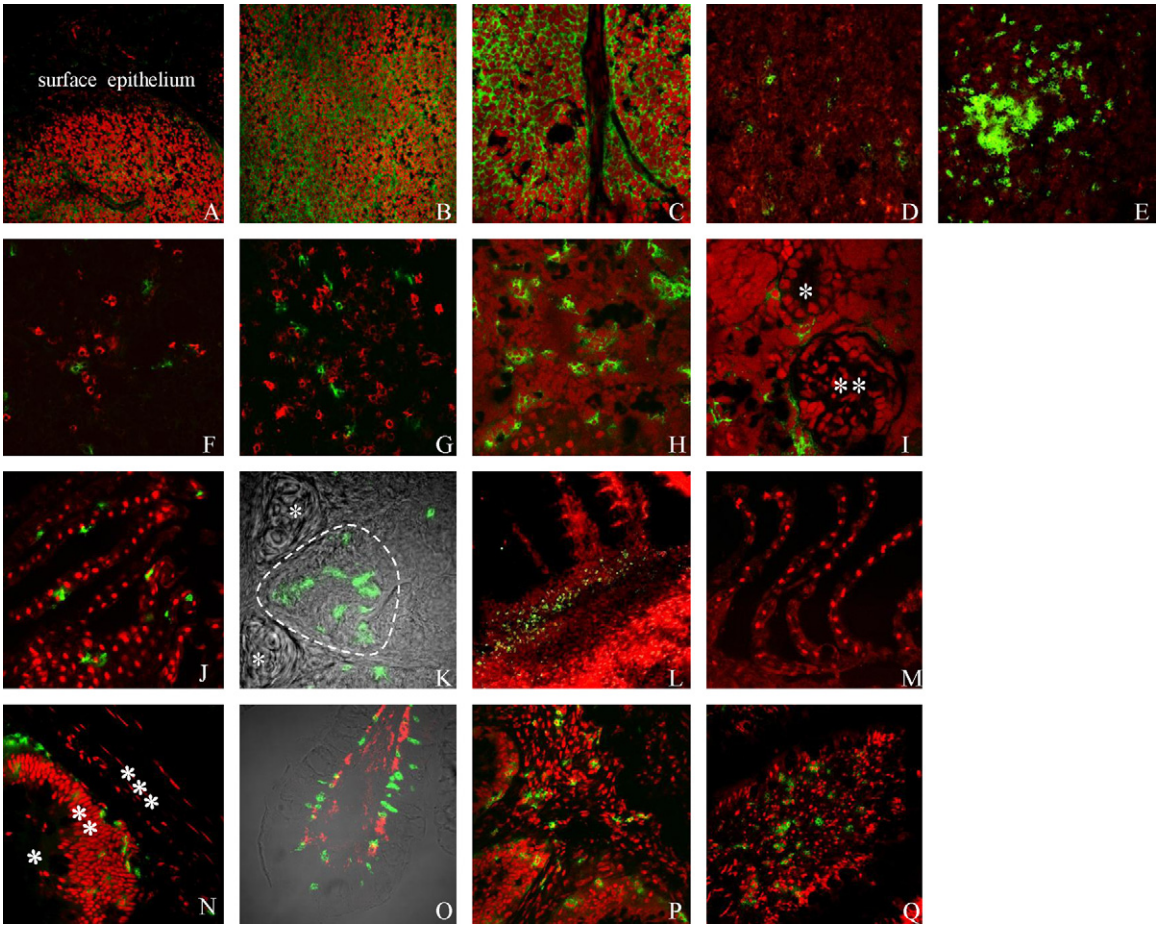

Fig. 4. Organ distribution of CD8α$^+$ cells *in situ*. (A–C) Thymus, single labeling (green = CD8α, red = PI/nucleic acid); (A) thymus surface covered by epithelium; (B and C) numerous positive cells with high abundance in the deeper layers. Frame size (FS of length × width) in μm: (A and B) = 307.4; (C) = 136.8. (D–G) spleen; (D and E) single labeling (green = CD8α, red = PI/nucleic acid); (D) scattered CD8α$^+$ cells in the parenchyma; (E) occasionally detected clusters of CD8α$^+$ cells; (F) double-labeling with anti-IgM mAbs (green = CD8α, red = IgM), no double-stained cells detected; (G) double-labeling with anti-thrombocyte mAb (green = CD8α, red = thrombocytes), no double-stained cells detected. FS in μm: (D and F) = 230.3; (E) = 325.8; (G) = 179.1. (H) Pronephros, single labeling (green = CD8α, red = PI/nucleic acid). FS in μm: 230.3. (I) Mesonephros, single labeling (green = CD8α, red = PI/nucleic acid), * = CD8α$^-$ tubule, ** = CD8α$^-$ malpighian corpuscle. FS in μm: (I) = 162.9. (J–M) gill, single labeling (green = CD8α, red = PI/nucleic acid; grey = DIC); (J) secondary lamellae with intraepithelial CD8α$^+$ cells; (K) accumulation of CD8α$^+$ cells in the ILT (encircled), * = CD8α$^-$ cartilage; (L) numerous subepithelial and intraepithelial CD8α$^+$ cells in a lymphoid structure of the gill arch; (M) conjugate control, secondary lamellae. FS in μm: (J, L and M) = 230.3; (K) = 209.4. (N–Q) Intestine; (N, P and Q) single labeling (green = CD8α, red = PI/nucleic acid); (N) midgut, * = crypt lumen, *** = CD8α$^-$ lamina muscularis, ** = intra- and subepithelial CD8α$^+$ cells, (O) midgut, double-labeling with anti-IgM mAbs (green = CD8α, red = IgM, grey = DIC), no double-stained cells detected; (P) hindgut, numerous intra- and subepithelial CD8α$^+$ cells; (Q) hindgut, numerous CD8α$^+$ cells in the lamina propria; FS in μm: (N) = 230.3; (O and Q) = 325.8; (P) = 305.4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sistent with the existence of CD8α$^+$ CTLs as known in mammals, and suggest the presence of CD4$^+$ Th cells in the CD8α$^-$ lymphocyte population. Intriguing are the very few CD8α$^+$ lymphocytes in the blood, and the high abundance of CD8α$^+$ lymphocytes in respiratory tissue, which is very different from the situation in mammals.

Rainbow trout CD8α$^+$ cells appeared as small ungranulated cells (FSC^{low}/SSC^{low}) which is a typical feature of both lymphocytes and thrombocytes in fish. They are clearly distinct from thrombocytes or IgM$^+$ B cells since they are not stainable by anti-IgM or anti-thrombocyte antibodies. Furthermore, sorted CD8α$^+$ cells lacked *IgT* transcripts, excluding the B cell subset expressing IgT but not IgM (Hansen et al., 2005; Li et al., 2006; Zhang et al., 2010). These results are somewhat in contrast with a recent report of Araki et al. (2008), describing that an anti-fugu CD8α serum also binds to cells of the myeloid lineage (monocytes/macrophages and neutrophils). In marine species fugu, which is from a different teleost superorder than salmonids, almost half of fugu PBL that had morphological properties of monocytes/macrophages/granulocytes expressed CD8α molecules. In rainbow trout, only very few cells with morphological features of doublets, lymphoblasts and monocytes/macrophages (FSC^{high}/SSC^{low}) or granulocytes (FSC^{high}/SSC^{high}) were stained by our anti-CD8α mAbs, even in the peritoneal lavage from fish inoculated with *Aeromonas salmonicida* (data not shown). It would be interesting to determine if CD8α receptor is indeed expressed by different lineages of leukocytes in different fish species, or if these conflicting observations between rainbow trout and fugu are due to non-specific reactions of the antiserum with molecules other than CD8α.

Rainbow trout extrathymic CD8α$^+$ lymphocytes strongly expressed *CD8α* and *-β* and very low amounts of both *CD4-1* and *-2*, while extrathymic CD8α$^-$ lymphocytes expressed *CD4s* but not *CD8s*. This pattern is in good accordance with the presence in fish of both CD8$^+$CD4$^-$ and CD4$^+$CD8$^-$ T cell subsets. The expression, though low, of *CD4-1* and *-2* in rainbow trout extrathymic CD8α$^+$ lymphocytes may indicate the existence of mature CD4$^+$CD8αα$^+$ T cells as found in mammals. In human and mouse, CD4$^+$CD8αα$^+$ T lymphocytes are relatively few and mostly restricted to the intestine (Kunisawa et al., 2007), while in swine this cell population is abundant in blood and lymphoid tissues (Saalmüller et al., 1987). The RT-PCR data suggest that trout CD4$^+$CD8αα$^+$ cells are few like

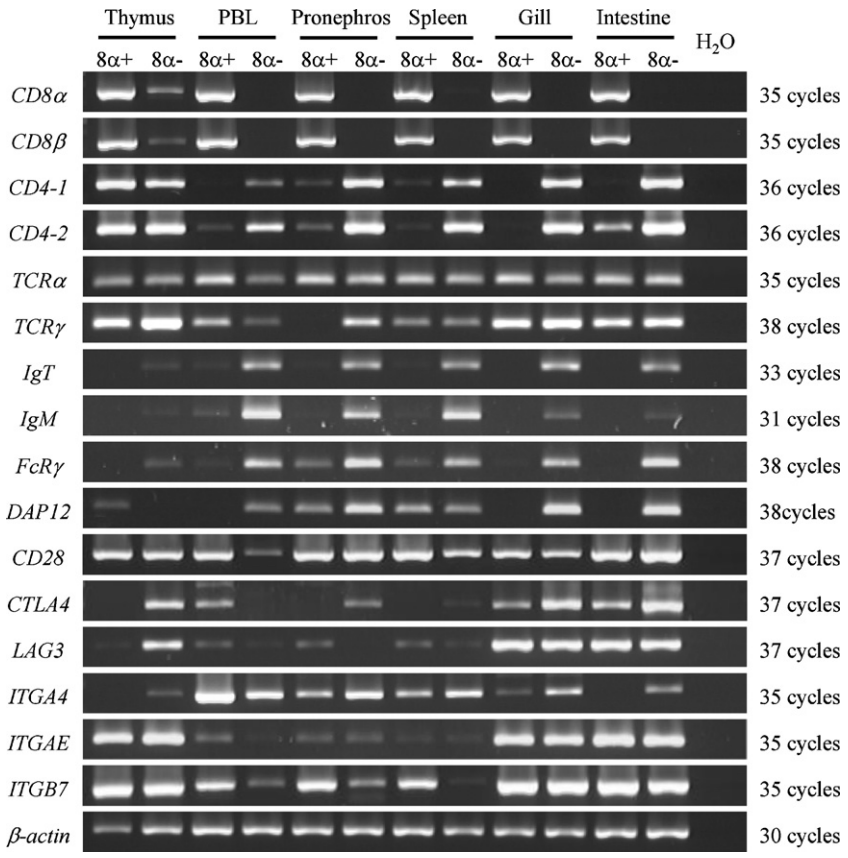

Fig. 5. Expression profile of flow-sorted rainbow trout CD8α$^+$ and CD8α$^-$ lymphocytes from blood, lymphoid and mucosal tissues. Rainbow trout leukocytes from thymus, PBL, pronephros, spleen, gill and intestine were stained with anti-CD8α mAb and FITC-conjugated goat anti-rat IgG, and then were sorted into CD8α$^+$ and CD8α$^-$ cells. RNA was extracted from both populations and analyzed by RT-PCR using primers for lymphocyte cell-specific marker genes. Data shown are from a single representative of three independent experiments. In each experiment, leukocytes from three different individuals were pooled.

in human and mouse, without indicating their predominance in intestine found in those species.

Importantly, CD8α$^+$ thymocytes express high amounts of *CD4-1* and *-2* transcripts, reminiscent of the CD4/CD8 double-positive cortical thymocytes of mammals (Ellmeier et al., 1999; Laky et al., 2006). In trout thymus, *CD8α* and *-β* transcripts were also found in CD8α$^-$ lymphocytes, be it at low levels. This may result from thymocytes that express too little CD8α for efficient antibody staining. The abundance of trout cells with bright CD8α$^+$ staining was high in the lower (basal) thymic layers but moderate in the subepithelial parts of the thymus, as in mammalian cortex and medulla (Van Ewijk et al., 1990), respectively. According to the mammalian affinity model of thymic selection, cells with very high levels of CD8 expression are deleted during positive selection, while lymphocytes with moderate levels are selected for maturation in the medulla (Lee et al., 1992). It will be very interesting to see in the future whether thymocytes include a CD4-1$^+$CD4-2$^+$CD8αβ$^+$ population, since that might imply "competition" between three different TCR co-receptors that individually have LCK binding capacity. Taken together, our results suggest that the expression of CD8 and CD4 markers defines distinct subsets in fish as in mammals, as well as differentiation stages of T cell precursors. The appearance of these subsets would have therefore predated the common ancestor of fish and tetrapods.

Mammalian CD8α molecules can be expressed as CD8αα homodimers on various leukocytes such as NK cells, αβ T cells, γδ T cells, regulatory T cells, DCs and macrophages. NK and myeloid cells express the transmembrane adaptor molecules DAP12 and FcRγ, which mediate intracellular signaling via ITAM motifs in their cytoplasmic tails. In zebrafish, both transcripts were detected in lymphoid cells, while *FcRγ* was also found in the myeloid cells (Yoder et al., 2007). Moreover, zebrafish DAP12 induced active signaling in combination with NITR9 (Novel Immune Type Receptor 9), which may be a functional equivalent of human KIRs (Wei et al., 2007). In the present study, we have identified rainbow trout *DAP12* and revealed that rainbow trout *DAP12* and *FcRγ* are predominantly expressed in CD8α$^-$ lymphocytes. By contrast, the expression of both molecules in CD8α$^+$ lymphocytes, especially in secondary lymphoid organs (pronephros and spleen), suggests the presence of rainbow trout CD8α$^+$ NK cells reminiscent of human CD8αα$^+$ NK cells (Cheroutre and Lambolez, 2008). Other mammalian lymphoid cells displaying CD8αα homodimers are γδ T cells. Human and murine γδ T cells are abundantly found in intestine but barely exist in blood and secondary lymphoid organs while some artiodactyls, e.g. bovine and swine, have abundant γδ T cells in their circulation, indicating high interspecies variability. Although the function of fish γδ T cells is largely unknown, their involvement in antigen recognition and mucosal immunity was suggested in terms of their recombinational diversity and expression (Boschi et al., 2010; Yazawa et al., 2008a,b). Our expression data reveal that *TCRγ* is most highly expressed in mucosal and thymic lymphocytes, in both CD8α$^+$ and CD8α$^-$ fractions, suggesting similarities in the distribution of γδ T cells between rainbow trout, human and mouse. In human and mouse γδ TCRs, tissue-biased usage of variable gene segments was shown (Bonneville et al., 2010). In Atlantic salmon, the *TCRγ* and *-α/δ* loci have multiple variable (V) segments (Yazawa et al., 2008a,b), but V segment specific tissue-biases are unknown. However, relative expression of six different constant (C) region segments of Atlantic salmon *TCRγ* was found to vary between tissues (Yazawa et al., 2008b), and in rainbow trout this is probably

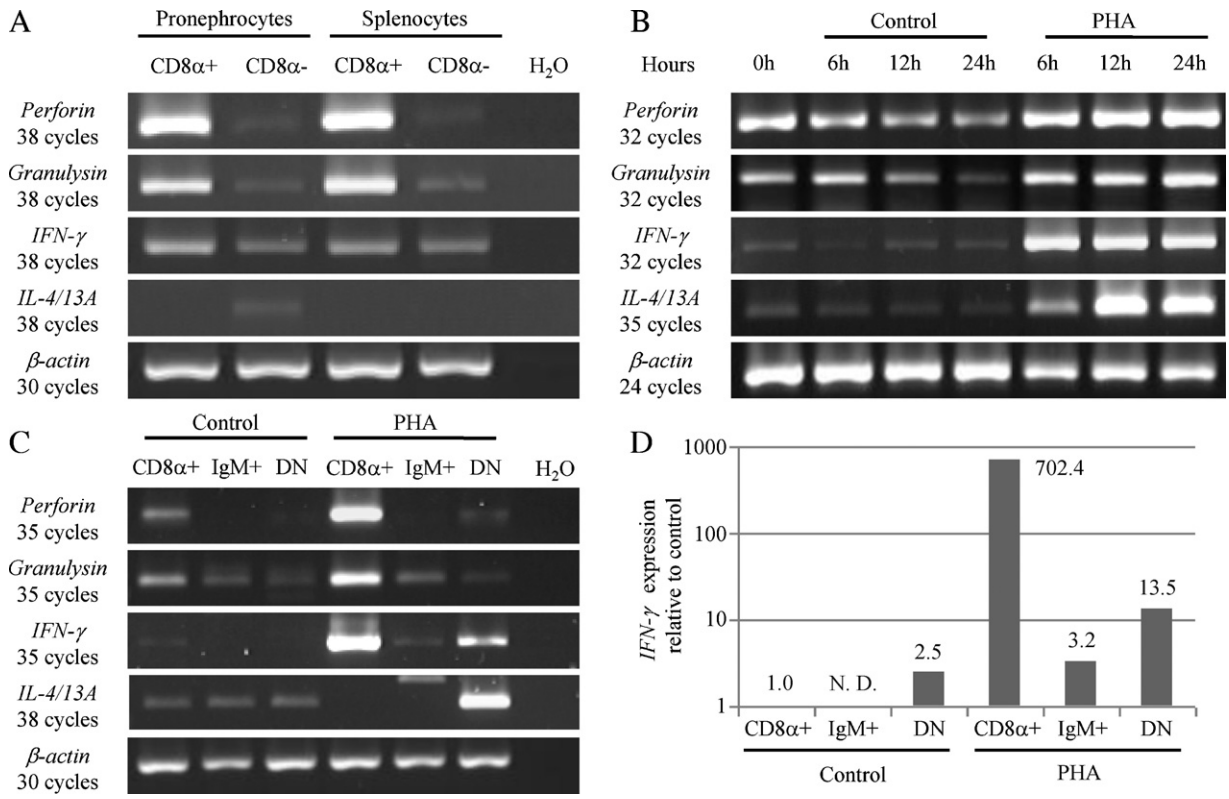

Fig. 6. mRNA expression analysis of effector molecules in CD8α⁺ cells. (A) RT-PCR analysis of *perforin*, *granulysin*, *IFN-γ* and *IL-4/13A* expression using RNA from sorted cells. Total RNA was extracted from CD8α⁺ and CD8α⁻ lymphocytes of pronephros and spleen, and was detected by one-step RT-PCR. Data are from a single representative of three independent experiments. In each experiment, leukocytes from three different individuals were pooled. (B) Time-course of mRNA expression in PHA-stimulated pronephrocytes. Pronephrocytes were incubated with 10 μg/ml of PHA-L. Total RNA was extracted at the indicated time points after incubation with or without PHA and used for RT-PCR analysis. Data are from a single representative of two different individuals. (C) RT-PCR using mRNA from sorted lymphocyte subpopulations with PHA stimulation. After flow-sorting of CD8α single positive (CD8α⁺), IgM single positive (IgM⁺) and double-negative (DN) lymphocytes, each subpopulation was cultured with or without 10 μg/ml of PHA-L. Total RNA was extracted 9 h after stimulation and used for RT-PCR analysis. Data are from a single representative of four independent experiments. In each experiment, leukocytes from two clonal trout were pooled. (D) Expression levels of *IFN-γ* mRNA in each population were determined by real-time RT-PCR. Expression levels are shown relative to the expression by unstimulated CD8α⁺ lymphocytes (set to 1). PCR reactions of each sample were run in triplicate. Data are from a single representative of two independent experiments. In each experiment, leukocytes from two different individuals were pooled.

similar (Supplemental Fig. 3). Our preliminary results suggest that the relative expression of rainbow trout TCRγ C segments also differs between CD8α⁺ and CD8α⁻ lymphocytes (Supplemental Fig. 3).

The proportions of CD8α⁺ cells in blood and secondary lymphoid organs were surprisingly and consistently low. The percentage of CD8α⁺ cells among naïve mouse splenocytes is around 10% (Shen et al., 2003) and in spleen biopsies of healthy humans it is around 19% (Martins-Filho et al., 1998), which is much higher than in rainbow trout. In the same line, the proportion of CD8α⁺ rainbow trout blood leukocytes is extremely low (approximately 0.3%) compared to 25% in human PBL (Norment and Littman, 1988). This is in agreement with our previous observations of a relatively low *TCRβ* and *CD8α* mRNA expression in PBL of naïve rainbow trout (Utke et al., 2008). In addition, these low expression levels are reflected by the low number of randomly spread CD8α⁺ cells in sections of secondary lymphoid organs. However, a few follicle-like accumulations of CD8α⁺ cells were observed. The number of those accumulations is remarkably increased in VHSV infected fish (to be published in detail elsewhere), suggesting clonal proliferation of CD8α⁺ cells. This is in line with earlier observations on TCR spectratyping of splenocytes from VHSV infected trout showing clonal T cell responses by TCRβ sequence analysis (Boudinot et al., 2001, 2002, 2004), and with findings made by Hetland et al. (2010) who described similar aggregations in pronephros after infection with infectious salmon anemia virus (ISAV) in Atlantic salmon.

In contrast to rainbow trout leukocytes from blood and secondary lymphoid organs, CD8α⁺ cells were highly abundant in the mucosal respiratory (gill) and digestive (intestine) tissues. This was evidenced by numerous intra- and subepithelial CD8α⁺ cells observed in cryostat sections. In leukocyte preparations of trout gill and intestine, the CD8α⁺ cells represented as much as 25% and 55% of the total leukocytes, respectively. In the gill, CD8α⁺ cells were not only present in the epithelium of secondary lamellae but also in lymphoid structures located at the surface of gill arches and primary lamellae, as well as at the basal junction between primary lamellae which is akin to the situation in healthy Atlantic salmon (Hetland et al., 2010). This latter structure has recently been described in salmonids (Haugarvoll et al., 2008; Koppang et al., 2010) and was termed by us as interbranchial lymphoid tissue (ILT) (Koppang et al., 2010). To provide an efficient gas exchange with the aqueous environment, gill epithelia and the protecting mucus layer are relatively thin, thus increasing the risk of pathogen entry. This risk is probably the reason for the high abundance of T lymphocytes in the teleost gill. In contrast to rainbow trout respiratory tissue, the percentage of CD8α⁺ cells among healthy murine lung and peribronchial lymph node leukocytes is rather low (~4%) (Jiang et al., 2009), suggesting differences in qualitative or quantitative uptake of airborne versus waterborne antigens. A fair proportion of rainbow trout intestinal leukocytes expressed CD8α molecules, which is also the case in humans and mice (Jabri and Ebert, 2007; Kunisawa et al., 2007).

Immunological differences between non-mucosal and mucosal lymphocytes were further indicated by the gene expression of immunosuppressive receptors and integrins. *CTLA4* and *LAG3,* which are related to inhibitory regulation of T cells and to regulatory T cells in mammals, were highly expressed in mucosal lymphocytes in contrast to PBL and to lymphocytes from secondary lymphoid organs. The intestinal epithelium is continuously exposed to a high concentration of commensal microflora and alimentary antigens, while the gill is in continuous contact with aqueous antigens including waterborne ubiquitous microorganisms. High expression of *CTLA4* and *LAG3* in intestinal and gill lymphocytes probably reflects the presence of regulatory T cells to maintain mucosal homeostasis. Strikingly, mucosal lymphocytes and thymocytes express high levels of the integrin genes *ITGAE* and *ITGB7* when compared to extrathymic non-mucosal lymphocytes. In mammals, ITGAE and ITGB7 are associated with the homing of T cells to the mucosal tissues and to the thymus. This observation suggests that a subpopulation of lymphocytes shows a tropism for mucosal tissues and thymus. However, while we could show homing of inflammatory cells to the inflamed site of DNA vaccine injection (Utke et al., 2008), others were unable to show homing to the intestine upon adoptive transfer of intraepithelial lymphocytes (IEL) (Bernard et al., 2006). Future experiments will have to clarify this.

CTL function in teleosts has been demonstrated, suggesting the presence of CTLs in different fish species, but the characteristics of cells involved are not always fully characterized (Fischer et al., 2006). Recently, Toda et al. (2009, 2011a,b) demonstrated that in ginbuna allospecific cytotoxic cells are CD8α$^+$ lymphocytes with perforin-mediated cytotoxic activity. In mammals, CTLs and NK cells exocytose granules containing perforin and granzymes into the intercellular cleft after specific recognition of target cells (Peters et al., 1991). Perforin forms pores allowing entry of granzymes into the cytoplasm of target cells finally resulting in apoptotic cell death and lysis (Smyth and Trapan, 1995). Granulysin, as does its homolog NK-lysin, colocalizes with perforin in cytotoxic granules of CTLs, NK cells (Andersson et al., 1995), and some CD4$^+$ cells (Davis et al., 2005), and shows a killing activity against bacteria (Andreu et al., 1999), protozoa (Jacobs et al., 2003) and fungi (Ma et al., 2002). We show in the present work that rainbow trout CD8α$^+$ cells express considerable amounts of *perforin* and *granulysin*. In contrast, a very low *perforin* and *granulysin* expression was detected in CD8α$^-$ lymphocytes, suggesting that cell-mediated cytotoxicity is predominantly executed by rainbow trout CD8α$^+$ lymphocytes. Moreover, rainbow trout CD8α$^+$ pronephrocytes stimulated with PHA, which is a mitogen that induces cytotoxic functions (Nelson et al., 1976), showed markedly enhanced *perforin* and *granulysin* expression. Expression analysis of *DAP12* and *FcRγ* suggests that most NK cells are among the CD8α$^-$ pronephrocytes, thus likely the cytotoxic gene expression signature of the CD8α$^+$ pronephrocytes is exhibited by CTL.

Another effector molecule of mammalian CTLs is IFN-γ, although it is also produced by CD4$^+$ Th1 cells, NK cells, NKT cells, some B cells and other professional APCs. In humans and mice, IFN-γ plays important roles in the regulation of adaptive immunity and cellular cytotoxicity (Schroder et al., 2004). An IFN-γ homolog with similar biological activities has been described in rainbow trout (Zou et al., 2005), where it was up-regulated during bacterial (Mulder et al., 2007), viral (Cuesta and Tafalla, 2009; Purcell et al., 2009) and parasitic (Raida and Buchmann, 2008) infections. In our study, both naïve rainbow trout CD8α$^+$ and CD8α$^-$ lymphocytes expressed *IFN-γ* transcripts. A remarkable induction of *IFN-γ* mRNA was observed in CD8α$^+$ pronephrocytes upon PHA stimulation. In contrast to IFN-γ, mammalian IL-4 and IL-13 are highly expressed by CD4$^+$ Th2 cells and trigger humoral immune responses. We recently identified rainbow trout *IL-4/13A* (Takizawa et al., in press), one of two *IL-4/13* genes in teleosts. In the present study, we have shown that the CD4$^+$ cell-enriched (CD8α$^-$IgM$^-$) lymphocytes, and not the CD8α$^+$ lymphocytes, clearly show enhanced *IL-4/13A* and a moderate induction of *IFN-γ* expression following PHA stimulation. These observations indicate that activated teleost CD8α$^+$ pronephrocytes are the major producers of IFN-γ as well as cytolytic molecules (*perforin* and *granulysin*), which is well compatible with a major role in cell-mediated cytotoxic immunity, and suggest that teleost CD4$^+$ cells play a pivotal role in cytokine production.

In conclusion, our study revealed the distribution and fundamental characteristics of CD8α$^+$ lymphocytes in the economically important teleost rainbow trout. The localization of CD8α$^+$ cells combined with the expression profiles of *CD8* and *CD4* transcripts suggests intrathymic T cell development similar to mammalian manner where αβ T cells mature through a CD4/CD8 double-positive stage. Quasi-exclusive expression of *CD8* and *CD4* in extrathymic CD8α$^+$ and CD8α$^-$ lymphocytes, respectively, suggests the subdivision of αβ T cells into CD8$^+$ CTLs and CD4$^+$ Th cells. CD8α$^+$ and CD8α$^-$ pronephrocytes, showed distinct gene expression profiles of cytolytic molecules (*perforin* and *granulysin*) and cytokines (*IFN-γ* and *IL-4/13A*), further supporting the presence of both CTLs and Th cells at the level of teleosts. Intriguingly, a high number of αβ T cells and γδ T cells, both consisting of CD8α$^+$ and CD8α$^-$ lymphocytes, seem to reside in mucosal tissues gill and intestine, which agrees with a recent report on abundant T cells (CD3ε$^+$ cells) in these organs (Koppang et al., 2010). Integrin gene expression patterns suggest dedication of specialized lymphocytes to the mucosal front, which according to abundant expression of *TCRγ* and inhibitory receptor genes presumably also include γδ T cells and regulatory T cells. There are some apparent differences in CD8α expression between species, e.g. trout may not possess CD8α$^+$ myeloid cells. However, the data support that compartmentalization of αβ T cells, γδ T cells and NK cells into CD8α$^+$ and CD8α$^-$ fractions, and the distinctive expression of effector molecules by CD8α$^+$ versus CD8α$^-$ lymphocytes, predate the separation between teleost and tetrapod ancestors. Future studies will concentrate on the functional characterization of CD8α$^+$ cells taking advantage of flow-sorted lymphocytes isolated from different organs of infected or immunized rainbow trout.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2011.02.008.

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Fish mucosal immunity: gill

5

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Chapter Outline

Mucosal Health in Aquaculture

Abstract

The gills of fish are covered with a thin mucous membrane, which has a large external surface area that is in continuous contact with ambient water irrigating the organ during respiration. Large amounts of soluble and particulate substances, including various pathogens, may penetrate this mucosal barrier to cause local and/or systemic infections followed by mucosal and systemic immune responses. While substances that do not pose a threat to the fish may be tolerated by the gill immune system, pathogens have been found to induce innate and adaptive immune responses when entering the fish through the gills. Adaptive responses provide the basis for immune memory and consequently vaccination. Recently, investigations on the molecular level have added to our understanding of how immune responses of the gills are triggered. Such studies have heightened our understanding of host–pathogen interactions and are enhancing the development of immersion vaccines. This chapter describes teleost gill development, physiology, cellular composition, and immune responses to selected pathogens known to affect the gills.

aluminium hydroxide gel or Matrix-Q. A control group also included non injected fish. Fish were sampled at several times after vaccination. The peritoneal cavity was washed with 5 mL of cold PBS to obtain the free peritoneal cells. Cells forming cell-vaccine masses and cells attached to the peritoneal mesothelium were also obtained and disaggregated by treatment with trypsin and EDTA. Cells were analysed by light microscopy, immunohistochemistry and flow cytometry. Gene expression was determined in free peritoneal cells by using a microarray rich in genes related to the immune response and to the cell cytoskeleton. In addition, gene expression was analysed by real-time PCR analysis of attached peritoneal cells and of spleen and kidney cells. The results indicate intense cell traffic to and from the peritoneal cavity, including free cells, cells attached to the mesothelium and others migrating to lymphoid organs. Gene expression analysis revealed intense upregulation of genes associated with cytoskeleton, cell adhesion and cell surface receptors. Apoptosis was observed from day one, with upregulation of genes involved in apoptosis and cell death. However, lymphocytes were scarce, and downregulation of genes related to lymphocyte activity and lymphocyte chemotaxis was observed. Finally, we compared the responses generated by the different adjuvants.

Keywords: Turbot, vaccination, immune response, peritoneal cells, gene expression, *Philasterides dicentrarchi*

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O-020.

Vaccination with outer membrane vesicles against intracellular pathogens for aquaculture the case of francisellosis in a zebrafish model

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Abstract
Vaccine development against many extracellular bacteria has been a success for the sustainability of the aquaculture industry. In contrast, infection of fish with intracellular pathogens remains largely an unresolved problem. Francisellosis is a bacterial disease in fish that presents nonspecific clinical signs and can cause high mortalities. *Francisella noatunensis (Fn)*, the causative agents of francisellosis, is a non-motile, Gram-negative, facultative intracellular bacterium. *Fn* consists of two subspecies; *F. noatunensis* subsp. *orientalis* (*Fno*) that causes disease in "warm-water" fish, like tilapia, while *F. noatunensis* subsp. *noatunensis* (*Fnn*) causes disease in fish living in colder waters, like cod. Production of membrane vesicles by cells is a conserved mechanism occurring throughout all domains of life. Outer membrane vesicles (OMVs) secreted from Gram-negative bacteria are spherical, 10-300 nm in diameter, and consist of a phospholipid bilayer with outer membrane proteins, endotoxin and a lumen with periplasmic proteins. Their secretion is associated with a variety of traits including the discharge of virulence factors during infections. Isolated OMVs are promising vaccine candidates against diseases caused by intracellular bacteria and have been used successfully as a vaccine against meningitis in humans. In the present work, we show that intact OMVs can be isolated from broth-cultured *Fno.* Proteomic analyses reveal that the vesicles include large sets of proteins involved in the host immune response, such as GroEL, OmpA and ClpB, which play an important role in the initial infection stages and the bacteria's overall virulence and survival within a host. Furthermore, *Fno* OMV was tested as a vaccine in an *Fno*-zebrafish infection model, confirming its capacity to significantly reduce the development of francisellosis, inducing the secretion of cytokines such as TNFα, IL-1β and IFNγ. Moreover, we report the transcriptional profile of important immune markers such as *mhcii* (major histocompatibility complex class 2) and *mpeg* (macrophage expressed gene), demonstrating that *Fno* mainly infect macrophages as their replication site in zebrafish. Our research unfolds new possibilities to study the pathogenesis and to develop treatments against intracellular pathogens by using zebrafish as an infection model.

Keywords: OMVs, vaccine, francisellosis, zebrafish, immune response

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O-021.

Adoptive transfer of immunity against red mouth disease in rainbow trout (*Oncorhynchus mykiss*)

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Enteric Red Mouth Disease (ERM) caused by *Yersinia ruckeri* is among the bacterial diseases in rainbow trout aquaculture that can be controlled by registered vaccines and part of these vaccines can be delivered by immersion which is the most convenient way of application inducing good antibody responses and ensuring decreased handling costs. However, the mechanisms by which fish are protected against *Yersinia ruckeri* are unclear. To shed light on this question we have bath vaccinated clonal rainbow trout donors by using a commercially available vaccine, and leukocytes have been isolated from vaccinated donors followed by adoptive cell transfer to naïve fish recipients of the same trout clone. Control recipients were injected with same numbers of leukocytes from non-vaccinated clonal donors. Transfer between genetically identical donors and recipients ensured that immune cells were not rejected by the recipients. After transfer, both groups of recipients were bath infected with a virulent field strain of *Yersinia ruckeri*. While rainbow trout that have received leukocytes from vaccinated donors showed no mortality, less than 40% of control recipients survived the challenge. Flow cytometry using leukocytes from recipient survivors revealed that the percentage of IgM+ B cells was considerably higher in recipients that were injected with leukocytes from vaccinated donors when compared with control recipients, while no differences in the percentages of CD8+ cells among groups were recorded. Being the first report on adoptive cellular transfer of immunity in a bacterial infection in fish these data suggest that immunity was rather transferred by IgM+ B cells, but not by CD8+ cytotoxic T cells. . It can be further suggested that bath vaccination has caused an effective systemic immune response since protection could be transferred by non-mucosal leukocytes.

Keywords: rainbow trout, *Yersinia ruckeri*, adoptive transfer, B cells, T cells

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O-022.

Oil adjuvant elevates protection of rainbow trout (*Oncorhynchus mykiss*) following injection vaccination against *Yersinia ruckeri*

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Contrasted TCRβ Diversity of CD8$^+$ and CD8$^-$ T Cells in Rainbow Trout

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Abstract

Teleost fish express highly diverse naive TCRβ (TRB) repertoires and mount strong public and private clonal responses upon infection with pathogens. Fish T cells express typical markers such as CD8, CD4-1 and CD4-2, CD3, CD28 and CTLA4. Fish CD8$^+$ T cells have been shown to be responsible for antigen-specific cell-mediated cytotoxicity in *in vitro* systems using histo-compatible effector and target cells. We compare here the complexity of TRB repertoires between FACS sorted CD8$^+$ and CD8$^-$ T cells from spleen and pronephros of rainbow trout. In contrast to human, while the TRB repertoire is highly diverse and polyclonal in CD8$^+$ T cells of naïve fish, it appeared very different in CD8$^-$ lymphocytes with irregular CDR3 length distributions suggesting a dominance of activated clones already in naïve fish or the presence of non conventional T cells. After infection with a systemic virus, CD8$^+$ T cells mount a typical response with significant skewing of CDR3 length profiles. The infection also induces significant modifications of the TRB repertoire expressed by the CD8$^-$ fraction, but for a different set of V/J combinations. In this fraction, the antiviral response results in an increase of the peak diversity of spectratypes. This unusual observation reflects the presence of a number of T cell expansions that rise the relative importance of minor peaks of the highly skewed distributions observed in unchallenged animals. These results suggest that the diversity of TRB expressed by CD8$^+$ and CD8$^-$ αβ T cells may be subjected to different regulatory patterns in fish and in mammals.

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Introduction

The adaptive immune response to infectious agents is characterized by initial priming and expansion of T and B cell clones specific of the pathogen. Antigens derived from the pathogen can be specifically recognized by the T cell antigen-specific receptor (TR), a disulfide-linked membrane-bound heterodimer expressed by T lymphocytes. TR comprises two chains, either α/β or γ/δ, each composed of two immunoglobulin superfamily domains (V for variable and C for constant), and a very short intra-cytoplasmic tail. The variable domain is highly diverse due to somatic rearrangements in variable (V), joining (J), and, in the case of the β or δ-chain, diversity (D) segments, occurring during T cell differentiation. This large diversity allows a specific recognition of any antigen by a few T cell clones in an individual, leading to activation and clonal expansion. The dynamics of antigen-specific lymphocyte responses *in vivo* during the course of infection follows a general pattern: the initial expansion of effector cells precedes a rapid contraction phase, leaving a relatively stable, small pool of memory cells that provide long-term immunity.

In mammals, protein antigens are recognized by αβ T cells as short peptides exposed at the surface of antigen (Ag)-presenting cells by Major Histocompatibility complex (MHC) molecules [1]. CD4 and CD8 co-receptors bind the side of MHC molecules on antigen presenting cells, and thereby increase their signalling capacity by intracellular recruitment of the lymphocyte specific kinase (LCK) [2]. These co-receptors determine on which class of MHC molecules αβ TRs recognize their specific peptides: CD4$^+$ T cells target peptides presented on MHC class II while CD8$^+$ T cells target peptides presented by the MHC class I. Once primed, CD4$^+$ T cells migrate to follicles to help B cells produce antibodies, and to peripheral sites of antigen exposure to fight incoming pathogens by inducing the appropriate type of effector cell function. CD4$^+$ T cells regulate the immune response through cytokine secretion and can be subdivided into different categories including regulatory CD25$^+$CD4$^+$ T cells, and helper T cells with various profiles of cytokine production [3,4]. Type 1 effector CD4$^+$ T helper (Th)-1 cells produce IFN-γ that promotes clearance of viruses and intracellular bacteria, while type 2 CD4$^+$ Th2 cells produce IL-4, IL-5 and IL-13 that promote clearance of

extracellular parasites. Another subset named Th17 is characterized by the capacity to produce IL-17, IL-21 and IL-22 and plays a key role in inflammation. Once antigen is eliminated, central memory and effector memory T cells persist in the memory pool to provide systemic immune surveillance in secondary lymphoid and in non-lymphoid tissues, to react promptly in case of secondary infection. CD8$^+$ T lymphocytes possess cytotoxic capacity and are responsible for the elimination of virus-infected and tumor cells. Following their initial expansion and subsequent clearance of the viral infection, most cytotoxic T lymphocytes (CTL) undergo apoptosis, leaving behind a small but stable pool of memory CD8$^+$ T cells. Thus, after early double-positive thymocytes express both CD4 and CD8 molecules and undergo differentiation into either CD8$^+$CD4$^-$ or CD8$^-$CD4$^+$ single-positive cells, CD4 and CD8 distinguish two basic lineages of αβ T lymphocytes representing T cells with heterologous functions [5].

Basic subsets of αβ T cells characterized by the expression of CD4 and CD8 have been found also in fish, indicating that these markers constitute fundamental molecules of the vertebrate T cell response, together with classical MHC class I and II, CD3, CD28 and CTLA4 co-receptors. In teleost fish, CD8α was discovered first in rainbow trout where it was expressed at high levels in the thymus and at lower levels in spleen, kidney, gut, and blood leukocytes [6]. CD8α and β were later found in a number of other teleost fish species, and also in Chondrichtyans [7–10]. Although trout CD8α lacks a typical LCK binding motif in its cytoplasmic tail, it has been recently shown that the CD8 co-receptor can bind LCK as in mammals [11]. Importantly, the expression level of CD8 transcripts increases during infections and allogenic stimulation [12], and is correlated with lymphocyte cytotoxic activity in ginbuna crucian carp [13]. Alloantigen-specific cytotoxicity is mediated by CD8$^+$ cells, but not by CD8$^-$ cells in the same species [14]. Also, several CD8$^+$ channel catfish leukocyte cell lines were identified as allospecific effectors, providing further evidence for the existence of cytotoxic T cells in teleosts [15]. Collectively, these observations indicate that cytotoxicity reported a long time ago in fish in graft experiments is mediated by CD8$^+$ T cells [16]. Regarding CD4, two copies have been found in fish: *cd4-1*, encoding a receptor with 4 Ig domains as in mammals, and *cd4-2*, encoding 2 Ig domains [17,18]. Both CD4-1 and -2 are mainly expressed by CD8$^-$IgM$^-$ lymphocytes, and their intracellular regions possess the canonical CXC motif required for the LCK binding, suggesting they are involved in similar mechanisms of T cell signaling as in mammals. Orthologues of the cytokines promoting the differentiation and effector functions of Th1 and Th17 cells have been found in fish including IL-2, IL-10, IL-12, IL-18, IFNα, IFNγ and IL-17, respectively, as well as the corresponding key transcription factors EOMES and T-bet [19,20]. Additionally, Th2 transcription factors GATA3 and STAT6 have been identified in several fish species [21–24], as well as Th2 cytokines belonging to the IL4/IL13 family [24]. These findings suggest that fish express a complex array of cytokines that has the capacity to regulate different functional types of CD4$^+$ T cells reminding mammalian Th1 and Th17. Hence, several lines of evidence indicate that CD8 and also probably CD4-1 and -2 identify two main lineages of T cells with conserved functions in vertebrates, at least since the common ancestor of teleost fish and mammals.

However, very little is known about the respective TR repertoires of CD8$^+$ and CD8$^-$ (including CD4$^+$) T cells in fish, and their respective responses to pathogens. We previously found that the TRβ (TRB) repertoire expressed by rainbow trout spleen leukocytes was diverse and polyclonal in healthy animals, and that viral infection induced a complex arrays of private responses and a public specific response retrieved in all clonal individual fish studied [25]. The TR diversity expressed by the different T cell subsets, which remains unknown, would be important to identify what arm(s) of T cell immunity is triggered during immune responses. Thus, a system set-up to follow the modifications of the TR repertoire expressed by CD8$^+$ versus CD8$^-$ T cells, would open the way to the characterization of the clonal complexity of cytotoxic and helper or regulatory responses, respectively. It would therefore lead to an accurate modeling of the impact of the route of infection, the adjuvant, the nature of Ag, and to a better understanding of the contribution of fish T cell immunity to the protection afforded by vaccination.

In primates, CD4$^+$ and CD8$^+$ αβ T cell repertoires of peripheral blood mononuclear cells (PBMC) are roughly of equal complexity, although it is generally accepted that the CD8$^+$ T cell subset is responsible for the majority of the observed skewing of complementary determining region (CDR)-3 length TRB spectratypes [26,27]. In the same line, the CD8$^+$ T cell repertoire was found highly restricted during the progression of Acquired Immuno-Deficiency Syndrome (AIDS), while no perturbation of CD4$^+$ T cell repertoire was obvious in the early stages of the disease [28]. Finally, both CD4$^+$ and CD8$^+$ T cell subsets are skewed in aging with frequent clonal proliferation observed in healthy elderly humans for a variety of TRB V (TRBV) families [29].

In this work, we compared the diversity of TRB expressed by CD8$^+$ and CD8$^-$ T cell subsets in rainbow trout using CDR3 length spectratyping. We show that the CD8$^+$ T cell population expresses a diverse polyclonal repertoire, which is skewed after response to a systemic viral infection as previously described from unsorted spleen leukocytes in [25,30]. In contrast, CD8$^-$ cells express a lower diversity of TRB with high inter-individual variations, even between naïve clonal individuals. Our observations suggest that rainbow trout spleen and pronephros contain a large repertoire of CD8$^+$ T cells in naïve fish, while the CD8$^-$ compartments comprise a significant proportion of expanded clones possibly produced during previous immune responses.

Materials and Methods

Ethics Statement

All animals were handled in strict accordance with good animal practice as defined by the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and by the Regional Paris South Ethics committee, and animal work was approved by the Direction of the Veterinary Services of Versailles (authorization number 78-28). The infection trials performed at the Friedrich-Loeffler-Institute were approved by the ethics commission of the German state Mecklenburg-Western Pomerania under the reference number LALLF M-V/TSD/7221.3-1.1-036/09.

Fish

Heterozygous isogenic rainbow trout, clonal line A36-A3, were produced at the INRA experimental fish farm (PEIMA, Sizun, France) by crossing two double haploid fish lines and sent as hatched eggs to FLI for hatching and further rearing. This clonal line is a cross between two fully homozygous individuals belonging to two distinct clonal lines that were originally established through two successive generations of gynogenetic reproduction (suppression of mitosis and meiosis in the first and second generations, respectively) [31]. Fish were at the age of two years during sampling. They were maintained in 400-liter tanks in a partially re-circulating water system and fed with commercial dry pellets.

Viral Challenge: Immunization Protocols and Sampling

Immunization and virus challenge were performed using the attenuated 25-111 variant of strain 07-71 of VHSV [32] through intraperitoneal injection. A first sub-lethal dose of 10^5 plaque forming units (PFU)/fish in 100 µl of sterile, endotoxin-free PBS was applied to each of the four fish in the infected group. This infection usually leads to good protection against a subsequent lethal infection. Four weeks later, fish received a second injection of the same virus (5×10^7 PFU/fish) and samples were collected 3 weeks later. Four control fish were injected with the same volume of PBS. Trout were sacrificed following approved procedures and subsequently exsanguinated.

Leukocyte Preparation

All following preparation steps were performed on ice or at 4°C. Pronephros and spleen were individually removed from the peritoneal cavity and homogenized with a Potter–Elvehjem homogenizer to prepare single cell suspensions. Cells were resuspended in mixed medium (MM, Iscove's DMEM/Ham's F12 (Gibco) at a ratio of 1:1, supplemented with 10% fetal bovine serum (FBS)) and layered onto an isotonic Percoll (Biochrome) gradient (? gradient (rad. After centrifugation at 650×g for 40 min cells at the interphase were collected and washed twice using MM.

Staining and Sorting of Lymphocytes

Pronephrocytes and splenocytes from individual fish each were pooled and double labelled with two monoclonal antibodies (mabs). For this purpose, a rat anti-trout CD8α mab [33] was combined with FITC-conjugated goat anti-rat IgG (H+L) (Jackson ImmunoResearch). The same cells were double stained with mab D11 labelling lymphocytes other than B cells and thrombocytes (manuscript in preparation) in combination with an R-PE conjugated F(ab')2 donkey anti-rat IgG (H+L) (Jackson ImmunoResearch) secondary antibody. Sorting was performed with a MoFlo high-speed cell sorter (Dako Cytomation). Only single (excluding doublets through pulse width gating) lymphocyte like cells (low FSC/low SSC) were considered in the sorting decisions. Macrophage-like cells (high FSC/low SSC), granulated cells (high FSC/high SSC) and cell debris were excluded from sorting. Three subpopulations were collected (30000 cells per population): CD8$^+$/D11$^+$ double positive cells, CD8$^-$/D11$^+$ single positive cells, and double negative cells. CD8$^+$/CD11$^-$ single positive cells were not detected. 3×10^4 cells of each subpopulation were directly sorted into 600 µl lysis buffer (RNeasy Micro Kit from Qiagen) containing 6 µl mercaptoethanol to prevent RNA degradation. A part of each subpopulation was sorted into MM to subsequently analyse their purity by flow cytometry at same analysis settings as for sorting.

RNA Preparation and cDNA Synthesis

Total RNA was purified and DNase treated using QIAgen RNA extraction kit. RNA (2 µg) was reverse transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies) with 2.5 µM oligodT_{25} primer in a final volume reaction of 20 µl. For sorted cells, total RNA was extracted from each cell population using RNeasy Micro Kit (Qiagen). Full-length cDNA was generated and amplified using the SMARTer^{TM} PCR cDNA synthesis kit (Clontech Laboratoires, Inc.), following manufacturer's instructions. The optimized protocol of this kit preferentially enriches for full-length cDNAs and retains true gene representation of genes in the original sample. The optimal number of PCR cycles determined for ds cDNA synthesis and amplification of S1, S2 and S3 samples was 23 cycles.

CDR3 Length Spectratyping Analysis

The spectratyping of TRB CDR3 length (Immunoscope analysis) first developed for mouse or human was previously adapted for rainbow trout [25]. A first amplification (PCR1) using a forward primer specific for a subgroup or a set of TRBV genes in combination with a reverse primer TRBC specific for the constant region was performed as follows: 1 µl cDNA was used as template for PCR1 using 0.4 mM of each dNTP, 0.4 µM of each primer (forward: $TRBV_{family\ specific}$, reverse: TRBC), and 0.025 u µl^{-1} of GoTaq DNA polymerase (Promega) in 1× reaction buffer with 2 mM of $MgCl_2$ (95°C for 5 min; 40 cycles of 95°C 45 s, 60°C 45 s, 70°C 45 s; 70°C 10 min) (**Table 1**). Primers specific for TRBV combined with a primer specific for TRBC amplify TRB sequences with TRBV from a given family but with different TRBJ content and diverse CDR3 lengths. In a second step, TRBV-TRBC PCR products were subjected to run-off reactions (PCR2) using 5′ 6-FAM- fluorescent C internal, reverse primers, or TRBJ reverse primers. Two µl of PCR1 product were applied as template using 0.4 mM of each dNTP, 10 pmoles of the fluorescent reverse primer, and 0.025 u µl^{-1} of GoTaq DNA polymerase (Promega) in 1× reaction buffer with 2 mM of $MgCl_2$. The reaction mixtures were amplified in the following conditions: 95°C for 5 min; 5 cycles of 95°C 1 min, 60°C 1 min, 70°C 2 min; 70°C 10 min. Two µl of the run-off products were mixed with 8 µl deionized formamide (Applied Biosystems) and 0.5 µl of the internal standard (GeneScanTM 500XL ROX, size standard, Applied Biosystems). The mix was then denatured at 95°C for 5 min and placed on ice before analysis in an ABI 3730HT sequencer (Applied BioSystems) at GeT-PlaGe core facility, Toulouse, France. Spectratyping data of each TRBV-TRBC and TRBV-TRBJ combinations were extracted using GeneMapper software (Applied BioSystems) and analysed with ISEApeaks software [34] [35]. Each spectratype or profile is composed of several peaks (typically 4 to 10) separated according to their corresponding length of run-off products, spaced by 3 nucleotides as expected for in-frame transcripts. Each peak corresponds to a given CDR3 length. For each profile associated with a TRBV-TRBC or TRBV-TRBJ combination, the area under each peak was calculated and stored in a peak database. These values were then computed to quantify the differences between CDR3 length distributions.

(1) In a given context, the "perturbation score" for a given TRBV-TRBC or TRBV-TRBJ profile was calculated as follows:

$$perturbation\_score = \frac{1}{2}\sum_{i=1}^{n} |p_{i,an} - p_{i,ref}|$$

where $p_{i,an}$ and $p_{i,ref}$ are the relative areas of the peak #i from the analysed and the reference profiles, respectively; n is the number of peaks detected in the reference profile.

An average perturbation was then computed for all the TRBV-TRBC or TRBV-TRBJ of a given sample, and/or for the different individuals of a dataset. As the intensity of CDR3 peaks was usually not comparable between different amplifications, we considered the percentage of use of each CDR3 length (i.e. the "relative area"), obtained by dividing the area of CDR3 peaks by the total area of all peaks within the profile. Profile perturbations range from 0 (analysed profile is identical to the reference), to 100 (no overlap at all).

(2) The repertoire diversity can be assessed by diversity measures based on the concept of Shannon entropy that provides a measure of the quantity of information encompassed in the repertoire. Given $P = (p_1,\ldots,p_S)$: a CDR3 profile where p_i is the relative proportion of the peak #i within S peaks and $\sum_{i=1}^{S} p_i = 1$.

The Shannon diversity index (SDI) is usually estimated using the following formula:

$$H = -\sum_{i=1}^{S} p_i \log_2 p_i$$

H reaches the maximum when p_i (i = 1,.,S) follows a uniform distribution, whereas a maximal TRB repertoire diversity is expected for a Gaussian-like distribution of CDR3 lengths. We therefore computed a modified version of Shannon entropy which reaches its maximum for a Gaussian-like CDR3 length profiles by transforming profile P into profile Q* according to a reference profile $Q = (q_1,\ldots,q_S)$ (considered to have a maximal diversity) and applied Shannon entropy to the transformed profile Q*.

First we calculated a profile Q'$(q'_1,\ldots,q'_S)$.

$$\text{with } q'_i = \frac{q_i - p_i}{1 - p_i} \text{ if } p_I \le q_I.$$

$$\text{and } q'_I = \frac{q'_i}{\sum_{i=1}^{S} q'_i} \text{ if } 0 \le q_I \le p_i.$$

To satisfy the normalization hypothesis of Shannon entropy, we normalized the profile Q' bydividing each relative proportion by its overall sum thus obtaining Q* with $q_i^* = \frac{q'_i}{\sum_{i=1}^{S} q'_i}$.

Finally, the Shannon entropy for the transformed and normalized profile Q* was computed as:

$$H = -\sum_{i=1}^{S} q_i^* \log_2 q_i^*$$

To assess a significant differences between groups, we performed statistical tests at level $\alpha = 0.01$ (type 1 error) on the perturbation scores and diversity index. Statistical significances of the differences for different group pairs were determined using empirical Bayes test from the limma (Linear Models for Microarray) packages (R/Bioconductor). We chose this test because it outperforms the classical Student t-test or Mann-Whitney-Wilcoxon non parametric test for small samples [36]. In addition, repertoire perturbation scores were used to perform Principal Component Analysis (PCA) analysis in order to compare the statistical dispersion of the samples on a multidimensional plan. Statistical and multivariate analyses were performed using R software (http://www.r-project.org/).

Table 1. Primer sequences used in this study.

Name	Sequence (5′-3′)
Repertoire	
TRBV1-F	AGACAGCTTCCAGGAGAGG
TRBV2-F	AAACACCTGCAGACCTGTAC
TRBV3-F	CAACAGTCAGCCCAAAACACT
TRBV4-F	AAGCCTAATGCCTCTGTCACA
TRBV5-F	ACCAGAAATCACAAGGAGAAACAG
TRBV6-F	CCAGGCTTCAGCATGCCCAGCTACA
TRBV7-F	AAGGAGATGCGTCAGTCACTCTA
TRBV8-F	AAGGACGGGCGCGAGGCGGACATC
TRBV9-F	GGCCACGAAACACCTTAAAGATG
TRBV10-F	AGCAGTCAATAGCTGATTCTAATC
TRBJ1_2-R	TAAAACAGTGAGTTTGGTTCCATT*
TRBJ2-R	CATTGCCAAAGAAGGCTGG*
TRBJ3-R	CAGAACAGTCAGTTTGGTTCCCG*
TRBJ4-R	GAGAACTGTTAATTTGGTGCCTTG*
TRBJ6-R	CCGGGTTCCTCCACCGAAGTC*
TRBJ7-R	ACGGTGAGTTTGGTGCCGG*
TRBJ8-R	TGCCGTTGCCGAAGTACG*
TRBJ9-R	GTGAGTCTGGAACCTGGA*
TRBJ10-R	ACTTCCCTCTCCAAAGTAGGC*
TRBC2-R	GTAGAAGCGGGTGGCTACAC
TRBC1-R	GTTTCTGTCTTCACACTTCTTAGC*
qPCR	
EF1-α-qF	CAAGGATATCCGTCGTGGCA
EF1-α-qR	ACAGCGAAACGACCAAGAGG
β-actin-qF	GGGAGAAGATGACCCAGATCATG
β-actin-qR	GGTGGTACGGCCAGAGGC
CD3γδF	CCTGATTGGAGTAGCTGTCTAC
CD3γδR	GCTGTACTCAGATCTGTCCATGC
CD4.1-F	AGCTTGAACGTGTTGCTGT
CD4.1-R	TCGAGTTACTTCACCAAACAC
CD8F	GAGTACACCTGCGCTGTGGAAT
CD8R	TCGAGTTACTTCACCAAACAC

*The 5′ 6-FAM- fluorescent version of the primer was used for immunoscope analysis.

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Real-time PCR Analysis of Gene Expression

For real time PCR, 3 µl of cDNA (1:3 diluted) was used as a template for amplification using gene specific primers (**Table 1**). PCR amplification was performed in a Mastercycler® ep realplex (Eppendorf), using ready prepared 2× master mix (Power SYBR Green PCR master mix, Applied Biosystems) with a final PCR volume of 25 µl, in white 96-well plates (Eppendorf). PCR conditions were 95°C for 10 min followed by 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The fluorescence signal output was measured and recorded at 80°C during each cycle for all wells for 40 cycles. A negative control (no template) reaction was also performed for each primer pair. QPCR calculations were based on the normalization of the expression of the genes of interest using the expression of the housekeeping gene trout elongation factor-1α (EF1-α). Equivalent EF1-α expression levels were obtained from populations S1, S2 and S3 for a given number of cells processed, confirming that the absence of expression of the T cell markers CD4, CD8 and CD3 in S3 was not due to RNA degradation. A sample from the serial dilution was run on a 2% agarose gel, stained with ethidium bromide and viewed under UV light to confirm that a band of the correct size was amplified. A

melting curve for each PCR was determined by reading fluorescence every degree between 60°C and 95°C to ensure that only a single product had been amplified. EF-1α and β-actin were used for normalization of expression. The relative expression levels of the genes of interest were determined using the Pfaffl method [37]. Efficiency of the amplification was determined for each primer pair using serial 10 fold dilutions of pooled cDNA, performed in the same plate as the experimental samples. The efficiency was calculated as $E = 10^{(-1/s)}$, where s is the slope generated from the serial dilutions, when Log dilution is plotted against ΔCT (threshold cycle number).

Results

Isolation of CD8α$^+$ and CD8α$^-$ Cell Subsets

Since the expression of the rainbow trout CD8 co-receptor appears to be correlated with T cell cytotoxic activity, we undertook the analysis of the TRB-repertoire of FACS-sorted CD8$^+$ and CD8$^-$ cell subsets to compare the respective TR diversity of cytotoxic and other αβ T cells in fish. The primary αβ T cell repertoire is shaped by negative and positive selection exerted in the thymus during T cell differentiation. These mechanisms depend on the presentation of self-peptides by MHC molecules at the surface of thymic stromal cells. It is therefore highly restricted by the MHC alleles expressed by the individual, as well as antigen-driven T cell responses occurring in periphery. To avoid fish-to-fish genetic variability that could lead to different TRB-repertoires, we used isogenic fish – i.e. a F1 cross between two fully homozygous fish belonging to gynogenetic clonal lines, which ensures that all individuals were genetically identical at all loci [31].

For flow sorting, two mabs were used: an anti-CD8α mab previously characterized in [33] and the mab D11, a newly established mab that labels 95% of thymocytes, about 15% of PBL and about 20% of spleen leukocytes. Although the exact nature of its target molecule still remains unknown, the mab D11 labels lymphocytes others than B cells, but not thrombocytes. Moreover, D11$^+$ lymphocytes from unstimulated fish do express *trb*, *cd4* and *cd8α* mRNAs but not *igm* or the thrombocyte marker *cd41* (manuscript in preparation). D11$^+$ represented 60% of the gated lymphocyte-like cells, approximately 10% of which were CD8α positive. The remaining D11$^-$ CD8$^-$ double negative lymphocyte-like cells represented 30–40% of the gate. Since all CD8α Since all CD8$^+$ there was no population of CD8α single positive cells (**Figure 1A**).

Three cell populations were sorted from the lymphocyte gate (FSC^{low}/SSC): a CD8$^+$D11$^+$ subset (S1) containing CD8$^+$ T cells only, a CD8$^-$D11$^+$ subset containing other lymphocytes among those most likely T helper cells (S2), and a CD8$^-$D11$^-$ subset containing other cells with lymphocyte scatter characteristics such as B cells and thrombocytes (S3). To validate the sorting conditions sorted subpopulations were checked by subsequent flow cytometry were purities of more than 97% were recorded (data not shown). Moreover, RT-QPCR experiments showed that the CD8α/D11 double positive lymphocytes (S1) expressed *cd3γδ* at a high level, while cells from S2 expressed about twice less, and S3 cells were *cd3γδ*$^-$ (**Figure 1B**). Accordingly, *cd8α* and *trb*, but not *cd4* transcripts were expressed in the cDNA for the S1 population while *cd4* and *trb*, but not *cd8α* transcripts were amplified from the D11 single positive cells (S2) (**Figure 1B**). The double negative cells (S3) did not express *cd8α* or *cd4* mRNA (**Figure 1B**). *Trb* transcripts were also undetectable (data not shown). Thus, the S1 population contained CD8α$^+$ T cells, the S2 contained CD4$^+$ and other T cells, and S3 contained all the other cells with lymphocyte morphology. Thus, the sorting procedure efficiently separated CD8α$^+$ and CD4$^+$ αβ T cells from trout leukocytes, allowing relevant comparisons of TRB repertoires expressed by these two cell subsets.

CD8α$^+$ and CD8α$^-$ T Cell Subsets Harbour Distinct TRB Repertoires in Naïve Fish

Previous studies have shown that "all-T cell" TRB-repertoires from lymphoid tissues of naive rainbow trout are highly diverse and polyclonal: CDR3 length spectratyping produced quasi-regular bell-shaped distributions of CDR3 length for all TRBV-TRBJ combinations from spleen, pronephros and thymus [25].

We first compared the TRB diversity of the different leukocyte fractions purified from spleen and pronephros of naïve fish using the same spectratyping approach. We studied the CDR3 length profiles of TRBV-TRBC PCR products, using an internal TVBC primer for the run-off step. This procedure integrates junctions with all TRBJ segments for a given TRBC, and provides a first synthetic measure of the diversity. These experiments generally resulted in typical bell-shaped profiles from both CD8$^+$ (S1) and CD8− (S2) fractions, as displayed for TRBV1-C, TRBV4-C, TRBV8-C and TRBV10-C (**Figure S1**). Although TRBV1-C profiles appeared less regular for the CD8$^-$ fraction compared to the CD8$^+$ sorted cells, the difference was not significant (Wilcoxon $p = 5.07E{-}02$; eBayes $p = 1.23E{-}01$). Importantly, since the TRBV primers are specific for TRBV gene families, each TRBV-TRBC profile aggregates the distribution for different TRBV genes and all TRBJ, which can strongly buffer differences between TRBV-TRBJ combinations.

To get a more detailed comparison, we then compared TRBV-TRBJ CDR3 length profiles between the CD8$^+$ and CD8$^-$ sorted fractions, using TRBJ specific primers for run-off reactions. We selected TRBV1, 4, 8 and 10 from our previous observations to study highly expressed low responsive TRBV family (TRBV1), less expressed TRBV families (TRBV4; 8 and 10) and responsive TRBV families (TRBV4 and 8). Many spectratypes were bell-shaped for both CD8$^+$ and CD8$^-$ fractions as observed for the whole T-cell population. However, differences in spectratypes appeared at this level of analysis between CD8$^+$ and CD8$^-$ subsets for a number of TRBV-TRBJ combinations as shown in **Figure 2A**. Indeed, CDR3 length distributions are often less regular and less complex (*i.e.* with less peaks and being more variable from fish to fish) in S2 compared to S1. Importantly, spectratyping was repeated from independent PCR amplifications and produced similar results, excluding that the expanded peaks observed in irregular profiles from S2 could be due to a random amplification of a few sequences, as frequently observed in experiments performed from cDNAs containing rare templates. To obtain a statistical validation of the differences observed between S1 and S2, we performed a quantitative analysis of all profiles using the ISEApeaks software [35]. ISEApeaks calculates the perturbation score of a given TRBV-TRBJ spectratype in comparison with a reference, summing the differences of areas calculated for each peak between the given and the reference profile [35]. Taking the average of the whole T cell population (i.e. both S1 and S2 groups), we computed the global perturbation score from all spectratypes for both S1 and S2 fractions of each individual. Principal component analysis of these perturbation values distinguishes two groups corresponding to S1 and S2, and indicates that S1 repertoires are very close to each other while S2 repertoires are much more dispersed, i.e. variable from fish to fish (**Figure 2B**). According to ebayes test, most of TRBV4-J and TRBV8-J are significantly different between both groups. A comparison of the matrices of perturbation values confirmed that

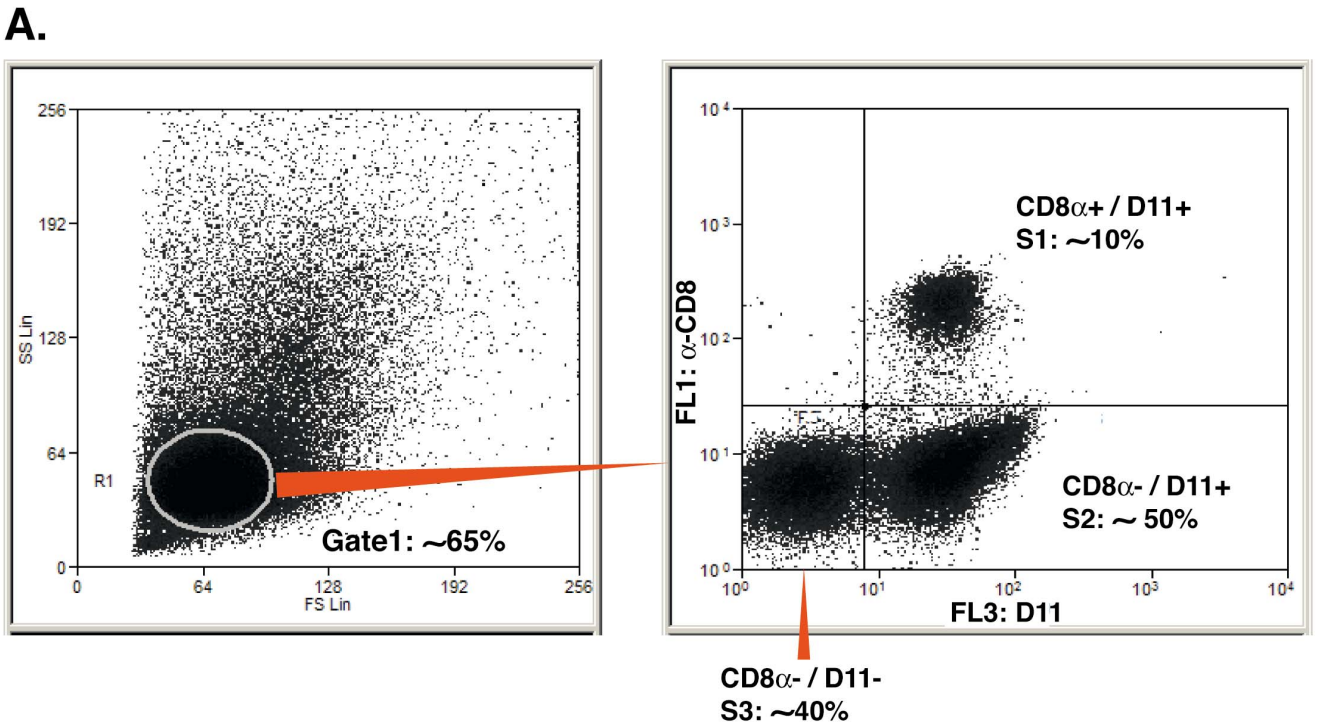

Figure 1. Separation of CD8α$^+$ and other T cells. Leukocytes were prepared from spleen and pronephros by density gradient centrifugation and stained with anti-CD8 mabs. (A) A lymphocyte gate (≈65% of events) was defined, and three subpopulations were sorted from it: a CD8$^+$D11$^+$ population (S1), a CD8-D11$^+$ population (S2), and a CD8$^-$D11$^-$ population (S3). Plots are from a single fish representing typical distributions of the lymphocyte subpopulations S1-3. (B) RNA and cDNA was prepared from each population (S1-3) and the expression of the T cell markers CD3γδ, CD4 and CD8α analysed by QPCR for individual fish (n = 4). The mean value is shown, and error bars represent the standard deviation among fish. The expression was normalized on the expression level of *Elf-1a* used as a housekeeping genes, and is represented as a proportion of the maximal expression level among S1-3 subsets.
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TRB CDR3 length spectratypes from CD8$^+$ and CD8$^-$ fractions were indeed significantly different for many TRBV-TRBJ combinations especially for families TRBV4 and 8 (see **Table II**). In addition, the PCA projection of S1 and S2 samples according to the first two components (respectively capturing 58.67% and 14.94% of the global variability) confirmed that both groups are apart and showed that the inter-individual variability is higher in the S2 subset compared to S1.

The degree of skewing of TRB CDR3 length distributions – the "regularity"- was objectively measured for all profiles using a diversity index derived from the Shannon index (SDI). Our adjusted Shannon diversity index (ASDI) is maximal for a high number of peaks distributed on a perfect theoretical Gaussian distribution. Comparing S1 (CD8$^+$) and S2 (CD8$^-$) T cell subsets, we found that the ASDI was lower for S2 compared to S1, indicating higher skewing and lower average peak numbers per profile in CD8$^-$ T cells (**Figure S2A**).

Taken together, these observations indicate that the CD8$^+$ T cell subset expresses a TRB repertoire more diverse and polyclonal

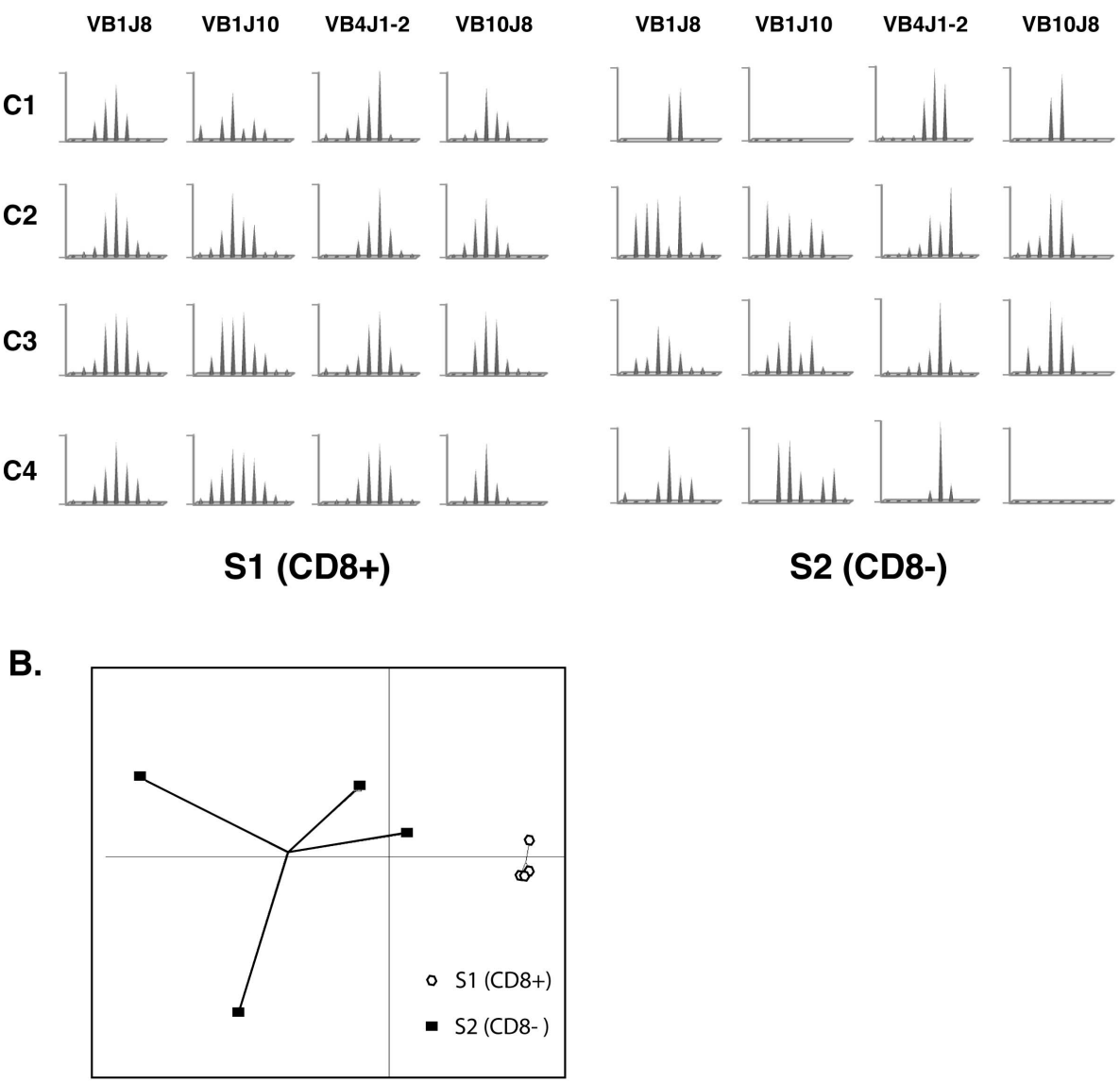

Figure 2. Comparison of CDR3 length spectratypes between S1 and S2 cell fractions in naïve fish. (A) CDR3 length profiles from CD8$^+$ (S1) and CD8$^-$ (S2) lymphocyte subsets are represented for selected TRBV-TRBJ combinations from four control fish (C1–4) (B) PCA projection of S1 and S2 samples according to the first two components (capturing respectively 58.67% and 14.94% of the global variability) using perturbation scores calculated for all spectratypes analysed.
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than the CD8$^-$ T cell subset(s) for which many TRB junctions show skewed spectratypes.

CD8$^+$ and CD8$^-$ Lymphocyte Repertoires in VHSV-challenged Fish

To understand the respective contributions of CD8$^+$ and CD8$^-$ T cells to the response against a pathogen, we analysed the TRB repertoire of S1 and S2 populations from fish infected by the Viral Haemorrhagic Septicaemia Virus (VHSV), a virus causing a systemic acute infection in rainbow trout. We have shown previously that rainbow trout mounts both public and private T cell responses during secondary infections with this virus [25,30]. However, whether the responding clones were mainly composed of CD8$^+$ or CD8$^-$ T cells remains unknown. To get insight into this question, clonal fish were vaccinated with an attenuated VHSV variant (strain 25–111), then subjected to a second booster injection three weeks later. Leukocytes were then harvested during the recall response and S1–3 populations were sorted as described above. The sorted fractions represented similar percentages as in control animals, and no significant differences in CD8 or CD4-1 expressions could be observed between control and infected groups (data not shown).

CD8$^+$ TRB repertoire shows a typical response after viral infection. Figure 3A depicts differences in CDR3 length distribution observed between CD8$^+$ fractions (S1) from infected and from control animals for representative TRBV-TRBJ, *i.e.* for a

Table 2. Statistical comparison of perturbation scores for all the TRBV-TRBJ profiles (Ebayes p-values).

	ctrl CD8$^+$ vs ctrl CD8$^-$	ctrl CD8$^+$ vs inf CD8$^+$	ctrl CD8$^-$ vs inf CD8$^-$
VB01C1	0,123	0,259	0,361
VB01J1-2	0,079	0,357	0,103
VB01J2	0,079	0,249	0,074
VB01J3	*0,014	0,235	0,898
VB01J4	0,074	-	0,483
VB01J6	0,146	0,622	0,730
VB01J7	*0,026	0,391	0,797
VB01J8	**0,008	-	**0,009
VB01J10	0,111	0,596	0,415
VB04C01	0,190	0,206	0,254
VB04J1-2	0,685	0,482	0,125
VB04J2	0,307	0,324	0,363
VB04J3	*0,027	**0,008	0,848
VB04J4	0,073	-	0,838
VB04J6	*0,017	0,411	0,600
VB04J7	0,149	0,116	0,814
VB04J8	*0,040	0,235	0,955
VB04J10	*0,034	0,125	0,135
VB08C01	0,538	0,088	0,742
VB08J1-2	**0,009	0,847	*0,014
VB08J2	-	0,175	**0,000
VB08J3	**0,001	**0,001	0,093
VB08J4	-	*0,011	**0,000
VB08J6	0,476	0,077	**0,000
VB08J7	*0,014	0,429	0,221
VB08J8	**0,005	0,062	0,478
VB08J10	**0,006	**0,002	**0,001
VB10C01	0,289	0,118	0,173
VB10J1-2	0,312	0,115	*0,012
VB10J2	0,174	0,292	0,095
VB10J3	0,386	0,096	**0,006
VB10J4	*0,038	*0,040	0,106
VB10J6	0,115	0,419	0,524
VB10J7	0,470	0,203	0,160
VB10J8	0,669	0,406	**0,004
VB10J10	*0,043	0,395	**0,004
#<0.05 *	14,000	5,000	10,000
#<0.01**	5,000	3,000	8,000

doi:10.1371/journal.pone.0060175.t002

combination that is poorly responsive in the CD8$^+$ fraction (V1J2) as well as for combinations showing modification upon virus infection (V4J7, V4J10).

To compare the CDR3 length distributions in CD8$^+$ T cells (S1 fraction) from naive and infected fish and to produce a quantitative description of spectratype modifications, we computed the perturbation score for the TRB repertoire (TRBV-TRBJ combinations) expressed by CD8$^+$ T cells. For this purpose, we took the average repertoire of the CD8$^+$ fraction from control fish as a reference. As illustrated by the PCA, infected and control animals are clearly distinct (**Figure 3B**). Indeed, statistical test of the perturbation matrix indicated that a number of TRBV/TRBJ profiles were significantly skewed in infected animals compared to control fish (**Table II**, see *p*-values for TRBV4/J3, TRBV8/J3, TRBV8/J4, TRBV8/J10 and TRBV10/J4). These results indicate that the TRB repertoire expressed by CD8$^+$ T cells is modified by the secondary viral infection.

Perturbation score indicates that CD8$^-$ TRB repertoire is also skewed by viral infection. We next computed the perturbation score of the CD8$^-$ (S2) fractions from infected fish taking the average repertoire of the control fish as a reference. Although profile inter-individual variability in naïve animals was

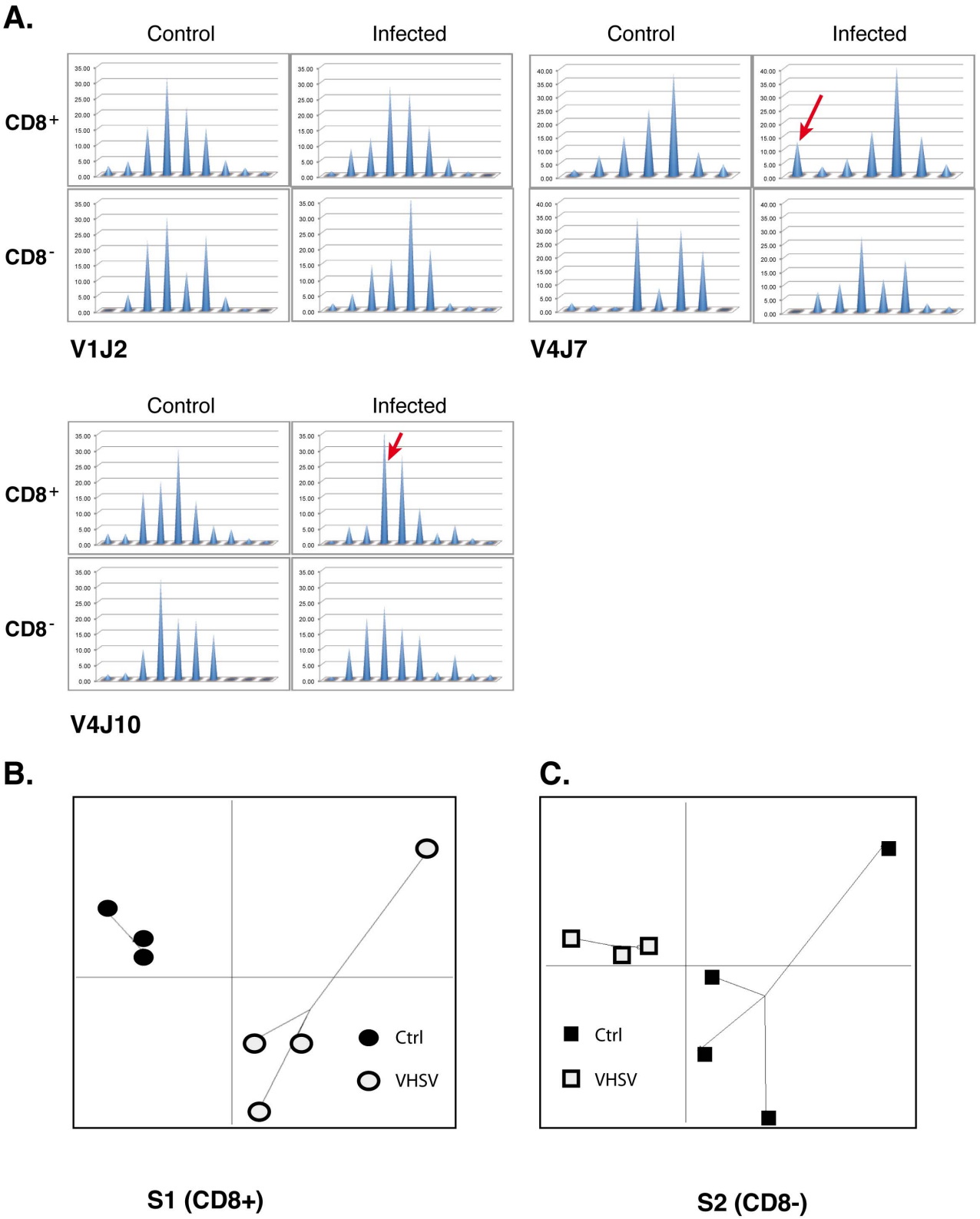

Figure 3. Virus infection induces modifications of the TRB repertoire expressed by CD8$^+$ and CD8$^-$ T cells. This figure depicts the CDR3 length distribution in CD8$^+$ fractions (S1) isolated from infected and from control animals for representative TRBV-TRBJ combinations that are poorly responsive in the CD8$^+$ fraction (e.g. V1J2) as well as for combinations showing CDR3 length modification upon virus infection (e.g. V4J7, V4J10). The arrows point to those peaks that represent CDR3 lengths with an increased abundance after viral infection. PCA projection of control and infected samples according to the first two components using perturbation scores calculated for all spectratypes analysed in S1 (B; PC1:40.26% & PC2:20.44% of the global variability) and S2 (C; PC1:31.66% & PC2:22.18% of the global variability) fractions.
doi:10.1371/journal.pone.0060175.g003

considerable, PCA analysis performed on these scores clearly discriminates TRB repertoires of CD8$^-$ T cells from control and infected fish (**Figure 3C**). We tested differences between perturbation values of each TRBV/TRBJ profile from CD8$^-$ (S2) subset in control and infected fish, respectively. We found significant differences between naïve and infected fish TRBV1/J8, TRBV8/J1-2, TRBV8/J2, TRBV8/J4, TRBV8/J6, TRBV8/J10, TRBV10/J1-2, TRBV10/J3, TRBV10/J8 and TRBV10/J10 (**Table II**). Interestingly, these combinations did not match those for which significant differences had been found for the S1 fraction.

Different impact of the viral infection on the TRB CDR3 length diversity in CD8$^+$ and CD8$^-$ T cell subsets. Selected CDR3 length spectratypes illustrate that the VHSV infection has a different impact on the TRB repertoire expressed by CD8$^+$ and by CD8$^-$ T cells, respectively (**Figure 3A**). To better qualify these modifications, we computed ASDIs for each profile in CD8$^+$ as well as CD8$^-$ fractions, using the corresponding average control repertoire as reference. While the perturbation analysis had identified unambiguous responses in CD8$^+$ T cells from virus infected fish, compared to the controls, ASDIs were not significantly different between CD8$^+$ T cells from infected and control fish, for any of the TRBV-TRBJ combinations considered. (**Figure S2B**).

In contrast, CD8$^-$ T cells showed a different behaviour upon viral infection: the median diversity of TRB CDR3 length estimated by ASDI calculated in reference to the average repertoire of the CD8$^-$ control was in fact higher in infected fish compared to the controls (**Figure S2C**). This ASDI was significantly higher (p-values <0.05) in infected fish for some TRBVTRBJ combinations (TRBV1/J6, TRBV4/C1, TRBV4/J1-2, TRBV4/J03, TRBV4/J10, TRBV8/J1-2; data not shown). In fact, antigen-driven clonal responses typically induce expansion of one or a few peak(s) of initially bell-shaped spectratypes, hence reduce their ASDI. In the case of the CD8$^-$ fraction of trout T cells, TRB CDR3 length profiles are highly skewed in unchallenged animals and the expansion of additional virus-specific peaks therefore increases the peak diversity, thus reducing the spectratypes skew.

Discussion

In this work, we used TRB CDR3 length profiling from CD8$^+$ and CD8$^-$ sorted fractions of spleen and pronephros leukocytes in order to examine the diversity and complexity of the corresponding repertoires in a teleost fish, the rainbow trout. Using anti-CD8α mabs to sort and characterize CD8$^+$ and CD8$^-$ T cells in different fish species, several reports have recently established that the CD8$^+$ fraction comprises cytotoxic T cells, which do not express the receptor CD4, as typically known in human and in the mouse. In contrast, the CD8$^-$ fraction contains CD4-1$^+$ and CD4-2$^+$ cells, which do not possess efficient killing capacity but have properties reminding mammalian T helper cells [14][38–40]. However, the TR repertoires expressed by the CD8$^+$ and CD8$^-$ T cell lineages in fish remained uncharacterized so far. We therefore undertook cell sorting followed by TRB CDR3 length profiling in healthy fish as well as in animals infected by a fish rhabdovirus, the VHSV.

In PBMCs from healthy humans, TRB CDR3 length follows a bell-shaped Gaussian-like distribution for most of expressed TRBV-TRBJ combinations [27,41]. This was also observed for both CD4$^+$ and CD8$^+$ subsets, although a few genes especially TRBV16 were often associated with skewed profiles [26]. Overall, the frequency of skewed profiles appeared slightly higher for CD8$^+$ T cells compared to CD4$^+$ T cells. This is in contrast with our observations in rainbow trout, where the CD8$^+$ T cell subset expresses a TRB repertoire more diverse and more regular than the CD8$^-$ fraction. We did not analyse rainbow trout PBMC because T cells are very scarce in the blood of this species, which hindered sorting experiments [42]. In fact, skewed TRB spectratypes are frequent in PBMCs of healthy macaques, not only in CD8$^+$ T cells but also in CD4$^+$ T cells while it is rarely seen in humans. Thus, CD4$^+$ T cells were significantly contributing to the repertoire skewing in macaques, and were found for a fair number of TRBVTRBJ combinations [27]. Hence, the respective structure of the TRB diversity in CD8$^+$ versus CD4$^+$ T cells appears to be variable between species even among closely related ones such as primates. This observation suggests that different situations will be likely found among teleost fish, which represent a highly complex and diversified group of lower vertebrates.

Since skewed profiles were found for different TRBV-TRBJ combinations in the CD8$^+$ and CD8$^-$ fractions of rainbow trout lymphocytes, they should not be primarily determined by constraints at the rearrangement level. Such constraints would depend on the genes involved and would likely lead to skewed profiles in both CD8$^+$ and CD8$^-$ T cells. Hence, skewed profiles likely represent tracks of previous antigen-driven expansions, which are expected to affect different TRBV-TRBJ combinations in both the CD8$^+$ and CD8$^-$ fractions. A frequent artefact in spectratyping analysis is observed when profiles are produced from cDNAs containing very low amounts of TRB templates. In such conditions, a few sequences are randomly amplified and produce a CDR3 length profile with only one or a few peaks, which does not provide a good representation of the junction diversity [43]. To exclude the possibility that skewed spectratypes – especially from CD8$^-$ cells - were due to such random amplification of rare TRB sequences, we checked the spectratypes produced from independent amplifications from the same cDNA templates. Those always showed similar patterns (data not shown) suggesting that spectratype changes reflected true TR repertoire changes. Therefore, it can be proposed that while trout CD8$^+$ T cells express a highly diverse, naïve repertoire of TRB that is available to mount cytotoxic responses, the CD8$^-$ T cell fraction of leukocytes would rather contain many large clones that have already accumulated during previous antigen-driven responses. We cannot completely rule out that the injection of endotoxin-free PBS to which control fish were subjected might have an impact on the TCR repertoire. However, such injection does not modify the expression level of the activation marker CTLA4 in leukocytes [44 and unpublished data], and we do not consider this is the most likely explanation to the difference between CD8+ and CD8− T cell repertoire.

Our observations are reminiscent of the accumulation of large T cell clones during aging, observed in elderly humans for both CD8$^+$ and CD4$^+$ lineages, leading to skewed profiles with comparable frequencies in the two subsets [29]. Alternatively, the CD8$^-$ subset may be enriched in non conventional T cells with restricted TRB diversity, such as mucosal-associated invariant T (MAIT) or natural killer T (NKT)-like cells [45,46]. Further characterization of the trout TRα repertoire will be necessary to clarify this issue.

As previously reported for the whole trout leukocyte population, we retrieved here that viral infection leads to a significant skewing of TRB CDR3 length profiles of CD8$^+$ subpopulation. Both visual comparison and perturbation analysis identified TRBV-TRBJ spectratypes that are skewed after viral infection in several fish. The combinations for which skewing was significant involved TRBV4, TRBV8 and TRBV10, but not TRBV1. Interestingly, in

whole-leukocyte analysis, TRBV4 has been retrieved in public responses in several trout genetic backgrounds, and TRBV8 and TRBV10 were found responsive while TRBV1 was never clearly involved in anti-VHSV response ([25,30] and unpublished data). The diversity index did not reveal any particular pattern in infected animals; in fact, the intensity of the spectratype skewing associated to the response against VHSV in the clonal fish used in this study appeared more moderate than the modifications of the TRB repertoire we observed previously with other genetic backgrounds [25,30,47]. Whether this moderate response was due to the genetic background or to environmental factors is difficult to determine unambiguously, but in absence of any sign of unwanted disease in the fish used for the project we favour the first interpretation.

The analysis of the CD8$^-$ response induced by the infection was more difficult due to the high frequency of skewed profiles and inter-individual variability among naïve fish regarding this fraction. However, a clear response was evidenced by the PCA, indicating that both CD8$^+$ and CD8$^-$ αβ T cells contribute to the anti-VHSV response. The TRBV-TRBJ combinations for which the profiles were significantly affected did not match those identified for the CD8$^+$ fraction, confirming that the response to the virus does not involve cells expressing similar TRB from CD8$^+$ and CD8$^-$ fractions. Intriguingly, TRB CDR3 length profiles were more regular and more complex after viral infection compared to the controls, as indicated by the diversity (Shannon modified) index. It appeared that the antiviral response lead to multiple expansions of CD8$^-$ T cells, sometimes reducing the relative importance of large peaks observed in the skewed profiles of the unchallenged controls. This may provide a first observation in favour of different impact of infections on CD8$^-$ T cells between trout and mouse (or human). However, further investigations would be required to link these preliminary observations about CD8$^+$ and CD8$^-$ leukocyte fractions to the immune memory and to functional properties of cytotoxic and helper T cells in fish. In particular, the clonal complexity of CD8$^+$ and CD8$^-$ T cell responses will have to be characterized by a thorough sequencing analysis of their respective TRs. Also, the availability of an antibody against additional markers including CD4 would be very important to sort T cell fractions from the thymus and other lymphoid tissues and track the distribution of T cell subsets during development and after immune responses. Thus, these studies will have both basic interest for deciphering the evolution of the immune system in an important branch of vertebrates, and a practical value for improving vaccination in aquaculture.

Supporting Information

Figure S1 Selected TRBV/C spectratypes from S1 and S2 T cell populations purified from control rainbow trout.
(PDF)

Figure S2 Comparison of spectratype diversity index. A. ASD index distribution for S1 and S2 fractions from control group. The reference used is the average repertoire through both groups. B: ASD index distribution for S1 fractions from control and infected groups. The reference used is the average repertoire of control groups. C: ASD index distribution for S2 fractions from control and infected groups. The reference used is the average repertoire of control groups.
(PDF)

Author Contributions

Conceived and designed the experiments: RC FT UF PB. Performed the experiments: RC FT AL UF PB. Analyzed the data: RC FT WC AS UF PB. Contributed reagents/materials/analysis tools: BK EQ THD. Wrote the paper: RC WC EQ AS UF PB.

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Abstract
Mucosal surfaces are multifunctional barriers that need to orchestrate a variety of physiological functions. One of the mechanisms by which mucosal surfaces carry immunological functions without compromising other physiological roles is the establishment of regional immunity. Regional immunity can be generated by selective expression of chemokines, chemokine receptors and adhesion molecules within mucosal microenvironments. In the olfactory organ of animals, immune responses may result in damage to the olfactory sensory neurons and, as a consequence, compromise olfaction. The olfactory organ of teleost fish is histologically divided into two distinct areas, a sensory or neuroepithelial region in the lateral regions and valleys if the lamellae and a non-sensory region located at the tip of each lamella characterized by presence of goblet cells. Thus, we hypothesized the presence of two unique immune microenvironments corresponding with these two regions in rainbow trout. In line with our hypothesis, we found clusters of CD8+ T cells at the mucosal regions of lamellae whereas few numbers were found in the neuroepithelium. Nasal CD8+ T cells displayed a mucosal phenotype similar to those found in the gut. Laser capture microdissected tip versus lateral samples of control trout olfactory tissue were collected and RT-qPCR revealed a higher expression of the chemokine and chemokine receptor CCL19 and CCR7, the adhesion molecules L-selectin, ICAM, VCAM, and integrin β2 and several T cell markers in the mucosal tip compared to the neuroepithelium. Moreover, MHC-II immunostaining indicated that the mucosal tip preferentially participates in antigen presentation within the trout nasal mucosa. Finally, in response to nasal vaccination, cell proliferation occurs in the tip mucosal region and not the neuroepithelium. Our results support the idea of compartmentalized immune responses within the nasal mucosa of teleosts that likely evolved as a sparing mechanism to protect olfactory sensory function.
Keywords: Regional immunity, NALT, trout, nasal immunity, mucosal immunity

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O-070.

Characterisation and bioactivities of type I interferon subtype E and F in salmonids

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Abstract
Salmonids are important commercial species in the European aquaculture industry. Many viral diseases can impact on the industry, leading to economic losses. Interferons (IFNs) are cytokines involved in antiviral defence, and study of their regulation and function may shed light on how fish can defend themselves against viral infection. Fish type I IFNs are classified into two groups based on cysteine residues in the mature peptide, namely group I and II IFNs, each of which is further divided into 3 subtypes in salmonids. The latest new subtypes, IFN-e and IFN-f, have only recently been identified and their biological activities have still to be characterised. In this presentation, IFN-e and −f genes in Atlantic salmon (*Salmo salar*) and their bioactivities were reviewed and compared to previous studies of other type I IFN subtypes (a-d). The type I IFN subtypes showed different expression patterns between the 2 groups in cell lines and primary head kidney cells stimulated with poly I:C and in heart and head kidney of cardiomyopathy syndrome (CMS) infected salmon. Transcript levels could be correlated to the antiviral response and the IFN-e and IFN−f recombinant proteins were shown to induce Mx gene expression which is known to mediate antiviral activity. This data together with the reactivity of specific antibodies to the subtypes provides a better understanding of the antiviral effects of type I IFNs in salmonids.
Keywords: Salmonids, type I interferons, bioactivities

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O-071.

CK9, an ancient rainbow trout chemokine that attracts and regulates B lymphocytes and macrophages

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Abstract
CK9 is a rainbow trout (*Oncorhynchus mykiss*) CC chemokine phylogenetically related to mammalian CCL25. Although CK9 is known to be transcriptionally regulated in response to inflammation particularly in mucosal tissues, its functionality has never been revealed. In the current work, we have demonstrated that CK9 has a strong chemotactic capacity for both B cells (IgM+ and IgT+) and macrophages. Along with its chemotactic capacities, CK9 modulated the MHC II turnover of B lymphocytes and upregulated the phagocytic capacity of both IgM+ cells and macrophages. Although CK9 had no lymphoproliferative effects, it increased the survival of IgT+ lymphocytes. Furthermore, we have established that the chemoattractant capacity of CK9 is strongly increased after pre-incubation of leukocytes with a T-independent antigen, whereas B cell receptor (BCR) cross-linking strongly abrogated their capacity to migrate to CK9, indicating that CK9 preferentially attracts B cells at the steady state or under BCR-independent stimulation. These results point to CK9 being a key regulator of B lymphocyte mucosal trafficking in teleost fish, able to modulate innate functions of teleost B lymphocytes and macrophages.
Keywords: Chemokine; CK9; trout; B lymphocytes; macrophages.

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O-072.

Characterization of antigen sampling cells in rainbow trout gill epithelium

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Abstract
In mammalian mucosal surfaces, microfold cells (M cells) take up antigens and deliver it to antigen presenting cells in lymphoid tissues such as Payer's patches. In rainbow trout, a part of gill epithelial cells take up *Aeromonas salmonicida* subsp. *salmonicida* (*A.s.s.*) bacterin during bath-vaccination. Antigen-sampling cells in gill epithelium could be stained

with lectin *Ulex europaeus* agglutinin 1 (UEA-1) but not with wheat germ agglutinin (WGA); this staining pattern is characteristic for mammalian M cells. In this study, we sorted *A.s.s.*+ UEA-1+ cells from rainbow trout gill epithelium and performed a comprehensive gene expression analysis using next generation sequencing to characterize the corresponding antigen sampling cells.

Rainbow trout (approx. 150 g) were bath-vaccinated with Syto61-stained *A.s.s.* bacterin. Epithelial cells were isolated from the gills and stained with FITC-labeled UEA-1 and subjected to flow cytometry followed by sorting. Two cell populations, *A.s.s.*+ UEA-1+ and *A.s.s.*+ UEA-1- were isolated and total RNA was extracted from each cell population. The cDNA library of each cell population was sequenced using a MiSeq system and gene expression analysis based on the read counts was performed using Trinity assemble software.

Flow cytometry revealed that there were two distinct cell populations containing the *A.s.s.* bacterin in the epithelium: *A.s.s.*+ UEA-1+ and *A.s.s.*+ UEA-1-. The percentage of each cell group was 22% in *A.s.s.*+ UEA-1+, and 5% in *A.s.s.*+ UEA-1- population. These two populations also showed different scatter characteristics in flow cytometry, where the *A.s.s.*+ UEA-1+ population exhibited a relatively high side scatter as a measure for pronounced granulation. Remarkably, the *A.s.s.*+ UEA-1+ population highly expressed annexin which is typical for mammalian M cells, and interleukin (IL)-20 which is typical for mammalian epithelial cells. In contrast, *A.s.s.*+ UEA-1- cells showed high expression levels of CD83 and IL-12 that are typical for mammalian macrophages and dendritic cells. MHC class II related genes were highly expressed in both of the cell populations from rainbow trout gills. These results suggest that almost of the bacterin are taken up by the UEA-1+ antigen-sampling cells which show properties of epithelial cells during bath-vaccination. In addition, macrophage/dendritic cell lineage may also take part in the uptake of the bacterin.

Keywords: antigen up take, antigen sampling cell, mucosal immunity, rainbow trout, next generation sequencer

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O-073.

Characterization of dendritic-like cells generated from hematopoietic spleen cultures from atlantic salmon (*Salmo salar* L.)

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Abstract

Dendritic cells (DCs) are the main organizers of the immune system in mammals bridging innate and adaptive immunity. DC-like cells are now also identified in teleost fish. At present there is little knowledge of fish DCs properties and functions due to lack of tools to isolate and analyze them. We have developed a protocol for generation of DC-like cells from hematopoietic spleen cultures from Atlantic salmon (*Salmo salar L.*). In the culture flasks, a stromal layer appeared and after 2 weeks incubation hematopoietic foci were formed. We will describe phenotype and morphology of both adherent and non-adherent MHCII+ DC-like cells. Upon challenge with bacteria IL12p40, CD83 and CD80/86 were significantly upregulated in the non-adherent DC-like cells. These results support that the cells produced from the hematopoietic cultures are professional antigen cells with potential to activate naïve T-cells belonging to the dendritic cell linage.

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O-074.

Neutrophil granulocytes in atlantic salmon (*Salmo salar* L.) are MHCII positive antigen-presenting cells

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Abstract

In fish, like in mammals, professional antigen presenting cells like dendritic cells, macrophages and B cells are phagocytic cells that ingest, process and present antigens for T cells via MHCII. Neutrophils are professional phagocytic cells with an oxygen-dependent degradation mechanism. By use of a monoclonal antibody against neutrophils in Atlantic salmon, we have shown that the neutrophil granulocytes are MHCII-positive cells. The neutrophils were isolated by magnetic cell sorting and morphological characterization and phenotypic analyses were performed. After in vitro challenge with bacteria, IL12p40, which is essential for proliferation of naïve T cells, were upregulated. Further, Atlantic salmon was bath challenge with the same bacteria as the in vitro challenge experiment and the fraction of neutrophil granulocytes, B cells and MHCII positive cells in primary and secondary immune organs were measured by flow cytometry. Antigen-presentation in fish will be discussed.

Keywords: Neutrophils, antigen-presenting cells, bacteria challenge, MHCII

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O-075.

Atlantic salmon physiological and immune response to amoebic gill disease and insight into the biology of the amoeba

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Abstract

Amoebic gill disease (AGD) is an emerging disease in North European Atlantic salmon (*Salmo salar*) aquaculture caused by the amoeba *Paramoeba perurans*. Non-optimal environmental conditions such as increasing water temperature may affect AGD progression. To understand the role of predisposing environmental conditions on the biology of the parasite and on the host physiological and immune response, different clonal cultures of *P. perurans* and AGD infected Atlantic salmon were exposed, respectively, *in vitro* and *in vivo* to two different temperatures, 10°C and 15°C. Outputs from the *in vitro* experiment are 1) an improved understanding of the impact of temperature on amoebae growth rate during conditions that potentially influence disease development and 2) characterisation of changes in bacterial communities at different temperatures for different clonal cultures. Negative binomial analysis in R (R software, version 3.0.1) showed significant differences in growth rate among clonal cultures over time. Moreover, for each clonal culture, the fraction of amoebae in suspension in seawater showed a higher increase over time at 10°C, whereas the fraction of amoebae attached to the malt yeast agar (MYA) showed a higher increase over time at 15°C. 16S MiSeq analysis was also performed to characterise the changes in bacterial communities present in the cultures at the two different temperatures of the *in vitro* experiment.

The host physiological and immune response to AGD infection during predisposing seasonal weather conditions (summer period) is still not well understood. Atlantic salmon smolts were exposed *in vivo* to the same

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Transcriptomic responses in rainbow trout gills upon infection with viral hemorrhagic septicemia virus (VHSV)

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ABSTRACT

It has been previously demonstrated that even though the fin bases constitute the main portal of entry of viral hemorrhagic septicemia virus (VHSV) in rainbow trout (*Oncorhynchus mykiss*), an important number of chemokine genes are up-regulated in the gills upon bath exposure to the virus. Because chemokines mediate the recruitment of leukocytes through the action of specific chemokine receptors, in the current study, we have studied the transcription of several immune genes in response to a VHSV bath infection in the gills, focusing both on chemokine receptor genes and on genes characteristic of distinct leukocyte populations such as IgM, IgD, IgT, CD4, CD8, perforin and MHC-II. We have studied the response to the virus in naïve fish as well as in fish that had been previously intramuscularly (i.m.) injected with a VHSV DNA vaccine. Additionally, we have sorted both IgM^+ and $CD8^+$ cells from the gills of naïve and infected animals to study some of these up-regulated genes in specific leukocyte populations. Our results indicate that despite the low replication level, VHSV provokes an up-regulation of IgM, IgT, CD3 and perforin transcription together with the up-regulation of CCR7, CCR9, CXCR3B and CXCR4 mRNA levels. Interestingly, MHC-II mRNA was up-regulated and CCR7 was down-modulated in IgM^+ cells from infected gills, whereas perforin, CCR7 and CXCR4 mRNA levels were higher in sorted $CD8^+$ cells from infected animals. Surprisingly, when fish had been previously injected with either the empty plasmid or the VHSV DNA vaccine, these up-regulations in immune gene transcription were no longer observed. Our results point to the gills as an important site for innate and acquired viral defense.

1. Introduction

Viral hemorrhagic septicemia virus (VHSV) belongs to the *Novirhabdovirus* genus within the *Rhabdoviridae* family, and is the etiological agent of a lethal disease for many aquacultured fish species worldwide, including rainbow trout (*Oncorhynchus mykiss*). The fin bases constitute the main portal of entry for both VHSV and a very closely related salmonid pathogen, the infectious hematopoietic necrosis virus (IHNV) (Harmache et al., 2006; Quillet et al., 2001, 2007). Upon bath exposure, viral replication is already visible as early as 8 h post-infection in this area, whereas no replication is observed at this point in the gills (Harmache et al., 2006). Despite this, some authors have detected VHSV replication in the gills at early time points (Brudeseth et al., 2002) and a previous study demonstrated that VHSV bath infection up-regulated an important number of chemokine genes in the gills (Montero et al., 2011). In fact, the induction of chemokine genes in the gills was much stronger than that observed in the fin bases, suggesting a viral interference mechanism when the virus actively replicates in the fin bases (Montero et al., 2011).

Chemokines attract immune cells and modulate them through the action of chemokine receptors, members of a family of seven transmembrane domain G-coupled receptors. In rainbow trout, the sequences of various chemokine receptors have been reported to date: CCR6 (Dixon et al., 2013), CCR7 (Ordas et al., 2012), CCR9 (Daniels et al., 1999), CCR9B (Dixon et al., 2013), CCR13 (Dixon et al., 2013), CXCR1 (Zhang et al., 2002), CXCR3A (Wang et al., 2008), CXCR3B (Wang et al., 2008) and CXCR4 (Daniels et al., 1999). However, there is no information concerning the chemokines that signal through these receptors and the patterns of transcription have mostly been assessed in complete tissues and not in individual cell types, with some exceptions. For example, rainbow trout IgM^+ cells from blood, kidney, spleen, gills, intestine and liver constitutively transcribe CCR9, CCR9B, CCR13, CXCR1 and CXCR4, whereas only IgM^+ cells from gills, intestine and liver and not those from blood, kidney or spleen, transcribe CCR6 and CCR7 (Abos

Abbreviations: i.m., intramuscularly; VHSV, viral hemorrhagic septicemia virus.

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et al., 2013). Additionally, CCR7 has been suggested to be implicated in the recruitment of B cells to mucosal surfaces during infection, since CCR7 transcription was up-regulated in both IgM^+ and IgT^+ cells from the intestine of fish infected with *Ceratomyxa shasta* in comparison to naïve fish (Ordas et al., 2012).

In the current study, we have evaluated the levels of transcription of known chemokine receptor genes in the gills in response to a VHSV bath infection, because regardless of whether the gills are a major target of early VHSV replication or not, their huge surface is in direct contact with water-borne antigens. Thus, gills are potential key sites for antigen sensing and may trigger important local and systemic antiviral immune responses. As chemokine induction should go along with the recruitment of leukocytes to the site of inflammation, we have also studied the transcription of marker genes for distinct leukocyte subpopulations such as IgM, IgT, IgD, CD4, CD8, perforin and MHC-II. These genes have been studied after VHSV exposure of naïve fish as well as in challenged fish that had been previously vaccinated with a VHSV DNA vaccine. Intramuscular delivery of DNA encoding the glycoprotein of VHSV has been proven as very effective (Lorenzen et al., 2000), able to confer high levels of protection, even though the mechanisms through which this protection is achieved are still unknown. Thus, correlating the effects of vaccination to protection will help us understand which immune responses need to be triggered to confer protection when developing future vaccines.

2. Materials and methods

2.1. Fish

Female rainbow trout (*O. mykiss*) adults of approximately 5–10 g (5–7 cm) were obtained from Centro de Acuicultura El Molino (Madrid, Spain) and maintained at the animal facilities of the Centro de Investigación en Sanidad Animal (CISA-INIA, Spain) in a re-circulating water system at 16 °C, with 12:12 h light:dark photoperiod. These fish were used for DNA vaccination followed by bath challenge with VHSV. Female rainbow trout of approximately 25 g (7–10 cm) used for bath infection and subsequent sorting of leukocyte subpopulations were of strain A36/A03 (kindly provided by Edwige Quillet, INRA, France) and hatched at the Friedrich-Loeffler-Institut (FLI, Germany). They were kept at the FLI at 16 °C, with 12:12 h light:dark photoperiod. All fish were fed twice a day with a commercial diet (Skretting, Spain). Prior to any experimental procedure, fish were acclimatized to laboratory conditions for at least 2 weeks.

All the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and have been approved by the INIA Ethics Committee and the Ethics Commission for Animal Experiments of the German State Mecklenburg-Western Pomerania.

2.2. VHSV DNA vaccine

The VHSV DNA vaccine used in this study, designated pVHSV, has been previously described (Lorenzen et al., 1998, 2005) and contains the gene encoding the full length VHSV G protein from isolate DK-3592B downstream of the cytomegalovirus promoter in the expression vector pcDNA3 (Invitrogen). The empty pcDNA3 plasmid was used as a control.

2.3. DNA vaccination and VHSV in vivo infection

Rainbow trout were divided into three groups (24 fish each) and injected intramuscularly (i.m.) with either 1 μg of pVHSV plasmid, 1 μg of the pcDNA3 control plasmid in PBS (50 μl) or the same volume of PBS in the epaxial muscle below the dorsal fin by use of a 27 G needle.

Thirty days post-vaccination, half of the rainbow trout in each of these three groups were challenged with VHSV through bath infection while the other half was mock-infected. This time point was chosen because previous studies had determined that acquired immunity in response to DNA vaccination is established at this time (Lorenzen et al., 2000). The VHSV challenge was performed as previously described (Montero et al., 2011). Briefly, fish were transferred to 4 L of a viral solution containing 5×10^5 - $TCID_{50}$/ml of the VHSV strain 0771 (kindly provided by Pierre Boudinot, INRA, France). After 1 h of viral adsorption with strong aeration at 16 °C, each experimental group was transferred to an individual water tank. Mock-infected groups were also transferred to 4 L tanks containing an equivalent amount of non-infected culture media. After 1 h of strong aeration, they were also moved to their corresponding tanks. At days 1 and 3 post-infection, six trout from each group were sacrificed by over-exposure to MS-222 and gills and spleen were sampled and placed in Trizol (Invitrogen) for RNA isolation.

For the sorting experiments, rainbow trout were bath infected with VHSV strain 0771 at FLI at a dosage of 10^5 $TCID_{50}$/ml water. Twelve fish were exposed to the virus in 20 L of aerated water and after 1 h, the water volume was increased to the final tank volume of 200 L, while an equivalent group of fish was mock-incubated with the same amount of virus free cell-culture medium in 20 L rearing water.

2.4. Sorting of IgM^+ and $CD8^+$ cells from infected gills

Gills were sampled from 4 fish per sampling day at days 0, 1, 3 and 7 post-infection. For this, all gill arches from separate fishes were pooled and homogenized using scissors in combination with a Potter-Elvehjem homogenizer. Cell suspensions were filtered through a nylon mesh to remove remaining aggregates and layered onto Percoll (1.075 g/ml) density gradients. Interphases were collected after centrifugation for 40 min at 500×*g* and washed. Cells were double-labeled using rat and mouse monoclonal antibodies (mabs), respectively, against rainbow trout CD8α (Takizawa et al., 2011) and against rainbow trout IgM using mab 4C10 (Thuvander et al., 1990) followed by double staining using anti-rat and anti-mouse species specific TriColor and Alexa488 conjugated secondary antibodies, respectively. Positive and negative cell populations were separately sorted directly into Trizol LS ($1–3 \times 10^4$ cells/sample) using a MoFlo (Dako-Cytomation) high-speed cell sorter. A small amount of cells from the same sort was recovered in cell culture medium and their purity confirmed by post-sort flow cytometry. At the end, two distinct subpopulations of gill leukocytes were received: $CD8^+$ and IgM^+ cells, together with the respective negative (unstained) populations.

2.5. RNA extraction and cDNA preparation

Total RNA was extracted from the gill samples using a combination of Trizol (Invitrogen) and RNAeasy Mini kit (Qiagen). In summary, samples were mechanically disrupted in 1 ml of Trizol using a disruption pestle. Then, 200 μl of chloroform were added and the suspension centrifuged at 12,000×*g* for 15 min. The clear upper phase was recovered, mixed with an equal volume of 100% ethanol and immediately transferred to RNeasy Mini kit columns. The procedure was then continued following manufacturer's instructions, performing on-column DNase treatment. Finally, RNA pellets were eluted from the columns in RNase-free water and stored at −80 °C until used. Two μg of RNA were used to obtain cDNA in each sample using the Bioscript reverse transcriptase (Bioline Reagents Ltd) and oligo $(dT)_{12-18}$ (0.5 μg/ml) following manufacturer's

instructions. The resulting cDNA was diluted in a 1:5 proportion with water and stored at −20 °C.

In the case of the sorted cells from the gills, 1–3 × 10^4 sorted cells were placed in 350 μl of Trizol LS and combined with 450 μl of Trizol and 200 μl of chloroform. The suspension was centrifuged at 12,000×*g* for 15 min. The clear upper phase was recovered, mixed with an equal volume of 100% ethanol and immediately transferred to RNAeasy Micro kit columns. The procedure was then continued following manufacturer's instructions, performing on-column DNase treatment. cDNA was obtained from 0.25–0.5 μg of RNA using the Bioscript reverse transcriptase as above. The resulting cDNA was diluted in a 1:2 proportion with water and stored at −20 °C.

2.6. Evaluation of immune gene expression by real time PCR

To evaluate the levels of transcription of the different genes, real-time PCR was performed in a LightCycler® 480 System instrument (Roche) using SYBR Green PCR core Reagents (Applied Biosystems) and specific primers (shown in Table 1). The efficiency of the amplification was determined for each primer pair using serial 10 fold dilutions of pooled cDNA, and only primer pairs with efficiencies between 1.95 and 2 were used. Each sample was measured in duplicate under the following conditions: 10 min at 95 °C, followed by 45 amplification cycles (15 s at 95 °C and 1 min at 60 °C). The expression of individual genes was normalized to relative expression of trout EF-1α and the expression levels were calculated using the $2^{-\Delta Ct}$ method, where ΔCt is determined by subtracting the EF-1α value from the target Ct. Negative controls with no template were included in all the experiments. A melting curve for each PCR was determined by reading fluorescence every degree between 60 and 95 °C to ensure only a single product had been amplified.

Table 1
Oligonucleotides used for real time PCR in this study.

Gene	Primer	Sequence
EF1α	rtEF1α F	GATCCAGAAGGAGGTCACCA
	rtEF1α R	TTACGTTCGACCTTCCATCC
CCR7	CCR7 4F	TTCACTGATTACCCCACAGACAATA
	CCR7 4R	AAGCAGATGAGGGAGTAAAAGGTG
CCR6	CCR6 F	TGCAGAGGAAACAGTTAACAATTCA
	CCR6 R	CCAGTAAACCCAGGATACAGATGAC
CCR9	CCR9 F	TCAATCCCTTCCTGTATGTGTTTGT
	CCR9 R	GTCCGTGTCTGACATAACTGAGGAG
CCR9B	CCR9B F	AATATTTCCAACGTCTGAAACAGGA
	CCR9B R	CTCACCCAGGACTTATCACACATTC
CCR13	CCR13 F	GTTCTGTACAACGTCTGGAAGGATT
	CCR13 R	ATGGCCAAAGGAAGTAGAAAGAAGA
CXCR1	CXCR1 F	CCTGATATCCAGAAGCTCTTTGTGT
	CXCR1 R	TTGCATCCAGCTCTATGATAATGAA
CXCR3A	CXCR3A F	CAAGGCAACCACAAATTACTATATTTATGATG
	CXCR3A R	CAGCACACACAGCACCAGGAT
CXCR3B	CXCR3B F	CACTGGAGCCATGTTTACAATCAACT
	CXCR3B R	CCCTCACAGACTCCAGGAAGTG
CXCR4	CXCR4 F	GTGCATGTGATCTACACCATC
	CXCR4 R	GAGCTGTGGCAAACACTATGT
IgM	rtIgM F	TGCGTGTTTGAGAACAAAGC
	rtIgM R	GACGGCTCGATGATCGTAAT
IgDm	mIgD F	CAGGAGGAAAGTTCGGCATCA
	mIgD R	CCTCAAGGAGCTCTGGTTTGGA
IgT	rtIgT F	AACATCACCTGGCACATCAA
	rtIgT R	TTCAGGTTGCCCTTTGATTC
CD3	CD3l F	CCTGATTGGAGTAGCTGTCTAC
	CD3l R	GCTGTACTCAGATCTGTCCATGC
CD8α	rtCD8α F	AGTCGTGCAAAGTGGGAAAG
	rtCD8α R	GGTTGCAATGGCATACAGTG
CD4-1	rtCD4-1 F	CCTGCTCATCCACAGCCTAT
	rtCD4-1 R	CTTCTCCTGGCTGTCTGACC
MHC-IIα	rtMHCIIα F	ACACCCTTATCTGCCACGTC
	rtMHCIIα R	TCTGGGGTGAAGCTCAGACT
Perforin	rtPerf F	GGAACGACGACCTGTTAGGA
	rtPerf R	TCATAGGGGAGGGCACATAG
VHSV N	VHSV-N-F	GAGAGAACTGGCCCTGACTG
	VHSV-N-R	CCCGAGTTTCTTGGTGATGT

2.7. Statistics

Data handling, analyses and graphic representation was performed using Microsoft Office Excel 2010. Statistically significant differences were determined using a Mann–Whitney *U* test ($p < 0.05$) using the SPSS program (Version 15).

3. Results

3.1. Effect of VHSV bath infection on immune gene transcription in gills

We first determined the levels of transcription for different immune genes in non-vaccinated fish exposed to a single VHSV bath infection, focusing on marker genes for different leukocyte subpopulations and chemokine receptor genes.

When fish were bath infected with the virus, the levels of transcription of IgM, IgT, CD3 and perforin significantly increased in comparison to levels observed in mock-infected controls at day 1 post-infection (Fig. 1A). At day 3 post-infection, these significant up-regulations were no longer observed (Fig. 1B).

Concerning chemokine receptor genes, the virus induced a significant up-regulation of transcription levels for CCR7, CCR9, CXCR3B and CXCR4 at day 1 post-infection (Fig. 2A), whereas only CXCR3B remained significantly up-regulated at day 3 post-infection (Fig. 2B).

3.2. Effect of VHSV bath infection on immune gene transcription in sorted $CD8^+$ cells from gills

To understand which cell types in the gills were contributing to the modulation of immune genes in response to the virus, we studied gene expression in sorted cells from virus and mock-infected fish in a separate experiment.

Sorted $CD8^+$ cells from the gills constitutively transcribed CD8, perforin, CCR7, CCR9 and CXCR4, but no CXCR3B mRNA was ever detected in these cells (Fig. 3). When fish were infected, the transcription levels of perforin significantly increased in sorted $CD8^+$ cells at day 3 post-infection. At day 7 post-infection, the levels of transcription of CCR7 and CXCR4 were significantly up-regulated in sorted $CD8^+$ cells from virus-infected fish in comparison to the levels observed in cells from mock-infected animals. No significant differences in the mRNA levels of CD8 or CCR9 were detected between infected and control fish at any of the time points studied.

3.3. Effect of VHSV bath infection on immune gene transcription in sorted IgM^+ cells from gills

Sorted IgM^+ cells from the gills constitutively transcribe IgM, IgD, MHC-II, CCR7, CCR9 and CXCR4, but no CXCR3B transcription was detected (Fig. 4). Although the levels of transcription of IgM, IgD, CCR9 and CXCR4 were not significantly affected by the viral infection, the mRNA levels of MHC-II in sorted IgM^+ from infected fish were significantly higher than in cells from mock-infected trout at all the time points studied. On the other hand, the virus infection provoked a significant down-regulation of CCR7 at day 7 post-infection.

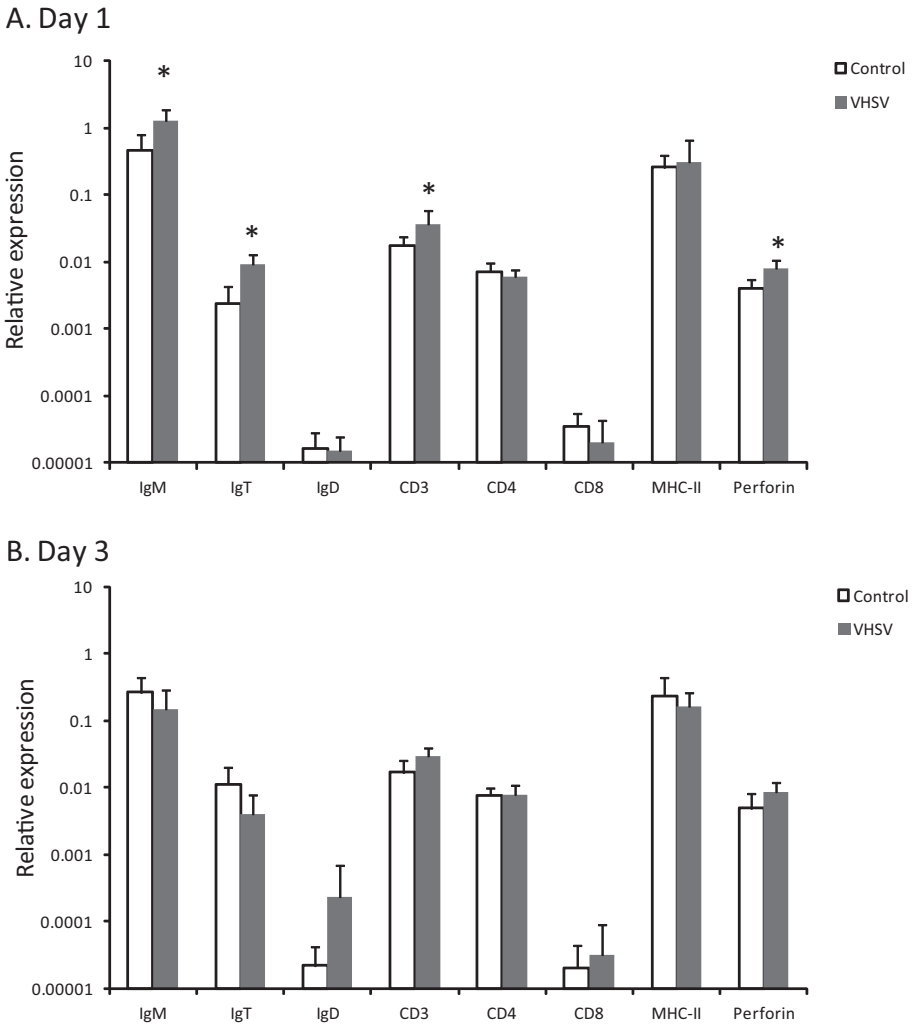

Fig. 1. Levels of transcription of immune genes characteristic of different leukocyte subpopulations in gills in response to VHSV. Rainbow trout were bath infected with VHSV (5×10^5 $TCID_{50}$/ml) or mock-infected. At days 1 (A) and 3 (B) post-infection six trout from each group were euthanized and the gills sampled to determine the levels of expression of a selection of immune genes by real-time PCR. Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± SD. *Levels of expression significantly different to those observed in mock-infected fish ($p < 0.05$).

3.4. Levels of transcription of immune genes in response to VHSV in DNA vaccinated fish

Additionally, we have studied the transcription of the genes that showed up-regulation in the gills in response to VHSV, in fish that had been previously injected with a VHSV DNA vaccine or injected with the empty plasmid. As a first step, we determined the levels of viral transcription in all gill samples at days 1 and 3 post-infection and compared them to the viral transcription levels in spleen. In unvaccinated fish, viral transcription was detected in the gills of 3 out of 6 individuals at day 1 and in 2 out of 6 at day 3 post-infection, while no viral transcription was detected in any of the infected fish that had been previously injected with the VHSV vaccine or with the vector backbone (Fig. 5A). However, viral transcription was detected in spleen samples from all infected groups both at day 1 and day 3, with non-significant differences among groups (Fig. 5B). Concerning marker genes for different leukocyte subsets, the virus up-regulated the transcription of IgM, IgT, CD3 and perforin at day 1 post-infection in non-vaccinated fish. However, this significant up-regulation was no longer observed in fish that had been injected with a VHSV DNA vaccine 30 days prior to the infection (Fig. 6). Surprisingly, the mRNA levels in response to VHSV in fish injected with the empty plasmid were also lower than mRNA levels in non-vaccinated VHSV-infected fish for all genes except IgT. Only for IgT, the transcription in response to the virus in VHSV DNA vaccinated fish was significantly lower than the transcription observed in response to VHSV in both control groups.

Concerning chemokine receptor genes, the virus significantly induced the transcription of CCR7, CCR9, CXCR3B and CXCR4 genes in non-vaccinated controls, whereas these up-regulations were no longer observed neither in fish injected with the empty plasmid or in fish injected with the VHSV DNA vaccine (Fig. 7). In this case, only CXCR3B was transcribed in virus-infected vaccinated fish at significantly lower levels than virus-infected fish that had been previously injected with the empty plasmid. In the other genes studied, the response observed in fish injected with the VHSV DNA vaccine was not significantly different to the one observed in fish that had been injected with an empty plasmid.

4. Discussion

Despite the importance of the gills in fish immunity to waterborne pathogens, not many studies have addressed the immune responses elicited in this tissue by viral infections (Grove et al., 2013; Montero et al., 2011). In the current work, following our previous report on chemokine modulation in the gills in response to VHSV (Montero et al., 2011), we have focused on studying what

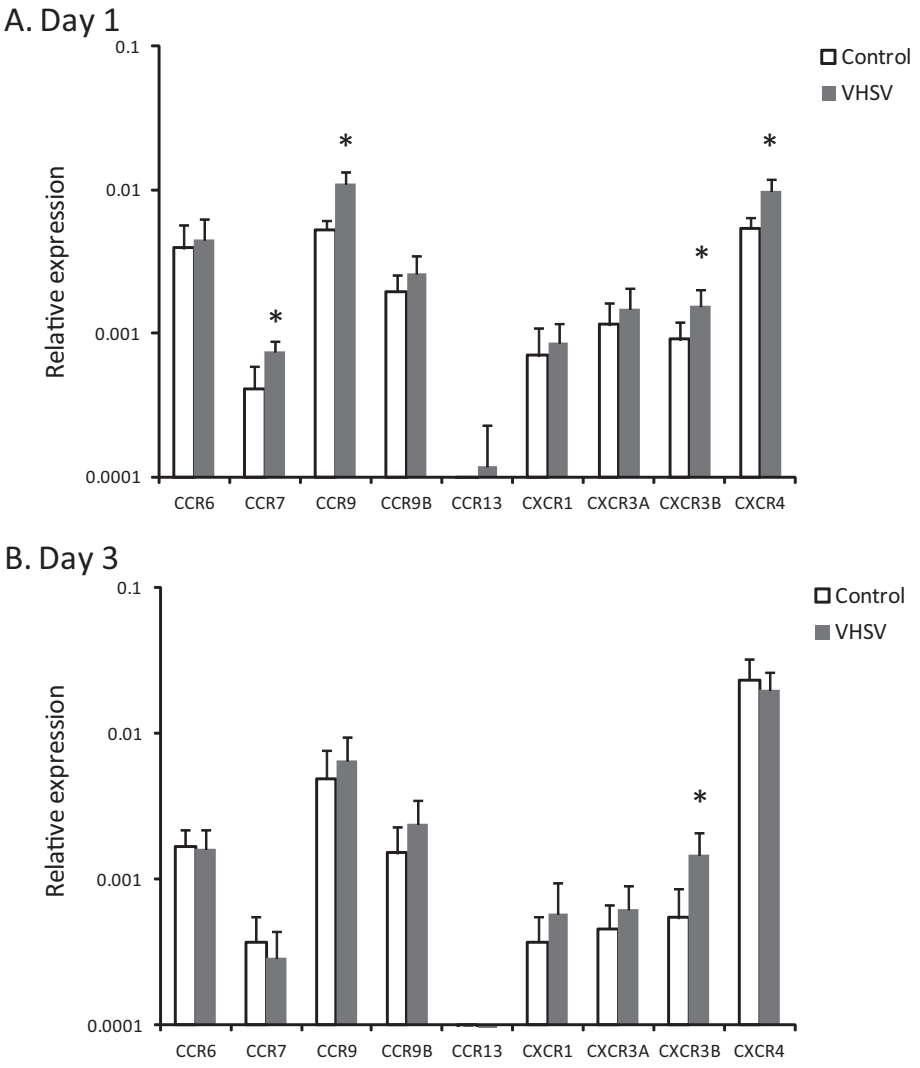

Fig. 2. Levels of transcription of chemokine receptor genes in gills in response to VHSV. Rainbow trout were bath infected with VHSV (5×10^5 $TCID_{50}$/ml) or mock-infected. At days 1 (A) and 3 (B) post-infection six trout from each group were euthanized and the gills sampled to determine the levels of expression of chemokine receptor genes by real-time PCR. Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± SD. *Levels of expression significantly different to those observed in mock-infected fish ($p < 0.05$).

chemokine receptor genes are modulated in response to VHSV exposure together with the analysis of marker genes specific for different leukocyte subsets.

Our results revealed that both IgM and IgT were up-regulated in response to the virus, indicating that at early time points post-infection, both IgM^+ and IgT^+ cells are involved in the antiviral response. The implication of IgM^+ cells, together with IgT^+ cells, in fish mucosal antiviral immunity has also been recently established in the digestive tract of rainbow trout after oral IPNV vaccination (Ballesteros et al., 2013). To further demonstrate if mucosal IgM^+ cells respond to the viral infection, we have sorted IgM^+ cells from gills of both infected and mock-infected fish and performed transcriptional studies. Even though IgM, IgD, CCR9 and CXCR4 transcription levels were not significantly modulated by VHSV, the virus provoked a significant up-regulation of MHC-II mRNA levels at all the time points studied. It is well known that B cells can be effective antigen presenting cells in mammals (Rodriguez-Pinto, 2005), and it has been speculated that this role might even be more important in fish since fish B cells are able to phagocytose and probably also to process and present antigens through MHC class II (Li et al., 2006; Zhang et al., 2009). Accordingly, our results strongly suggest a role of gill IgM^+ cells in antigen presentation. Additionally, VHSV provoked a significant down-regulation of CCR7 transcription at day 7 post-infection, but the biological significance of this effect is still unknown.

On the other hand, CD3 and perforin mRNA levels were also up-regulated in the gills in response to VHSV. Even though mRNA encoding CD8 remained almost undetectable in these samples, the increase in perforin suggests an implication of $CD8^+$ cells and maybe other cytotoxic cells such as NK cells in the early response. Thus, we separated $CD8^+$ cells from the gills of infected and mock-infected fish using flow sorting, and analyzed the levels of expression of relevant genes, including those up-regulated in complete gill tissues. Despite the fact that we could not detected an increased number of $CD8^+$ cells in the gills in response to the virus (data not shown), these cells significantly responded to the infection up-regulating perforin, CCR7 and CXCR4 mRNA levels. These results demonstrate that cytotoxic $CD8^+$ cells in the gills play a role in the early mucosal immune response to VHSV. The consequence for the up-regulation of the two chemokine receptors is still unknown. CCR7 has been shown to be regulated during mucosal responses in trout (Ordas et al., 2012), however, the chemokine ligands that signal through this receptor and their effect on the target cells are still unknown. In mammals, the effects of CCL19 and CCL21, the ligands for CCR7, have been widely studied in T cells (Moschovakis and Forster, 2012). Interestingly, it has been

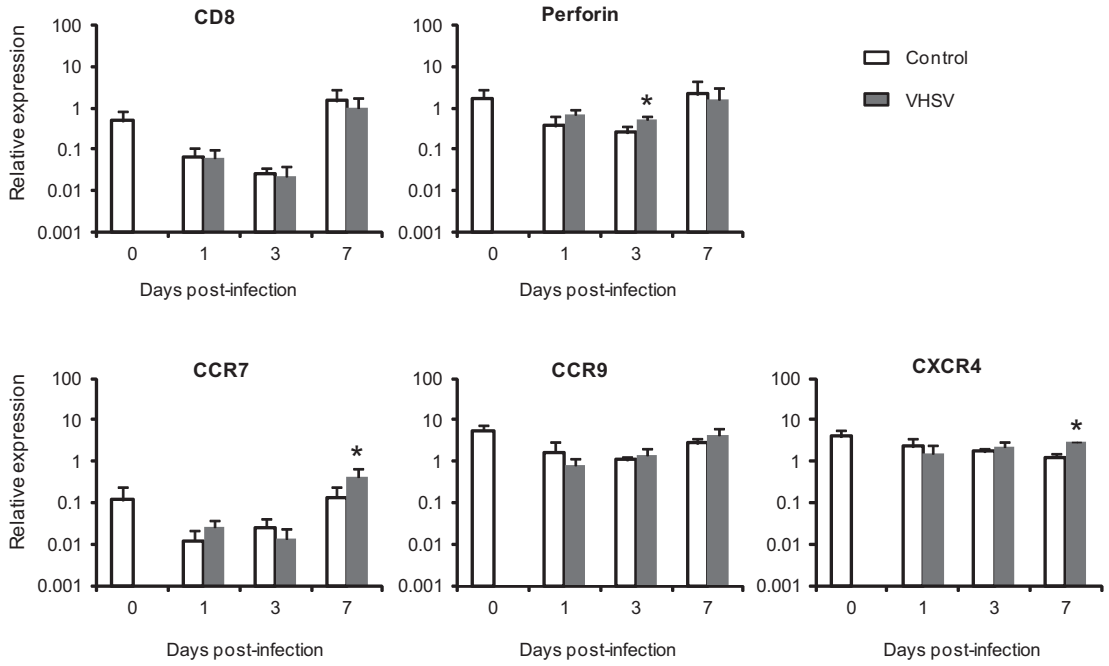

Fig. 3. Levels of transcription of different immune genes in sorted CD8$^+$ cells from control and infected fish. Fish were bath infected with VHSV or mock-infected. At different times post-infection, four fish in each group were euthanized and the gills sampled for leukocyte isolation. CD8$^+$ cells were sorted for RNA extraction and the transcription of immune genes determined through real time PCR in sorted cells ($N = 4$). Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± SD. *Levels of expression significantly different to those observed in mock-infected fish ($p < 0.05$).

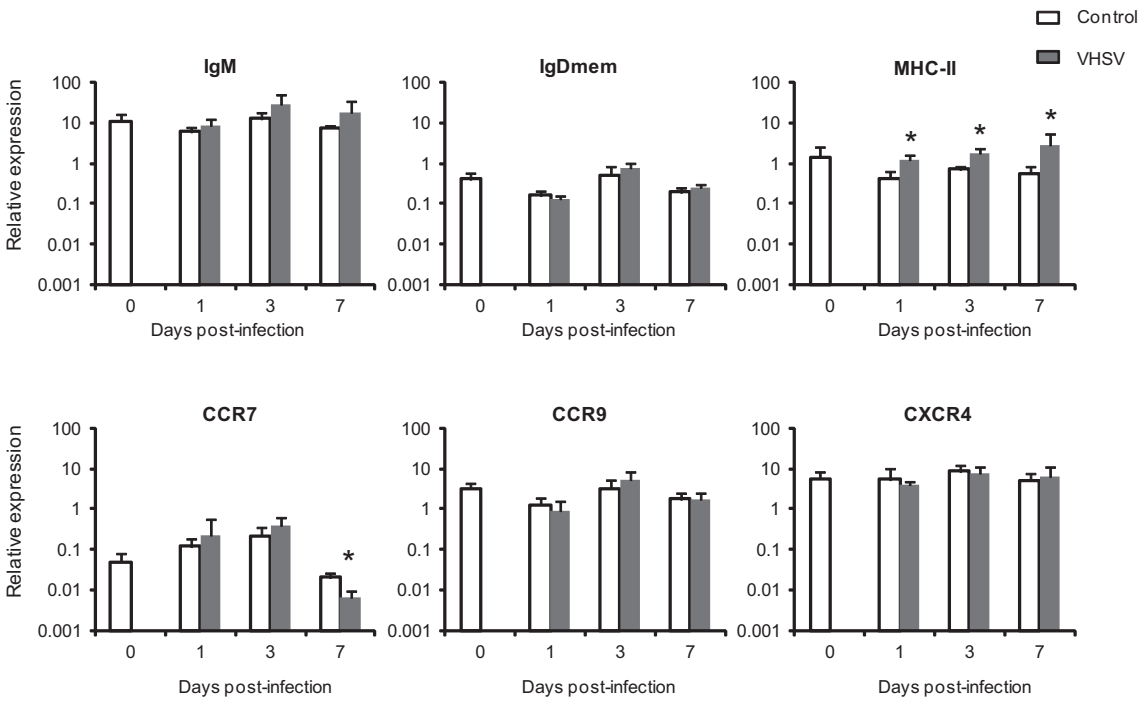

Fig. 4. Levels of transcription of different immune genes in sorted IgM$^+$ cells from control and infected fish. Fish were bath infected with VHSV or mock-infected. At different times post-infection, four fish in each group were euthanized and the gills sampled for leukocyte isolation. IgM$^+$ cells were sorted for RNA extraction and the transcription of immune genes determined through real time PCR in sorted cells ($N = 4$). Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± SD. *Levels of expression significantly different to those observed in mock-infected fish ($p < 0.05$).

demonstrated that in addition to effects on cell motility, these chemokines can produce diverse effects on CD8$^+$ T cells, such as promoting survival (Link et al., 2007), preventing them from apoptosis (Kim et al., 2005) or acting as a co-signal for cell expansion (Flanagan et al., 2004). On the other hand, CXCR4 in mammals is the receptor for CXCL12, a chemokine with diverse roles in inflammation and homeostasis (Karin, 2010). Interestingly, CXCL12 has been shown to activate CD8$^+$ T cells for the production of NO in humans (Choy et al., 2008), thus it would be interesting to determine the effects of CXCL12 on trout CD8$^+$ cells.

Finally, concerning those genes for which significant up-regulations were detected in response to VHSV bath infection,

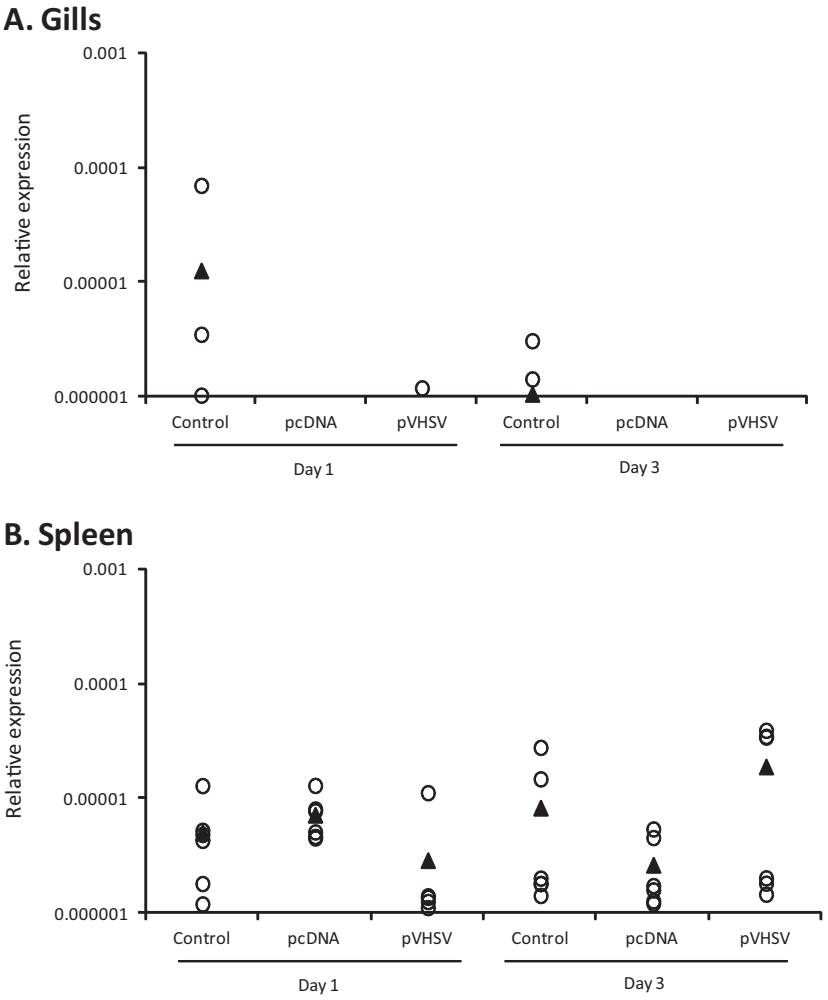

Fig. 5. VHSV N transcription in fish injected with pVHSV and then infected with VHSV. Rainbow trout were intramuscularly injected with a VHSV DNA vaccine (pVHSV), with an empty plasmid (pcDNA) or with PBS (Control). At day 30 post-vaccination, half of the trout in each group were bath infected with VHSV (5×10^5 $TCID_{50}$/ml) while half were mock-infected. At day 1 and 3 post-infection, six trout from each group were euthanized and the gills (A) and spleen (B) sampled to determine the levels of transcription of the VHSV N gene by real-time PCR. Data are shown as gene expression relative to the expression of endogenous control EF-1α obtained in individual fish (circles). Bars indicate mean values in each group.

transcriptional studies were also conducted in fish injected with a VHSV DNA vaccine and VHSV challenged. It has been already established that DNA vaccinated rainbow trout mount an effective immune response within one month at 16 °C (Lorenzen et al., 2000). Despite this, we observed no significant transcriptional differences between pVHSV-injected and subsequently VHSV infected fish and fish injected with the DNA vector backbone and virus challenged one day after challenge for most of the genes tested. The plasmid backbone of DNA vaccines is known to contain multiple CpG motifs that contribute to its immunogenicity (Coban et al., 2005), but the mechanisms through which it suppresses the viral responsiveness in gills should be further elucidated. However, this partial unresponsiveness in these two groups contrasted with the mRNA responses observed in unvaccinated fish that were exposed to viral bath challenge. These results suggest that both the injection of the plasmid DNA backbone and the DNA vaccine trigger mechanisms that suppress selected mRNA responses as well as virus replication in the gills early after bath infection with VHSV. On the other hand, viral transcription in the spleen was not affected by DNA injection and all infected groups showed an active viral transcription in the spleen. Only in the case of IgT and CXCR3B, the responses to the virus in VHSV-vaccinated fish were significantly different than the responses observed in fish injected with the empty plasmid, pointing to these two genes as important elements in mucosal immunity. IgT has been suggested as a main element in mucosal immunity (Zhang et al., 2010), whereas CXCR3B, the receptor for CXCL9, CXCL10, and CXCL11 (Clark-Lewis et al., 2003) is known to be absent in naïve B cells, present in a fraction of memory B cells expressing IgG and retained when these memory B cells differentiate into plasma cells (Muehlinghaus et al., 2005). In our studies, CXCR3B transcription was not detected in IgM^+ cells or $CD8^+$ cells, thus it might be possible that they precisely IgT^+ cells are the ones expressing CXCR3B. In mammals, it is expected that innate inflammatory responses are lower in response to a pathogen in vaccinated animals, and this seems to be the case in our studies, where once protection has been established through vaccination, the gills are rendered, at least partially, unresponsive to the virus. It might be possible, however, that different results are observed if we challenge fish with a lower viral dose or if we sample at later times post-infection, and this is something we should address in the future.

In summary, we have established that infection with VHSV up-regulates the transcription of IgM, IgT, CD3, perforin, CCR7, CCR9, CXCR3B and CXCR4 in the gills at early times post-infection. Specifically, perforin, CCR7 and CXCR4 were up-regulated in $CD8^+$ cells from infected fish, whereas MHC-II was up-regulated and CCR7 was down-modulated in sorted IgM^+ cells from infected. Surprisingly, these up-regulations were no longer observed when fish

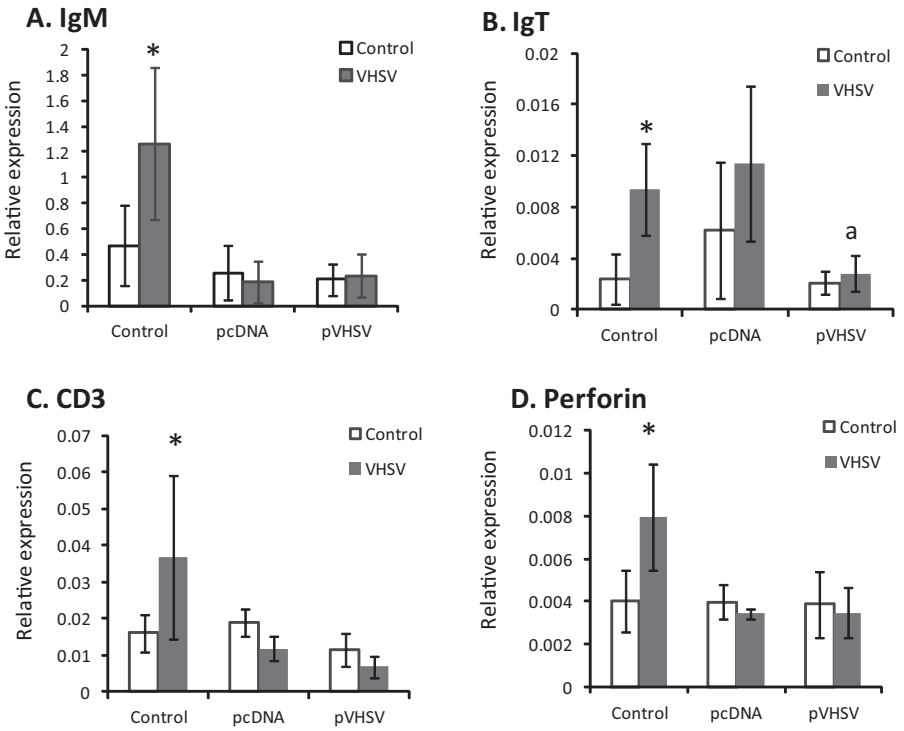

Fig. 6. Levels of transcription of immune genes characteristic of different leukocyte subpopulations in gills in response to VHSV after DNA vaccination. Rainbow trout were treated as described in the legend of Fig. 5 and gills sampled at day 1 post-infection. Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± SD.*Levels of expression in infected samples significantly different than corresponding mock-infected controls. An "a" denotes that values from virus-infected vaccinated fish are significantly different than values obtained in virus-infected fish that were previously injected with the empty plasmid or with PBS ($p < 0.05$).

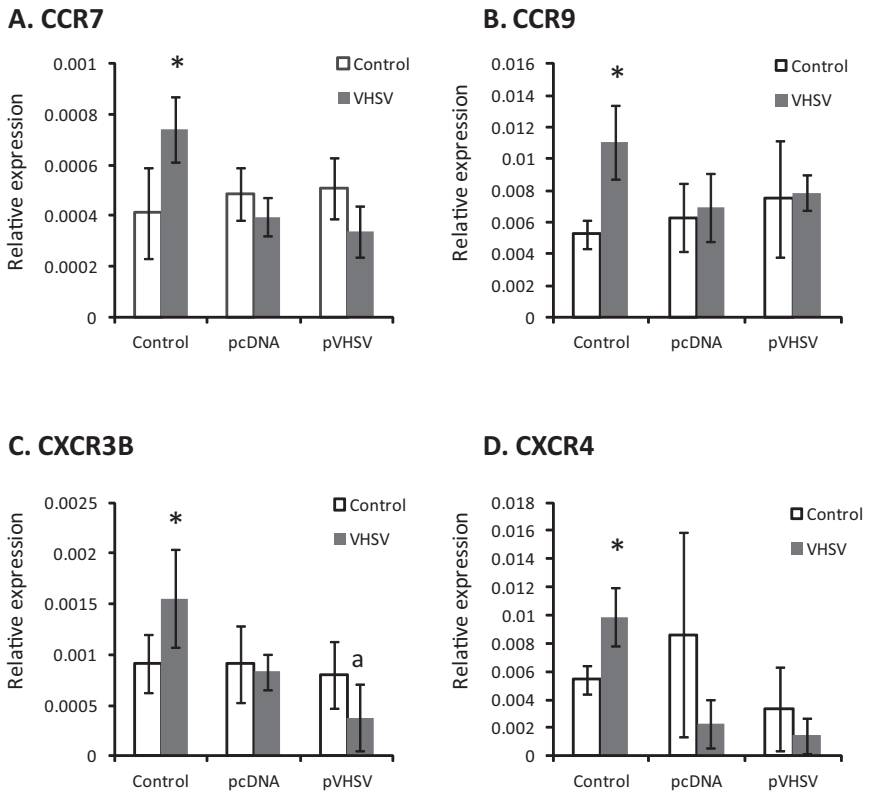

Fig. 7. Levels of transcription of chemokine receptor genes in gills in response to VHSV after DNA vaccination. Rainbow trout were treated as described in the legend of Fig. 5 and gills sampled at day 1 post-infection. Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± SD. *Levels of expression in infected samples significantly different than corresponding mock-infected controls. An "a" denotes that values from virus-infected vaccinated fish are significantly different than values obtained in virus-infected fish that were previously injected with the empty plasmid or with PBS ($p < 0.05$).

were injected with a plasmid DNA no matter if the DNA encoded for the VHSV glycoprotein or not. Finally, our data suggests that IgT and CXCR3B are important players in the mucosal response to VHSV.

Acknowledgements

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Full length article

Transcription analysis of two *Eomesodermin* genes in lymphocyte subsets of two teleost species

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ABSTRACT

Eomesodermin (Eomes), a T-box transcription factor, is a key molecule associated with function and differentiation of $CD8^+$ T cells and NK cells. Previously, two teleost *Eomes* genes (*Eomes-a* and *-b*), which are located on different chromosomes, were identified and shown to be expressed in zebrafish lymphocytes. For the present study, we identified these genes in rainbow trout and ginbuna crucian carp. Deduced Eomes-a and -b amino acid sequences in both fish species contain a highly conserved T-box DNA binding domain. In RT-PCR, both *Eomes* transcripts were readily detectable in a variety of tissues in rainbow trout and ginbuna. The high expression of *Eomes-a* and *-b* in brain and ovary suggests involvement in neurogenesis and oogenesis, respectively, while their expression in lymphoid tissues presumably is associated with immune functions. Investigation of separated lymphocyte populations from pronephros indicated that both *Eomes-a* and *-b* transcripts were few or absent in IgM^+ lymphocytes, while relatively abundant in $IgM^-/CD8\alpha^+$ and $IgM^-/CD8\alpha^-$ populations. Moreover, we sorted trout $CD8\alpha^+$ lymphocytes from mucosal and non-mucosal lymphoid tissues and compared the expression profiles of *Eomes-a* and *-b* with those of other T cell-related transcription factor genes (*GATA-3*, *T-bet* and *Runx3*), a Th1 cytokine gene (*IFN-γ*) and a Th2 cytokine gene (*IL-4/13A*). Interestingly, the tissue distribution of *Eomes-a/b*, *T-bet*, and *Runx3* versus *IFN-γ* transcripts did not reveal simple correlations, suggesting tissue-specific properties of $CD8\alpha^+$ lymphocytes and/or multiple modes that drive IFN-γ expressions.

1. Introduction

In mammals, a number of transcription factors involved in T cell maturation and differentiation have been identified. Th-POK and Runx3, for instance, regulate the commitment of common progenitor $CD4^+CD8^+$ double-positive cells to helper and cytotoxic lineages, respectively [1]. Naïve $CD8^+$ and $CD4^+$ T cells can further differentiate into effector cytotoxic T lymphocytes (CTL) and helper T (Th) cells, respectively, expressing lineage-specific transcription factors. The T-box transcription factor, Eomesodermin (Eomes), was originally discovered as a key molecule in *Xenopus* mesodermal development [2], and the involvement of Eomes in T cell functions was only later discovered in mammals [3]. Eomes controls the expression of cytolytic effector molecules and IFN-γ in orchestra with another T-box transcription factor T-bet, and both T-box transcription factors coordinately regulate the differentiation of $CD8^+$ T cells and NK cells [4,5]. In $CD4^+$ T cells, the lineage-specific transcription factors T-bet, GATA-3, RORγt, BCL-6 and Foxp3, control differentiation of Th1, Th2, Th17, T_{FH} and Treg cells, respectively [6].

Specific cell-mediated immunity, which is executed by $CD8^+$ CTL in mammals, has been studied in several teleost species, especially ginbuna crucian carp (*Carassius auratus langsdorfii*), channel catfish (*Ictalurus punctatus*) and rainbow trout

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(*Oncorhynchus mykiss*) [7–10]. The functional conservation of teleost CD8α^+ T cells has been reported in ginbuna by using monoclonal antibodies (mAbs) against CD8α. Ginbuna CD8α^+ T cells can execute alloantigen-specific perforin-mediated cytotoxicity [11–13], and an important role of CD8α^+ T cells in the graft-versus-host reaction (GVHR) was also shown in ginbuna [14]. From expression studies on the transcriptional level, it has been suggested that virus-specific cytotoxic activity can be mediated by CD8α^+ cells in both rainbow trout and ginbuna [15–18]. These findings indicate the presence of cytotoxic T lymphocytes (CTL) in fish similar to those of higher vertebrates. However, the mechanisms of differentiation and maturation of T cell subsets are still largely unknown although T cell-related genes such as TCR, CD3, CD4, CD8, CD28 and CTLA-4 have been identified in several fish species [19].

Around 350 million years ago a fish-specific whole genome duplication (FS-WGD) occurred [20], and some genes, situated as single copy in mammals, are represented by two copies in teleosts. Among T-brain1 subfamily genes, *T-brain1*, *Eomes* and *T-bet*, only *Eomes* was found represented by two loci in teleosts [21]. Eomes-a in zebrafish was shown to play an important role in the embryogenesis, as known for mammalian Eomes [22–26], but the precise function of teleost Eomes-b has not been investigated yet. Interestingly, zebrafish *Eomes-a* shows high expression levels throughout ontogeny, while the *Eomes-b* expression was found to increase gradually during embryonic development [27]. Moreover, the expression of both *Eomes-a* and *-b* was detected in immune cells, especially in lymphocytes [21]. Recently, it was revealed that overexpression of Atlantic salmon (*Salmo salar*) Eomes-a can induce *IFN-γ* and *granzyme A* transcripts [28]. In addition, a positive correlation of salmon *Eomes-a* expression with copy numbers of infectious pancreatic necrosis virus suggested the involvement of Eomes-a in immune responses to viral infection [29]. These observations imply the importance of Eomes-a and -b for the immune system as also known for mammalian Eomes.

To date aforementioned transcription factors associated to T cell development have been identified in several fish species, but the expression analysis in teleost T cell subsets remained to be investigated. In the present study, we have cloned two *Eomes* genes, *Eomes-a* and *-b*, from rainbow trout and ginbuna crucian carp, which serve as experimental models for teleost cell-mediated immunity. Expression analysis comparing several organs of rainbow trout and ginbuna showed that both *Eomes* genes are mainly transcribed in lymphoid organs. Moreover, the expression profiles of both *Eomes* genes as well as T cell-related transcription factors (*T-bet*, *Runx3* and *GATA-3*) and cytokines (*IFN-γ* and *IL-4/13A*) were examined in rainbow trout CD8α^+ lymphocytes from non-mucosal and mucosal lymphoid tissues, revealing inconsistencies between Th1 transcription factors (*T-bet*, *Eomes-a* and *-b*) and *IFN-γ* expression, suggesting that the CD8α^+ lymphocyte populations are distinct between individual tissues.

2. Materials and methods

2.1. Fish

Homozygous isogenic rainbow trout strain C25, established at Nagano Prefectural Experimental Station of Fisheries, Japan, and clonal triploid ginbuna crucian carp from Lake Suwa (S3N clone) were used for *Eomes-a* and *-b* sequence analysis. Rainbow trout strain "Born" obtained from the Landesforschungsanstalt für Landwirtschaft und Fischerei, Born, Germany, and clonal triploid ginbuna from the island of Okushiri (OB1 clone) were used for expression analysis. Rainbow trout were maintained in 400 l tanks at 15 °C in a partially recirculating water system and fed with commercial dry pellets. Ginbuna were maintained in a recirculation system with filtered water disinfected by ultraviolet light at 25 °C and fed pelleted dry food once daily.

2.2. cDNA preparations and sequencing of Eomes-a and -b in rainbow trout and ginbuna

Total RNA was extracted from splenocytes of rainbow trout and ginbuna by using NucleoSpin RNA II system (Macherey–Nagel) and RNeasy mini kit (Qiagen), respectively. One microgram of total RNA was reverse-transcribed into cDNA for 5′- and 3′-RACE PCR with a SMARTer RACE cDNA Amplification Kit (TAKARA BIO INC). PCR reactions for RACE and ORF cloning were performed with Phusion Hot Start High-Fidelity DNA Polymerase (New England BioLabs) according to the manufacturer's guidebook. Cloning and sequence analysis were performed as described in previous reports [30,31]. The sequence of primers for cDNA cloning and expression analysis is noted in Supplementary Tables 1 and 2. The Genbank accession numbers are as follows: rainbow trout *Eomes-a-1*, JF719911; rainbow trout *Eomes-a-2*, JF719912; rainbow trout *Eomes-b*, JF719913: ginbuna *Eomes-a-1*, JF719914; ginbuna *Eomes-a-2*, JF719915; and ginbuna *Eomes-b*, JF719916.

2.3. Expression analysis of two Eomes mRNAs in rainbow trout and ginbuna tissues

Rainbow trout tissues indicated in Fig. 2(A), Supplementary Figs. 3 and 4 were homogenized by using Tissue lyser (Qiagen), after which total RNA was extracted by NucleoSpin RNA II system (Macherey–Nagel). RT-PCR was performed with OneStep RT-PCR Kit (Qiagen), gene-specific primers (Supplementary Tables 1 and 2), and 10 ng total RNA per 12.5 μl reaction mixture according to the manufacturer's guidebook. Total RNA of ginbuna tissues indicated in Fig. 2(B) was extracted with RNAiso (Takara Bio Inc.). One μg of total RNA from the various tissues was reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression analysis by PCR with the specific primer sets was performed with BIOTAQ (Bioline).

2.4. Expression analysis of flow-sorted cells

Leukocyte preparation from rainbow trout and ginbuna is described in previous publications [30,32]. Single cell suspensions of rainbow trout pronephrocytes were incubated with a rat anti-CD8α mAb [32] and a combination of mAb 4C10 and N2 (mouse origin), that bind to IgM heavy chain [33] and light chain [34], respectively, for 30 min, followed by staining with FITC-conjugated goat anti-rat IgG (H + L) and R-PE-conjugated anti-mouse IgG subclass specific antibodies (Jackson ImmunoResearch) for 20 min, respectively. The stained leukocytes were separated into CD8α^+, surface IgM$^+$ (sIgM) and CD8α^-/sIgM$^-$ cells with a MoFlo high-speed cell sorter (Dako). Rainbow trout leukocytes from thymus, blood, pronephros, spleen and mucosal tissues were likewise stained with a rat anti-CD8α mAb and FITC-conjugated goat anti-rat IgG (H + L) and were subsequently sorted into CD8α^+ and CD8α^- lymphocytes using a MoFlo (DakaCytomation) high-speed sorter. Ginbuna pronephrocytes were separately stained with anti-IgM, anti-CD8α or anti-CD4-1 mAbs. After washing, cells were incubated with biotinylated anti-mouse IgG + M goat IgG (Jackson Immunoresearch) followed by staining with streptavidin-PE (Serotec). In ginbuna, separate single staining was necessary because fluorescence intensity of mAb$^+$ cells was not intense enough to properly discriminate more than one positively stained subpopulation in double staining. Six populations, sIgM, CD8α^+ and CD8α^- lymphocytes or CD4-1$^+$ and CD4-1$^-$ lymphocytes were

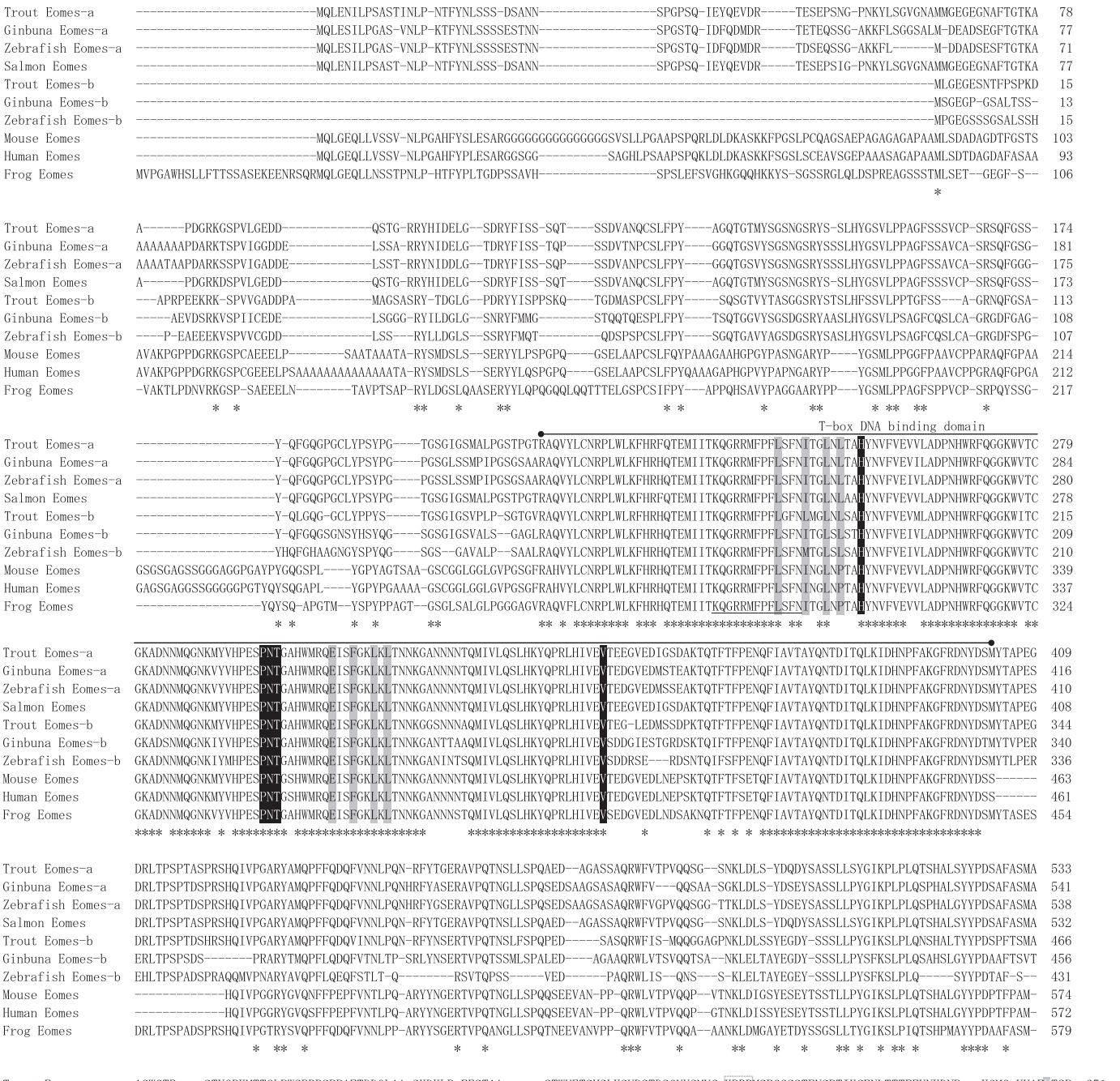

Fig. 1. Alignment of Eomes amino acid sequences. The solid lines above and below the alignment indicates predicted T-box DNA binding domains and NLSs, respectively. The black and gray shadings represent the conserved motifs for homodimerization and NES, respectively. Asterisks indicate positions that have a single, fully conserved residue.

sorted by using an EPICS ALTRA HyPerSort sorter (Beckman Coulter). After sorting of rainbow trout and ginbuna lymphocyte populations, total RNA was extracted from 30,000 and 10,000 cells of each cell population, respectively, by using RNeasy Plus Micro Kit (Qiagen). Conventional semi-quantitative RT-PCR was performed as described above. For gene expression analysis in sorted rainbow trout CD8α^{+} and CD8α^{-} lymphocytes, real-time RT-PCR was performed using the QuantiTect Probe RT-PCR Kit (Qiagen) and an

Mx3000P real-time PCR instrument plus analysis software (Stratagene), using gene-specific primers and probes (Supplementary Table 1). Target quantities were determined using a relative standard curve method with total RNA from gill or spleen. A normalized amount of target gene was calculated by dividing the amount of target gene by the amount of *EF-1α* as the endogenous house keeping control. Statistical significance was analyzed by unpaired Student's *t*-test with two-tailed distribution.

3. Results and discussion

3.1. Characterization of cDNA sequences of rainbow trout and ginbuna Eomes genes

Sequence data on the cloned full-length cDNAs of rainbow trout and ginbuna Eomes genes are depicted in Supplementary Fig. 1. Fig. 1 shows the amino acid sequences of *Eomes* genes in fish and other vertebrates. Both deduced Eomes proteins in rainbow trout and ginbuna contain a T-box DNA binding domain (Fig. 1; solid line above alignment). The amino acid sequences in T-box DNA binding domains are highly conserved among vertebrate Eomes while some substitutions and deletions in this domain were particularly found in Eomes-b. In contrast to the conservation of DNA binding domain, the identity of N- and C-terminus sequences is relatively low, and notably the deletion of the N-terminus which corresponds to the Eomes-a sequences from first to second methionine, was observed in Eomes-b. Amino acids involved in homodimerization of T-box genes [35] are fully conserved in teleost Eomes-a and -b (Fig. 1; black shading). Nuclear localization signals (NLS) have been identified in some T-box genes, and a typical NLS (K-K/R-x-K/R (x represents any amino acid): Fig. 1; boxed in dotted line) is also conserved among vertebrate Eomes [36]. These conserved amino acids suggest that both teleost Eomes proteins are homodimerized and localized in the cell nucleus. Interestingly, nuclear export signal (NES) with the consensus motif Φ-$(x)_{2\text{-}3}$-Φ-$(x)_{2\text{-}3}$-Φ-x-Φ, where Φ represents hydrophobic residues (L, I, F, V, M, P), was shown to play an important role in shifting Tbx5 between nuclear and cytoplasmic sites [37]. Two putative NESs were found in the T-box DNA binding domain of vertebrate Eomes (Fig. 2, gray shading). Although the initial position of the latter NES is occupied by a hydrophilic amino acid, glutamine acid, the former is composed with hydrophobic amino acids only. Moreover, in vertebrate T-bet and T-brain1, the homologous positions consist of hydrophobic amino acids, suggesting that the T-brain1 subfamily (T-brain1, T-bet and Eomes) utilizes former NESs for its export from the nucleus to the cytoplasm resulting in further signal transduction.

Intraspecies comparison of teleost Eomes-a with Eomes-b amino acids reveals 51.5–64.5% amino acid identities in their coding regions (Supplementary Table 3). Within teleost Eomes-a, the amino acid identities are 78.8–99.0%, while lower values of 56.8–67.4% were detected for Eomes-b. Similar lower identities were identified between teleost Eomes-a and Eomes-b (51.5–64.5%). The phylogenetic tree comprised of the T-brain1 subfamily shows that the intracluster distance of teleost Eomes-b is relatively large when compared to that of teleost Eomes-a (Supplementary Fig. 2). Despite the conserved synteny of the genomic region where teleost Eomes-b and other vertebrate Eomes locate [21], phylogenetic tree and aforementioned modifications of amino acid sequences suggest that teleost *Eomes-b* underwent a rapid evolutionary diversification after the duplication of teleost Eomes genes by FS-WGD around 350 million years ago.

3.2. Tissue distribution of two Eomes genes in rainbow trout and ginbuna

The tissue distribution of both *Eomes* transcripts of rainbow trout and ginbuna was examined by conventional RT-PCR (Fig. 2(A) and (B)). Rainbow trout and ginbuna brain tissues were found to express both *Eomes* transcripts at relatively high levels. In zebrafish and other vertebrates, *Eomes-a* and *Eomes* (also called *T-brain2*) as well as *T-brain1* are expressed during brain development [22,38], while the murine Eomes was shown to be involved in adult hippocampal neurogenesis [39]. These results suggest that both teleost Eomes take part in the neurogenesis not only of embryos but also of adults. In addition, both *Eomes* transcripts were expressed in ovary, corresponding to the *Eomes-a* expression in zebrafish oocytes [23].

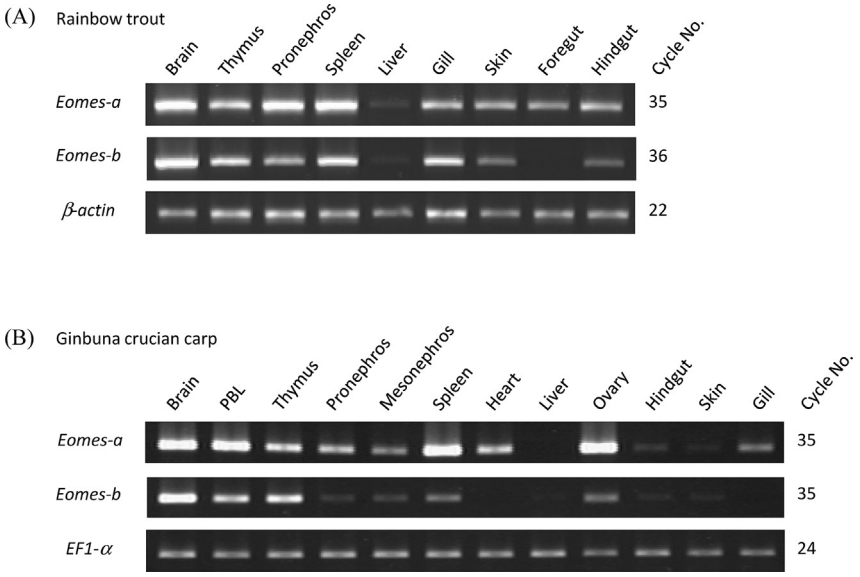

Fig. 2. Tissue distribution of *Eomes-a* and *Eomes-b* transcripts. Total RNA was extracted from rainbow trout and ginbuna tissues (indicated above the gels). (A) For expression analysis of rainbow trout tissues, extracted RNA was used for one-step RT-PCR with primer sets specific for trout *Eomes* genes. (B) For expression analysis of ginbuna tissues, the total RNA from indicated tissues was transcribed into cDNA, after which PCR was performed using primer sets specific for ginbuna *Eomes* genes. Rainbow trout *β-actin* and ginbuna *EF-1α* were used as internal controls, respectively. Data shown are representative of three independent fish.

However, in zebrafish ovary no *Eomes-b* transcripts were detected [21], while in trout (Supplementary Fig. 3) and ginbuna *Eomes-b* was still expressed but at lower levels than *Eomes-a*. Moreover, a different *Eomes-b* expression pattern from *Eomes-a* was shown during zebrafish embryogenesis [27]. Thus, their roles in oogenesis and embryogenesis may be distinct. In lymphoid organs, both *Eomes* transcripts of rainbow trout and ginbuna were readily detected. Rainbow trout *Eomes-a* in pronephros and spleen and ginbuna *Eomes-a* in PBL and spleen showed high expression levels similar to that in brain, while *Eomes-b* in both fish species was detected in lymphoid organs at lower levels when compared to that in brain. Although both *Eomes* isoforms were expressed in mucosal tissues from rainbow trout and ginbuna, their expression levels were relatively low compared to lymphoid organs.

3.3. Expression of teleost master transcription factor in lymphocyte subset shows similarities to mammals

Recently established antibodies reactive to teleost T cell co-receptors CD4-1 (ginbuna) and CD8α (ginbuna and rainbow trout) enabled us to discriminate and isolate teleost T cell subsets [32,40]. To examine the expression of the T cell-related transcription factors (*Eomes-a*, *Eomes-b*, *T-bet* and *GATA-3*) in lymphocyte subsets, rainbow trout pronephrocytes were simultaneously stained with anti-IgM and anti-CD8α mAbs, while ginbuna pronephrocytes were separately stained with anti-IgM, anti-CD8α and anti-CD4-1 mAb. Fig. 3 shows the expression analysis with templates from sorted lymphocyte subpopulations. A high sorting purity was confirmed by post-sorting flow cytometry (higher than 95%, data not shown) and PCR analysis with primer sets specific to the respective cell marker genes, *IgM*, *CD8a* and *CD4* (Fig. 3(A) and (B)). For rainbow trout *IgM* detection, we used a reverse primer which is specific to the transmembrane region of *IgM heavy chain*. Nevertheless, a few *IgM* transcripts were found in CD8α$^-$/sIgM$^-$ lymphocytes. This may be attributed to other sIgM$^+$ B cells, e.g. mAb 1.14$^+$ B cells [41] or sIgM^{low} or sIgM$^-$ B cells which would be homologous precursors to mammalian small pre-BII cells [42]. However, we describe mAb 4C10/N2-positive and -negative lymphocytes as sIgM$^+$ and sIgM$^-$ in this paper, respectively.

In both fish, all T cell-related transcription factors examined were predominantly expressed in sIgM$^-$ lymphocytes while low levels of *T-bet* transcripts were detected in sIgM$^+$ lymphocytes, similar to our previous observations [30,43]. In rainbow trout, two *Eomes* and *T-bet* transcripts were not only expressed in CD8α$^+$ lymphocytes but also in CD8α$^-$/sIgM$^-$ lymphocytes, while *GATA-3* was mainly detected in CD8α$^-$/sIgM$^-$ lymphocytes (while being expressed in CD8α$^+$ lymphocytes at low levels; Fig 3(A)). In ginbuna, both *Eomes* transcripts were found in CD8α$^+$ and CD8α$^-$ lymphocytes at similar expression levels, but predominantly in CD4-1$^-$ and not in CD4-1$^+$ lymphocytes. In contrast, ginbuna *GATA-3* was mainly expressed in lymphocytes other than sIgM$^+$ and CD8α$^+$. In comparison to CD8α$^-$ and CD4-1$^-$ lymphocytes, ginbuna *T-bet* expression levels were somewhat higher in CD8α$^+$ and CD4-1$^+$ lymphocytes. In mammals, both T-box transcription factors Eomes and T-bet are important for IFN-γ production by CD8$^+$ T cells, and also T-bet has essential functions as transactivator of IFN-γ in Th1 cells [44]. Mammalian GATA-3 is mainly expressed in CD4$^+$ T cells, especially in Th2 cells, to produce Th2 cytokines, IL-4, IL-5 and IL-13 [45]. In our analysis, these transcription factors were predominantly expressed in T cells, but not sIgM$^+$ B cells, suggesting their involvement in T cell function. Notably, CD8α$^+$ lymphocytes from both species strongly expressed *T-bet*, *Eomes-a* and *-b*, whilst ginbuna CD4-1$^+$ and rainbow trout CD8α$^-$/sIgM$^-$ lymphocytes (enriched in CD4$^+$ cells) expressed *T-bet* and *GATA-3* (Figs. 3 and 4). Therefore, teleost T cell-related transcription factors appear to show expression pattern resembling mammals.

Lower expressions of teleost *Eomes*, *T-bet* and *GATA-3* were found in CD8α$^-$, sIgM$^+$ and CD8α$^+$ lymphocytes, respectively. Mammalian Eomes and T-bet contribute to NK cell effector function and development [5,46]. In addition, it was suggested that Eomes and GATA-3 expressions are involved in optimal IFN-γ production of Th1 cells [47,48] and in Th2 cytokine production (IL-4 and IL-5) of Tc2 cells [49], respectively. Therefore, teleost Eomes expression in CD8α$^-$ lymphocytes may be attributed to NK and/or CD4$^+$ cells. Interestingly, carp *T-bet* and *IFN-γ1* were up-regulated in IgM$^+$ cells

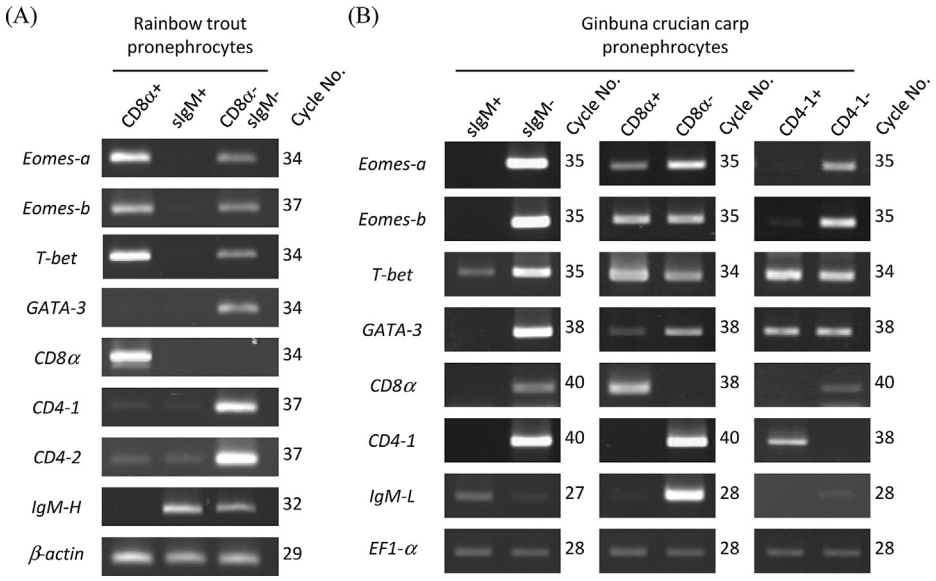

Fig. 3. Expression analysis of *Eomes-a*, *Eomes-b*, *T-bet* and *GATA-3* transcripts in sorted lymphocytes. Pronephrocytes stained with the antibodies reactive to the markers indicated above the gels were sorted into respective antibody positive and negative populations. Total RNA was extracted from sorted lymphocytes. (A) For expression analysis of sorted cells from rainbow trout, extracted RNA was used for one-step RT-PCR with specific primers for the indicated genes. (B) For expression analysis of sorted cells from ginbuna, total RNA was transcribed into cDNA, after which PCR was performed with the cDNA and specific primers for indicated genes. Data shown are representative of three independent fish.

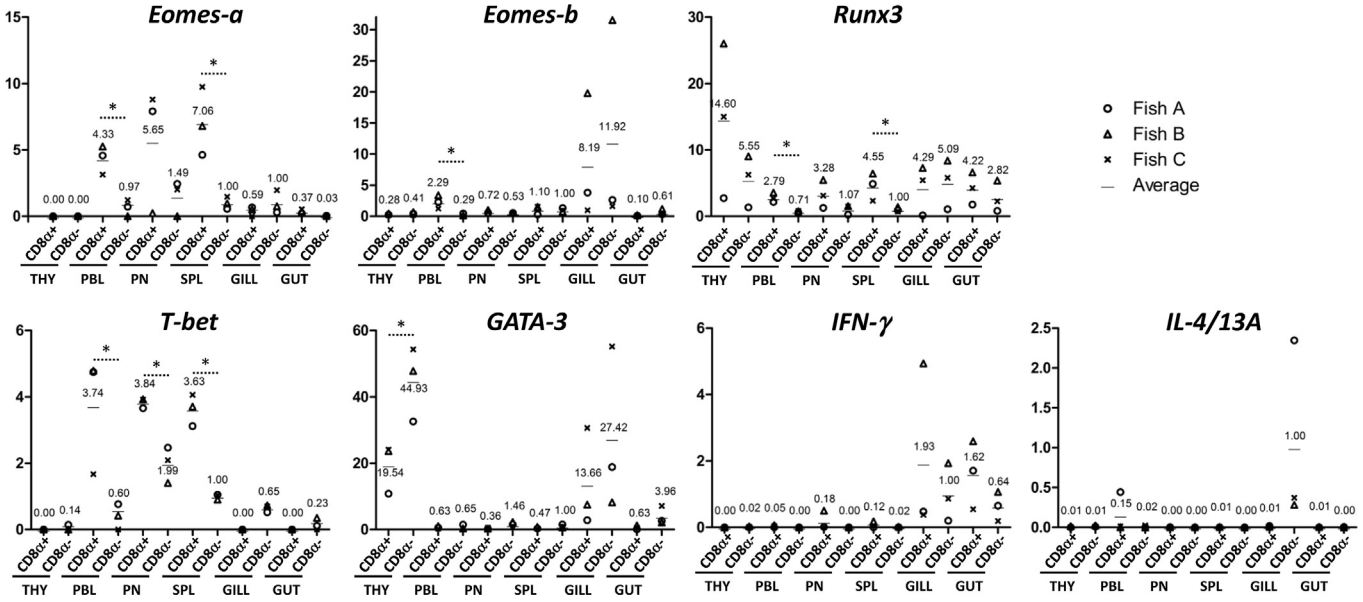

Fig. 4. Expression profile of transcription factors and cytokines in flow-sorted rainbow trout CD8α^+ and CD8α^- lymphocytes from blood, lymphoid and mucosal tissues. Rainbow trout leukocytes from thymus (THY), blood (PBL), pronephros (PN), spleen (SPL), gill (GILL) and intestine (GUT) were stained with anti-CD8α mAb and FITC-conjugated goat anti-rat IgG, and then were sorted into CD8α^+ and CD8α^- lymphocytes. RNA was extracted from both populations and used for one-step real-time PCR with primers for the genes of transcription factors (*Eomes-a*, *Eomes-b*, *T-bet*, *GATA-3* and *Runx3*) and cytokines (*IFN-γ* and *IL-4/13A*). Expression levels of transcription factors and cytokines are shown relative to the expression by CD8α^- lymphocytes in spleen and gill, respectively (set to 1). The values in each column (bar) are the average generated from the expression levels of three pooled samples while each pool was prepared from cells of three individuals. Respective symbols (circle, cross and triangle) represent the values from each pooled sample. The mean values of gene expression levels between CD8α^+ and CD8α^- lymphocytes in each tissue were statistically compared by an unpaired Student's *t*-test, and asterisk represent a *P*-value of <0.05 which was considered statistically significant.

by LPS stimulation [50]. Thus, T-bet expression in rainbow trout and ginbuna B cells seems consistent with situation of carp T-bet, suggesting the important role of T-bet in IFN-γ production in teleost B cells.

3.4. T-box transcription factor and IFN-γ in CD8α$^+$ lymphocytes are differentially expressed between rainbow trout non-mucosal and mucosal tissues

In the experiments on tissue distribution, we found both *Eomes* and *T-bet* transcripts expressed at low levels in mucosal tissues (Fig.2, Supplementary Fig. 4) [51]. However, in addition to spleen high levels of *IFN-γ* expression, which is transactivated by T-bet and Eomes in mammals, were detected in trout mucosal tissues too [52,53], and in salmonids, high levels of CD3ε$^+$ and CD8α$^+$ T cells have been shown in mucosal tissues [31,54,55]. These observations seem to be discordant in their association. However, *IFN-γ* and *T-bet* in rainbow trout pronephros were found to be simultaneously increased after *Tetracapsuloides bryosalmonae* infection [51], and salmon splenocytes stimulated with a T cell activator induced *Eomes-a* and *IFN-γ* expression [28]. In addition, overexpression of *Eomes-a* in Atlantic salmon cells resulted in *IFN-γ* up-regulation in splenocytes [28]. Therefore, we wonder whether this discrepancy can be explained by different mRNA expression patterns between tissues and single cell leukocyte suspensions which are often observed due to the composite population of cell types in each organ. Therefore, to determine the expression levels of these genes in leukocytes, we sorted equal numbers of CD8α$^+$ and CD8α$^-$ lymphocytes from rainbow trout lymphoid organs and analyzed the expression profile of T cell-related transcription factors and cytokines (*IFN-γ* and *IL-4/13A*) with real-time (Fig. 4) and conventional RT-PCR (Supplementary Fig. 5).

Corresponding to the observations on tissue distribution, the expression of rainbow trout *Eomes-a* and *T-bet* was much stronger in CD8α$^+$ lymphocytes from blood, pronephros and spleen than in those from mucosal tissues, while *IFN-γ* transcripts were found to be higher in mucosal CD8α$^+$ and CD8α$^-$ lymphocytes than in non-mucosal ones. In contrast, although CD8α$^+$ lymphocytes from blood constitutively expressed higher *Eomes-b* transcripts than CD8α$^-$ lymphocytes, *Eomes-b* was expressed at same or even lower expression levels in CD8α$^+$ lymphocytes from other tissues when compared to those in CD8α$^-$ lymphocytes. However, this obvious *IFN-γ* expression in mucosal lymphocytes is different from a paper described by Komatsu et al. (2009) [56], where no *IFN-γ* expression was recorded in isolated intraepithelial cells, and this discrepancy may be due to different compositions of intraepithelial lymphocytes (IELs) in the samples. In humans, some studies showed that a small proportion of CD8$^+$ lymphocytes in lung and intestinal spontaneously secrete IFN-γ [57–59]. In addition, resident CD8$^+$ cells in human intestine and lung further induce expression of IFN-γ upon stimulation [60,61]. Similarly, murine CD8$^+$ IELs, but not splenocytes, spontaneously secrete IFN-γ [62 and can rapidly synthesize IFN-γ through CD3-mediated signaling [63]. Importantly, murine IFN-γ serves as a regulatory factor in intestinal homeostasis and inflammation [64]. Therefore, the high expression levels of *IFN-γ* in trout mucosal lymphocytes suggest that mucosal CD8α$^+$ lymphocytes can spontaneously induce IFN-γ transcription using signal pathways independent of Eomes-a and T-bet.

3.5. Runx3 is a possible transactivator of IFN-γ in mucosal lymphocytes

In mammals, induction of CD8$^+$ T cells requires T-bet and Eomes expression in early and late developmental stage, respectively [44,65], while IFN-γ production by CD4$^+$ Th1 cells is regulated by two major signal pathways, IL-12-STAT4 and IFN-γ/IL27-STAT1-T-bet transduction [6], and for IFN-γ. In addition to these pathways, Runt-related transcription factor 3 (Runx3), which in thymus

silences CD4 transcripts to commit thymocytes toward CD8 single positive T cells, has been shown to play a critical role in IFN-γ transactivation in both Th1 and $CD8^+$ T cells [65–67]. In teleosts, it was shown that zebrafish Runx3 is involved in regulation of embryonic hematopoiesis [68] and that pufferfish *Runx3* is expressed in mucosal tissues at higher levels than in spleen and kidney [69]. Therefore, we examined how rainbow trout *Runx3* is expressed in lymphoid tissues and leukocytes. As shown by conventional RT-PCR, *Runx3* was strongly expressed in thymus, followed by other lymphoid and mucosal tissues (Supplementary Fig. 4). Similarly the *Runx3* transcripts were detected at similar levels in mucosal and non-mucosal $CD8\alpha^+$ lymphocytes while highest expression levels were observed in $CD8\alpha^+$ thymocytes (Fig. 4, Supplementary Fig. 5). In addition, mucosal $CD8\alpha^-$ lymphocytes expressed higher levels of *Runx3* than non-mucosal ones. The *Runx3* expression patterns in mucosal tissues, unlike the expression patterns of *T-bet* and *Eomes-a*, suggests that teleost Runx3 plays important roles in IFN-γ production by $CD8\alpha^+$ lymphocytes originating from non-mucosal as well as from mucosal tissues. However, the detailed mechanism of these transcription factors in teleost fish remains to be analyzed.

3.6. Gill $CD8\alpha^-$ lymphocytes from rainbow trout highly express the Th2-related genes, GATA-3 and IL-4/13A

Rainbow trout Th2 master transcription factor *GATA-3* was mainly expressed in mucosal $CD8\alpha^-$ lymphocytes (Fig. 4, Supplementary Fig. 5). Moreover, as recently reported [53], gill $CD8\alpha^-$ lymphocytes show high *IL-4/13A* transcripts, indicative of Th2 skewing of gill environment. Notably, $CD8\alpha^+$ lymphocytes from gill were likewise found to express *GATA-3* transcripts at relatively high levels when compared to those from other tissues. Mammalian $CD8^+$ Tc2 cells can produce Th2-type cytokines through a GATA-3 signaling pathway [49,70]. Therefore, teleost $CD8\alpha^+$ lymphocytes, especially in gill, may have the competence to produce Th2 cytokines in combination with GATA-3 expression although only negligible *IL-4/13A* expression levels were detected in rainbow trout naïve $CD8\alpha^+$ lymphocytes.

3.7. Rainbow trout Runx3 and GATA-3 are highly expressed in both $CD8\alpha^+$ and $CD8\alpha^-$ thymocytes

In thymus, both $CD8\alpha^+$ and $CD8\alpha^-$ lymphocytes showed highest transcript levels of *Runx3* and *GATA-3* while only a low transcript levels of *Eomes-a*, *Eomes-b* and *T-bet* were detected. GATA-3 and Runx3 are critical transcription factors for CD4/CD8 lineage selection in mammalian thymus, respectively [71]. Recently, the existence of $CD4^+CD8\alpha^+$ double-positive thymocytes has been proven in ginbuna on the protein level, suggesting similar developmental stages of T cells as in mammals [40]. That those double-positive thymocytes do likely exist in rainbow trout too was suggested after the finding that $CD8\alpha^+$ thymocytes express *CD4* genes [32]. Therefore, transcription factors Runx3 and GATA-3 appear to be essential in the development of teleost T cells.

4. Conclusions

The present study identified two *Eomes* cDNAs each in rainbow trout and ginbuna. Moreover, their mRNA expression as along with the expression of T cell-related transcription factors and cytokines in different lymphocyte subsets has been determined. The expression patterns of these transcription factors in pronephric T cell subsets were found to be similar to those of mammals, suggesting their involvement in development of teleost T cell subsets. In spite of many other similarities between mammalian and teleost immune systems, it has been discovered recently that the proportions of rainbow trout T and B cells in blood and lymphoid tissues is significantly different from those in mammals [53,72]. Moreover, we were able to demonstrate differences between non-mucosal and mucosal tissues not only in terms of $CD8\alpha^+$ lymphocyte distribution, but also of gene expression profiles regarding the cell surface markers, such as *TCRγ*, immunosuppressive receptors and integrins [32]. The data presented here further showed distinct expression patterns of transcription factors between non-mucosal and mucosal $CD8\alpha^+$ lymphocytes. Rather surprisingly, both $CD8\alpha^+$ and $CD8\alpha^-$ mucosal lymphocytes displayed high amounts of *IFN-γ* transcripts, while *IL-4/13A* expression was rather restricted to $CD8\alpha^-$ gill cells (probably $CD4^+$ cells). These observations imply that the lymphocytes in certain tissues have specialized characteristics, resulting in a unique immune response at that site, and that *IFN-γ* and *IL-4/13A* seem to keep homeostasis in mucosal tissues.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.11.004.

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Constitutive high expression of *interleukin-4/13A* and *GATA-3* in gill and skin of salmonid fishes suggests that these tissues form Th2-skewed immune environments

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ABSTRACT

Rainbow trout and Atlantic salmon *interleukin-4/13A* (*IL-4/13A*) genes were identified. They were found expressed at high level in thymus, gill, and skin, in concert with the transcription factor gene *GATA-3*. High expression levels of *IL-4*, *IL-13*, and *GATA-3* were also detected in murine thymus, suggesting similar importance of the fish and mammalian homologues for early T cell development. In mammals, combined high expression of IL-4/13 and GATA-3 in tissues other than thymus is mostly indicative of Th2 responses. Th2-skewage may protect fish skin and gill from parasites and from damage by inflammatory Th1 and Th17 responses. The immune milieus of fish gill and skin are relevant to aquaculture, because these tissues are preferred sites for vaccine administration. The similarities between the immune milieus of fish gill and thymus may reflect an evolutionary relationship, since these tissues map close together lining the gill cavity. Expression patterns of *IL-4/13A* and *interferon gamma* (*IFN-γ*) in isolated trout gill cells and pronephrocytes were consistent with Th2 identity of IL-4/13A.

1. Introduction

IL-4 and IL-13 are closely related cytokines important for Th2 responses (for reviews see Wynn, 2003; Nakajima and Takatsu, 2007; Paul and Zhu, 2010). Th2 responses are especially important for defense against parasites, while also involved in various allergies. The tandemly organized *IL-4* and *IL-13* genes map to human chromosome 5, and the IL-4/13 molecules form a subfamily of the short chain type I cytokines (Rozwarski et al., 1994; Ohtani et al., 2008). Both IL-4 and IL-13 can signal through the receptor IL-13R (IL-4Rα/IL-13Rα1), whereas the receptors IL-4R (IL-4Rα/IL-2Rγ) and IL-13R decoy (IL-13Rα2) specifically interact with IL-4 and IL-13, respectively. Intracellular IL-4R and IL-13R signaling involves signal transducer and activator of transcription 6 (STAT6) protein. IL-4 and IL-13 can stimulate B cell proliferation, regulate B cell class switching to IgE, induce epithelial cells to produce mucus, up-regulate VCAM-1 expression on endothelial cells, and stimulate macrophages into an anti-parasite mode (Martinez et al., 2009). IL-4 and IL-13 are strongly expressed by Th2 cells, and while IL-4 promotes Th2 development (positive feedback loop) it suppresses the development of Th1 and Th17 cells (Ansel et al., 2006; Zhu et al., 2009). A master regulator of Th2 responses is the transcription factor GATA binding protein 3 (GATA-3), which enhances transcription of the genomically clustered Th2 cytokine genes *IL-4*, *IL-13*, and *IL-5* (Ansel et al., 2006). In addition to T cells, IL-4 and IL-13 can also be expressed by NKT cells, mast cells, basophils, eosinophils, B cells, and some other leukocyte populations (e.g., Coffman, 2010); however, the present article will simply refer to IL-4/13-rich environments as "Th2-skewed".

In fish, Th2 responses have not been clearly shown, but genes of the *IL-4/13* family (Li et al., 2007; Ohtani et al., 2008; Mitra et al., 2010), *GATA-3* (Neave et al., 1995; Takizawa et al., 2008; Kumari et al., 2009; Wang et al., 2010), *IL-4Rα*, *IL-13Rα1*, *IL-13Rα2* (Lockyer et al., 2001; Liongue and Ward, 2007), and *STAT6* (Guo et al., 2009; Mitra et al., 2010) have been found. Fish *IL-5* has not been properly identified yet (Ohtani et al., 2008). Teleost fish have two genes of the *IL-4/13* family, *IL-4/13A* and *IL-4/13B*, which are situated on separate chromosomes in regions that duplicated during the fish-specific whole genome duplication (FS-WGD) around 350 million years ago (Jaillon et al., 2004; Ohtani et al., 2008). IL-4 and IL-13 in tetrapod species share a cysteine bridge (Powers et al., 1993; Eisenmesser et al., 2001) which is lacking in deduced fish IL-4/13A and IL-4/13B,

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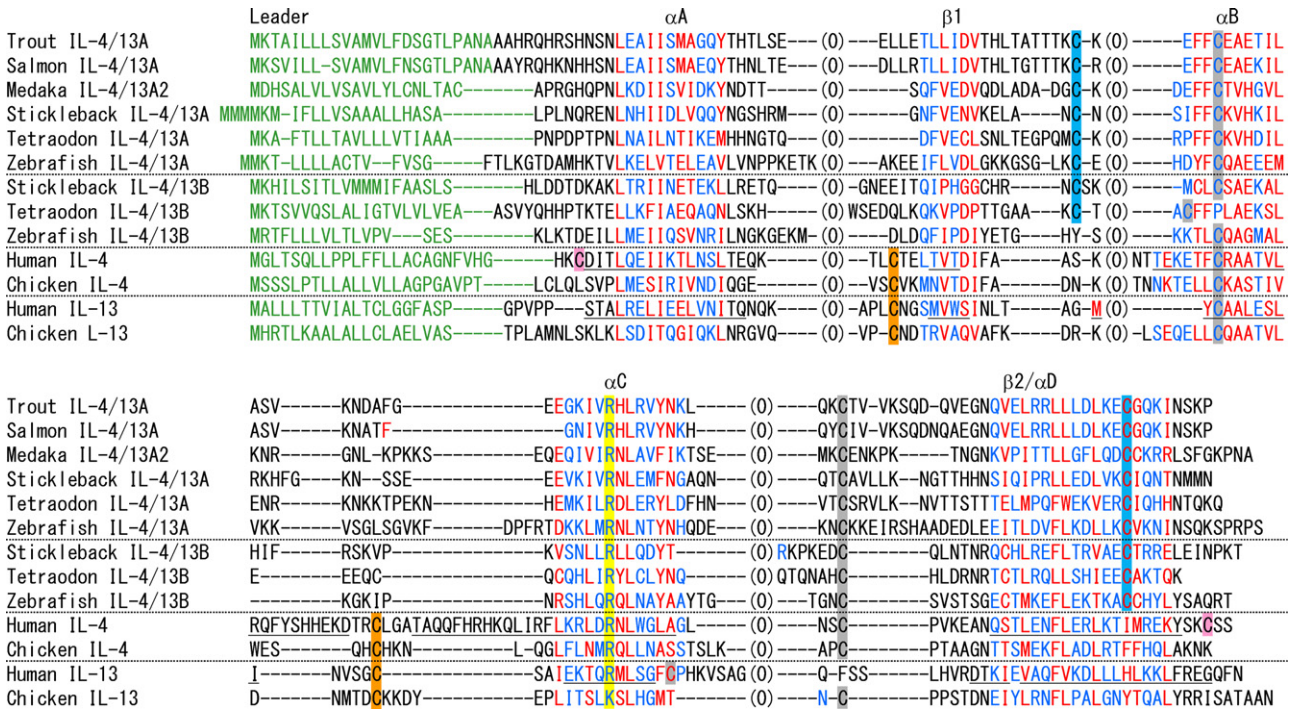

Fig. 1. Alignment of IL-4/13 cytokine amino acid sequences. Underlines indicate α-helix (αA-αD) and β-strand regions (β1 and β2) of human IL-4 (Powers et al., 1993) and IL-13 (Eisenmesser et al., 2001); the β-strand in the IL-4 CD loop comprises the residues QST. Predicted leader sequences are in green; blue and red fonts are explained in the manuscript text. Parentheses relate to intron positions with numbers indicating the intron phase (assumed for salmon IL-4/13A). The yellow-shaded basic residue in α-helix C is important for binding of human IL-4 to IL-4Rα (Hage et al., 1999). Cysteine pairs known, or expected, to form disulfide bonds are distinguished by color shading.

while the fish molecules share another cysteine pair which is absent in the mammalian molecules (Fig. 1; Ohtani et al., 2008). Therefore, the most likely evolutionary scenario is that fish *IL-4/13A* and *IL-4/13B* duplicated as part of the FS-WGD, and that tetrapod *IL-4* and *IL-13* derived from a tandem gene duplication within the tetrapod lineage (Ohtani et al., 2008).

A few functional studies on teleost *IL-4/13A* and *IL-4/13B* have been reported. Tetraodon *IL-4/13A* expression was up-regulated after stimulation with ConA/PMA/PHA (Li et al., 2007), T cell enriched Fugu PBL were found to express more *IL-4/13A* and *IL-4/13B* after stimulation with recombinant B7 molecules (Sugamata et al., 2009), recombinant zebrafish IL4/13B was shown to increase the number of IgT-positive and CD209-positive cells in blood (Lin et al., 2009; Hu et al., 2010), and in zebrafish spleen the expression of *IL-4/13B*, *GATA-3*, and *STAT6* was simultaneously enhanced after PHA stimulation (Mitra et al., 2010).

In the present study we report the identification of *IL-4/13A* genes in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). The tissue distribution of salmonid *IL-4/13A* and *GATA-3* expression was compared with the expression of *IL-4*, *IL-13*, and *GATA-3* in mouse. High levels of these transcripts were found in both salmonid and murine thymus, while constitutive *IL-4/13A*-richness of skin and respiratory tissue was found in salmonids but not in mouse. Experiments with isolated cells from gill and pronephros (head kidney) indicated that trout *IL-4/13A* is mainly expressed by surface IgM-negative cells, readily inducible by PHA but not by poly I:C, and regulated differently from the Th1 cytokine gene *IFN-γ*.

2. Materials and methods

2.1. Animals

Homozygous isogenic rainbow trout strain C25, established at Nagano Prefectural Experimental Station of Fisheries, Japan (Dijkstra et al., 2007), was used for *IL-4/13A* sequence analysis. Rainbow trout strains "Born" and "Steelhead" were obtained from the Landesforschungsanstalt für Landwirtschaft und Fischerei, Born, Germany (Verleih et al., 2010), and reared at the FLI, Insel Riems, Germany, in a partially recirculating water system at 12 °C; tissue sampling was performed when fish were 1–2 years old. Atlantic salmon smolts, 14–18 cm in fresh-water phase, were obtained in May at Solbergstrand Research Station, Norwegian Institute for Water Research, Drøbak, Norway, where they were kept at 10 °C in tanks with filtrated, UV-treated river water. All fish were apparently healthy at the time of sampling. Before tissue sampling, trout and salmon were anesthetized with benzocaine, and trout were also exsanguinated. For expression analysis, the tissues indicated in Figs. 2 and 3 were isolated and directly soaked into RNAlater (Ambion); the isolated part of the gill is shown in Fig. S1 and includes gill arch and filaments; the skin (surface ~1 × 2 cm) was sampled from the left side of the back with a scalpel.

Apparently healthy, 8-week-old Balb/c mice (Moellegaard, Germany) used for tissue sampling were kept under standard laboratory animal conditions at the FLI. Murine skin from the right-side lower abdomen was sampled after removing the hair. Submandibular, axillary, inguinal and popliteal lymph nodes were collected as systemic lymph nodes. For murine lung a part of a right pulmonary lobe was sampled.

2.2. Database searching for salmonid IL-4/13A

A conserved motif around the cysteine in α-helix B was deduced from various published teleost IL-4/13 sequences (Ohtani et al., 2008), and with that motif the NCBI database (http://www.ncbi.nlm.nih.gov/) was screened (tblastn analysis) at default settings for matching ESTs of salmonid fishes.

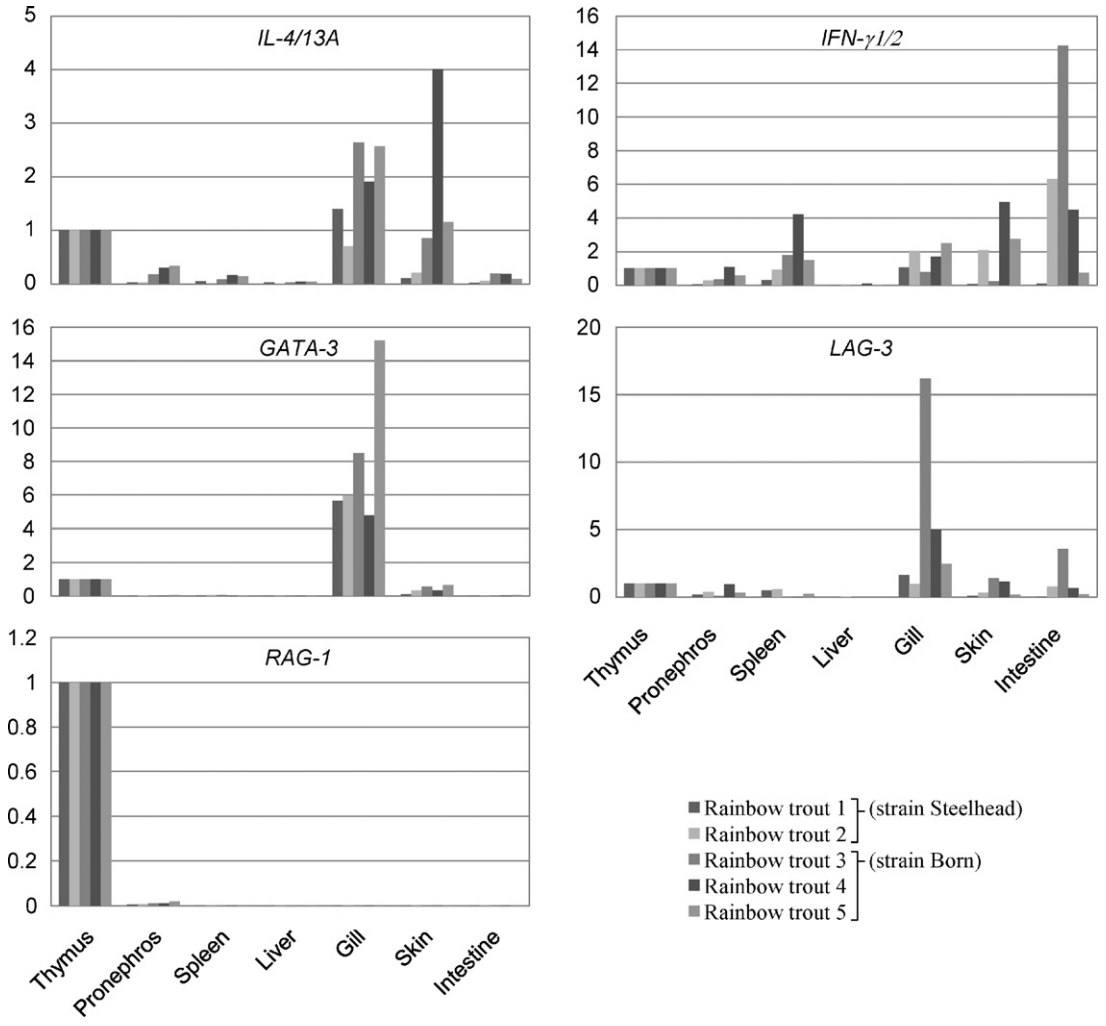

Fig. 2. Tissue distribution of *IL-4/13A* and other gene transcripts in five rainbow trout individuals (1–5), analyzed by real-time RT-PCR. The expression levels were determined by a relative standard curve method using *EF-1α* as endogenous reference, and are depicted in relation to thymus (set to 1). Fish pronephros is a lymphoid organ. For intestine a part of the hindgut was taken.

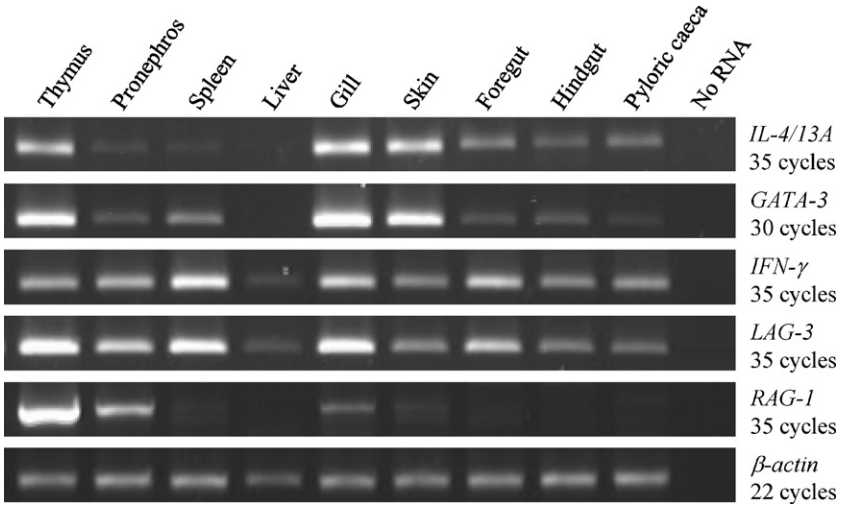

Fig. 3. Tissue distribution of *IL-4/13A* and other gene transcripts in Atlantic salmon, analyzed by conventional semi-quantitative RT-PCR. Data-sets shown are from a single representative salmon. Genes and cycle numbers are indicated at the right. Pyloric caeca are blind-end outpocketings of the anterior part of the intestine.

2.3. Sequence analysis of salmonid IL-4/13A; GenBank accession numbers

For salmonid *IL-4/13A* sequence analysis total RNA isolated from gill and genomic DNA isolated from erythrocytes were used. RNA and DNA isolation, cDNA synthesis, cloning and sequence analysis were performed similar as described before (Ohashi et al., 2010). Rainbow trout *IL-4/13A* cDNA was amplified by 3′RACE using primers *IL-4/13A-F1* and Adapter primer (Invitrogen), rainbow trout *IL-4/13A* genomic DNA was amplified using primers *IL-4/13A-F1* and *-R1*, and Atlantic salmon *IL-4/13A* open reading frame was amplified from cDNA with primers *IL-4/13A-F1* and *-R2*. For primers and their locations see Table S1 and Fig. S2.

GenBank accession numbers are: rainbow trout *IL-4/13A* cDNA, AB574337; rainbow trout genomic *IL-4/13A*, AB574338; Atlantic salmon *IL-4/13A* cDNA, AB574339.

2.4. Analysis of deduced IL-4/13A amino acid sequences

Nucleotide sequences were analyzed and translated with computer software (GENETYX version 7.0.6, Software Development, Tokyo, Japan). Leader peptides were predicted by SMART (http://smart.embl.de/). Alignment of amino acid sequences for Fig. 1 was performed by hand in consideration of sequence similarities, exon borders, relative phylogenetic distances and functional motifs, and was roughly in agreement with α-helix predictions for teleost IL-4/13A made by Phyre software (http://www.sbg.bio.ic.ac.uk/~phyre/; data not shown). Phylogenetic tree analyses by UPGMA and neighbor joining method were performed using GENETYX software with the sequences aligned as in Fig. 1. The non-salmonid teleost IL-4/13 sequences used for comparison were as described by Ohtani et al. (2008).

2.5. Expression analysis

Tissues of trout, salmon, and mouse were homogenized using Tissue Lyser (Qiagen), after which total RNA was extracted by NucleoSpin RNA II system (Macherey-Nagel). Conventional semi-quantitative RT-PCR was performed according to the OneStep RT-PCR Kit (Qiagen), gene specific primers (Table S1), and 10 ng total RNA per 12.5 μl reaction. For analysis of gene expression in rainbow trout also real-time RT-PCR was performed, with 25 ng total RNA per 20 μl reaction, using an Mx3000P instrument plus analysis software (Stratagene); these amplifications were performed according to the QuantiTect Probe RT-PCR Kit (Qiagen), using gene specific primers and probes (Table S1). Target quantity was determined using a relative standard curve method with total RNA from gill. A normalized amount of target gene was calculated by dividing the amount of target gene by the amount of *EF-1α* as endogenous control.

Reliability of the RT-PCR data shown in the present study is concluded from the similarities found for different individuals and species using different approaches. In addition the article conclusions were confirmed by obtaining similar RT-PCR results when using 16-fold diluted samples and four extra cycles for amplification (data not shown), arguing against possible effects of tissue-specific chemical contamination.

2.6. Stimulation of pronephrocytes and gill cells with PHA and poly I:C

Pronephros and gills were removed from rainbow trout "Born" individuals. The gills (including arches) were first cut by scissors into small pieces in a small amount of mixed medium (MM; Iscove's DMEM/Ham's F12 (Gibco) at a ratio of 1:1, supplemented with 10% fetal bovine serum) on ice. Tissues were homogenized with a Potter-Elvehjem homogenizer to make cell suspensions. For gill cells, remaining organ debris was removed by passing the suspension through a sieve (mesh size 100 μm), followed by centrifugation of the sieved cells (10 min, 650 × *g*, 4 °C) and resuspension of the resulting pellet into 2 ml of distilled water to lyze erythrocytes; then 48 ml MM was added, followed by two washes with MM using centrifugation (10 min, 650 × *g*, 4 °C). For pronephrocytes the cell suspension was layered onto an isotonic Percoll (Biochrome) gradient ($r = 1.075$ g/ml) and centrifuged for 40 min at 650 × *g*, after which pronephrocytes at the interphase were collected and washed twice with MM by centrifugation (10 min, 650 × *g*, 4 °C). One million pronephrocytes and 4×10^5 gill cells (only mononuclear leukocytes were counted) in 500 μl of MM were plated into 48-well microplates and stimulated with PHA (0.1 and 10 μg/ml; Biochrome) and poly I:C (5 and 50 μg/ml; InvivoGen) at 15 °C. At 0 h, 4 h, and 24 h, cells were lyzed and their RNA was isolated according to the NucleoSpin RNA II system (Macherey-Nagel); RNA of a single well was dissolved into 250 μl water, of which 2.5 μl was used in the conventional RT-PCR and real-time RT-PCR reactions as described above.

2.7. RT-PCR analysis of flow-sorted cells

Trout gill cells were prepared as described above and labelled with a mixture of anti-IgMmAbs (4C10 and N2; Thuvander et al., 1990; Fischer and Köllner, 1994), followed by flow-sorting of IgM^+ and IgM^- lymphoid cells using a MoFlo (Dako) high speed cell sorter, total RNA extraction from 30,000 cells of each fraction by RNeasy Plus Micro Kit (Qiagen), and conventional semi-quantitative RT-PCR as described above.

3. Results and discussion

3.1. Identification and analysis of salmonid IL-4/13A nucleotide sequences

By using a deduced/hypothesized ancestral teleost IL-4/13 motif (CEDEFFCKAEKVL) as query sequence, we were able to retrieve *IL-4/13A* ESTs of Atlantic salmon (GenBank EG860817, EG860818, EG837624, EG837625) and rainbow trout (GenBank CX038018) from the NCBI database. Primers derived from these ESTs (Fig. S2; Table S1) were used for PCR amplification of rainbow trout and Atlantic salmon *IL-4/13A* cDNA, after which the amplicons were cloned and sequenced. Trout and salmon *IL-4/13A* share 93% nt identity in their open reading frames (ORFs), and their 3′UTRs carry RNA destabilization motifs as found in previously identified teleost *IL-4/13A* and other cytokine genes (Fig. S2; Shaw and Kamen, 1986; Li et al., 2007; Ohtani et al., 2008). Genomic sequence of trout *IL-4/13A* was determined as well, revealing that the ORF was encoded by four exons separated by phase 0 introns (Fig. 1 and Fig. S2), which is common among short-helix type I cytokines (Rozwarski et al., 1994; Ohtani et al., 2008). Trout *IL-4/13A* EST CX038018 represents an unusual *RAD50* read-through hybrid transcript (*RAD50/IL-4/13A*; probably not functional), which suggests that salmonid *IL-4/13A* maps downstream of *RAD50* in head-to-tail fashion as known for *IL-4/13A* of other teleosts (Li et al., 2007; Ohtani et al., 2008).

Salmonid *IL-4/13B* ESTs were not found upon database mining, which may be related to lower expression levels as suggested by the presence of six zebrafish *IL-4/13A* ESTs in the absence of zebrafish *IL-4/13B* ESTs in the NCBI database (Ohtani et al., 2008).

3.2. Analysis of deduced salmonid IL-4/13A amino acid sequences

Deduced trout and salmon IL-4/13A consist of 145 and 142 amino acids, respectively, including putative leader sequences (Fig. 1). The salmonid molecules share 85% aa identity with each

other and <30% aa identity with IL-4/13A of other teleosts. In Fig. 1 the trout and salmon IL-4/13A sequences are aligned with IL-4/13A and -B of other teleosts, and with tetrapod IL-4 and IL-13. Short-helix type I cytokines are characterized by four α-helices A, B, C, and D in "up-up-down-down" orientation and in many family members, including in IL-4 and IL-13, the AB- with CD-loop interaction involves a small β-sheet (Rozwarski et al., 1994). In Fig. 1, α-helices and β-strands known in human IL-4 (Powers et al., 1993) and IL-13 (Eisenmesser et al., 2001) are underlined; the blue and red residues in these regions relate to a consensus structural framework among short chain type I cytokines which was distinguished by Rozwarski et al. (1994), with red indicating the positions of residues commonly exposed to the molecule interior and therefore often bearing hydrophobic side chains (which was helpful for making the alignment). Because of poor sequence conservation some parts of the alignment are highly discussable, but we deem the alignment of the (predicted) α-helix regions rather reliable and some motifs are well conserved (motifs similar to LxxII in αA, FCxA in αB, LxRxL in αC, and FLxxLxxxxR in αD; x signifies any amino acid).

The salmonid IL-4/13A molecules have typical teleost IL-4/13 features, including (1) two cysteine pairs (shaded blue and grey in Fig. 1; the blue pair is unique, the grey pair is also found in tetrapod IL-4 and IL-13), and (2) the I/LxR/KxLxxY/F motif in α-helix C. The conserved arginine in α-helix C (shaded yellow in Fig. 1) is the most important residue for binding of human IL-4 to IL-4Rα (Hage et al., 1999). There are no pronounced motifs distinguishing between teleost IL-4/13A and IL-4/13B, except for maybe the position of the cysteine in the CD loop (shaded grey in Fig. 1). However, phylogenetic tree analyses reliably cluster salmonid IL-4/13A with IL-4/13A of neoteleosts (Fugu, Tetraodon, medaka, stickleback) (Fig. S3), to which salmonids are closer related than to zebrafish (Nelson, 2006); that the phylogenetic tree analyses (Fig. S3) can not even detect the orthologous relationship between these neoteleost/salmonid sequences and zebrafish IL-4/13A (Ohtani et al., 2008) is yet another indication of the extraordinary rapid evolutionary diversification within this molecule family (Huising et al., 2006; see also Table S2).

3.3. High expression of IL-4/13A and GATA-3 in trout and salmon thymus, gill, and skin

Tissues of three rainbow trout of strain "Born", two rainbow trout of strain "Steelhead", and three Atlantic salmon were investigated for expression of *IL-4/13A* and several other genes. For trout this was done by real-time RT-PCR (Fig. 2) as well as by conventional semi-quantitative RT-PCR (Fig. S4), while for salmon this was only done by the latter technique (Fig. 3 and Fig. S4). The RT-PCR data consistently show that the sites of highest salmonid *IL-4/13A* expression are thymus, gill, and skin. Because this investigation was done in apparently healthy salmonids obtained from very different locations (trout from Germany, salmon from Norway), the high level of *IL-4/13A* expression presumably represents constitutive and not disease-associated expression. Moreover, the gill in naïve Tetraodon was also reported as a site of high *IL-4/13A* expression (Li et al., 2007; that study did not investigate thymus or skin).

Tissue distribution of salmonid *GATA-3* was similar to that of *IL-4/13A* (Figs. 2 and 3 and Fig. S4), which agrees with the importance of GATA-3 for expression of *IL-4/13* family genes (Pai et al., 2004; Ansel et al., 2006) and the presence of a GATA-3 binding motif in teleost *IL-4/13A* gene promoters (Ohtani et al., 2008). High constitutive expression of *GATA-3* in salmonid thymus, gill, and skin has been reported before (Kumari et al., 2009; Wang et al., 2010).

IFN-γ is a key molecule of Th1 responses. In trout two gene copies of *IFN-γ* are present, *IFN-γ1*and *IFN-γ2* (Purcell et al., 2009), which both match with the primers in each of our assays. For mammals, the ratio between *IFN-γ* and *IL-4* amounts is sometimes used as a measure for Th1/Th2 balance (e.g., Nan et al., 2007). Ratios between amounts of arbitrary units *IL-4/13A* and *IFN-γ*, determined by real-time PCR, are indicated for trout tissues in Table 1. On average, the thymus, liver, gill, and skin samples have a much higher ratio of *IL-4/13A* to *IFN-γ* than found in spleen and intestine, while the average value of the pronephros samples lays in between. These data suggest that the immune milieus of trout thymus, gill, and skin are Th2-skewed in comparison with those of pronephros, spleen, and intestine. The high ratio of *IL-4/13A* versus *IFN-γ* transcripts in liver (Table 1) should probably not be over-interpreted, since expression of either gene is very low (Figs. 2 and 3, and Fig. S4).

Table 1
Ratios between expression levels of *IL-4/13A* and *IFN-γ 1/2* arbitrary units.

	Trout 1	Trout 2	Trout 3	Trout 4	Trout 5	Average
Thymus	1.52	1.91	0.75	2.09	1.18	1.49
Pronephros	0.98	0.22	0.38	0.58	0.67	0.57
Spleen	0.26	0.04	0.04	0.08	0.12	0.11
Liver	4.05	0.76	2.31	1.01	1.72	1.97
Gill	2.00	0.65	2.52	2.34	1.22	1.75
Skin	2.38	0.20	2.63	1.69	0.50	1.48
Intestine	0.28	0.02	0.01	0.09	0.15	0.11

Data are from the same experiment as depicted in Fig. 2. White, ratios >0.7; light grey, ratios between 0.2 and 0.7; dark grey, ratios <0.2.

Th2-bias of gill and skin is very important for aquaculture, because these tissues are the preferred sites for vaccine administration due to low handling costs and non-invasiveness (e.g., during bath or spray immunization; Nakanishi and Ototake, 1997). Fish farmers should realize that the site of vaccine administration affects the type of immune response induced, and that targeting of the gill or skin may not be very effective for inducing a Th1 response. This is a point of concern because Th1 responses are critical for the control of many viral diseases.

3.4. High expression of IL-4/13 and GATA-3 in salmonid thymus resembles the situation in mammals

For comparison of the above salmonid data with tissue distribution of *IL-4*, *IL-13*, and *GATA-3* in mammals, we refer to literature (which appears very scarce on relative expression levels among various mammalian tissues) and our semi-quantitative RT-PCR analysis of two mouse individuals (Fig. 4 and Fig. S5). Expression of *IL-4*, *IL-13*, and *GATA-3* was high in murine thymus (Fig. 4 and Fig. S5) similar as found for salmonids (Figs. 2 and 3, and Fig. S4). Mammalian GATA-3 plays an important role in several stages of early T cell development in the thymus (Pai et al., 2003; Ho and Pai, 2007), which probably explains the high *GATA-3* levels in this organ (Fig. 4 and Fig. S5; Anderson et al., 2004). IL-4 and IL-13 are believed to have multiple functions in the thymus (Yarilin and Belyakov, 2004). The high expression levels of *IL-4/13* family and *GATA-3* transcripts add to the accumulating evidence that teleost and mammalian thymus are functionally similar (e.g., Danilova et al., 2004; Fischer et al., 2005).

Significant amounts of *recombination activating gene 1* (*RAG-1*) were only found in salmonid thymus and pronephros (Figs. 2 and 3, and Fig. S4), where RAG-1 is presumably involved in recombination of the T and B cell receptor genes, respectively (Hansen, 1997).

3.5. Salmonid gill may form a Th2/Treg-skewed immune environment

Teleost gill has a large surface area, covered by a thin layer of mucus, which is exposed to vast amounts of actively pumped water. Gill has important functions in gas and ion transport, nitrogenous waste excretion, and hormone production (Evans et al., 2005). Although high amounts of *IL-4/13A* and *GATA-3* were found in salmonid gill (Figs. 2 and 3, and Fig. S4), the amounts of *IL-4*, *IL-*

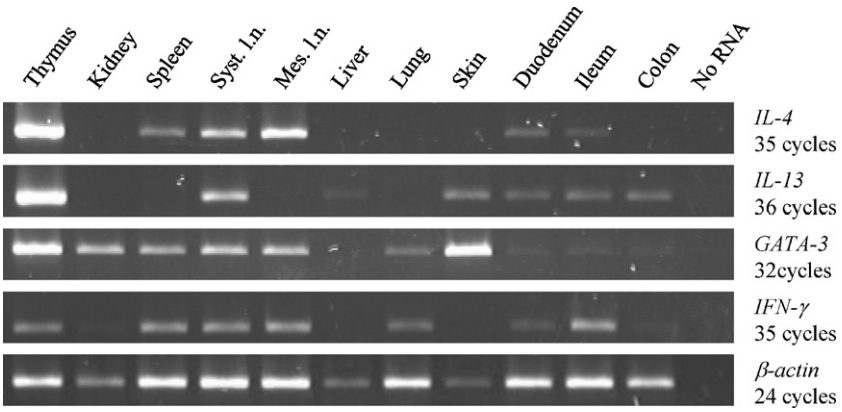

Fig. 4. Tissue distribution of *IL-4*, *IL-13*, *GATA3*, and *IFN-γ* expression in mouse, analyzed by semi-quantitative RT-PCR. *β-Actin* was analyzed as a measure for RNA quality. The data-set shown is from a single representative mouse. Genes and cycle numbers are indicated at the right. Syst. l.n., systemic lymph nodes; Mes. l.n., mesenteric lymph nodes.

13, and *GATA-3* in murine lung were not higher than those in other organs (Fig. 4 and Fig. S5). So the immune milieu of salmonid gill appears, at least superficially, very different from that of its murine functional counterpart. However, some data suggest that the mammalian lung also is Th2-biassed (e.g., Dodge et al., 2003), but if existent, this bias apparently is not pronounced enough (as exemplified by Fig. 4 and Fig. S5) for notification in mainstream scientific literature.

We wondered how an antigen-exposed tissue like fish gill can have high levels of *IL-4/13A* and *GATA-3* without being inflamed, and therefore investigated the expression of *lymphocyte activation gene 3* (*LAG-3*). In mammals LAG-3 is associated with inhibition of immune responses and with Treg cells (Workman and Vignali, 2003; Huang et al., 2004), and rainbow trout LAG-3 cytoplasmic tail carries an immunoreceptor tyrosine-based inhibitory motif (ITIM) suggestive of similar function (Ohashi et al., 2010). Substantial levels of *LAG-3* were found in gill of all investigated trout and salmon (Figs. 2 and 3, and Fig. S4), consistent with an immunosuppressive environment. Moreover, high levels of *IL-11* were previously reported for trout gill (Wang et al., 2005), another indicator of an anti-inflammatory environment (Trepicchio and Dorner, 1998). So we hypothesize that salmonid gill forms a "Th2/Treg-skewed" environment which protects from parasites and from inflammatory Th1 or Th17 responses. Dedication of the gill immune milieu to protection from parasites is supported by the presence of eosinophilic granule cells (Holland and Rowley, 1998). Th1 and Th17 responses in fish have not been proven yet, but there are indications for their existence and their ability to cause tissue damage (e.g., Zou et al., 2005; Gunimaladevi et al., 2006; Metzger et al., 2010; Mutoloki et al., 2010).

Although under discussion (e.g., Halonen et al., 2009), it is commonly believed that during pregnancy (e.g., Adkins, 2003; Chaouat, 2007) and in neonatals (e.g., Morein et al., 2007; Rose et al., 2007) the immune system is Th2-skewed in order to protect from damaging Th1 responses. So teleost gills and mammalian pregnancies/neonatals may be similar in using Th2-skewage to protect sensitive tissue.

3.6. High expression of IL-4/13 and GATA-3 in salmonid skin whereas in murine skin only GATA-3 was high

Fish skin differs from mammalian skin by having an outer layer of living epithelial cells which are covered with mucus (Shephard, 1994). Relatively high amounts of *IL-4/13A* and *GATA-3* were found in salmonid skin, although on average less than in thymus and gill (Figs. 2 and 3, and Fig. S4; Kumari et al., 2009; Wang et al., 2010). In contrast, murine skin only expressed high levels of *GATA-3*, while *IL-4* and *IL-13* were low (Fig. 4 and Fig. S5). Mammalian GATA-3 is involved in skin development, and its expression is particularly high in hair follicles (Kaufman et al., 2003). Explanation of the high amounts of *IL-4/13A* in salmonid skin is difficult, because forced expression of IL-4 in skin of transgenic mice caused inflammatory disease similar to human atopic dermatitis (Chan et al., 2001). Expression levels of indicators of immunosuppression such as *LAG-3* (Figs. 2 and 3, and Fig. S4), *IL-11* (Wang et al., 2005), *TGF-β*, and *IL-10* (preliminary data not shown) were low in salmonid skin, thus its immune environment appears different from the "Th2/Treg-skewed" environment of the gill. Possibly the high *IL-4/13A* presence in fish skin has functions outside the immune system, because mammalian IL-4 was found to stimulate proliferation of keratinocytes (Junghans et al., 1996).

3.7. IL-4/13A and IFN-γ expression by isolated gill cells and pronephrocytes are differentially regulated

Cells were isolated from trout gill and pronephros, *in vitro* stimulated with PHA (a mitogenic lectin) or poly I:C (synthetic double-stranded RNA), and assayed for *IL-4/13A* and *IFN-γ* expression by real-time RT-PCR (Fig. 5) and conventional RT-PCR (Fig. S6). The pronephrocyte preparation consisted of lymphoid cells, macrophages and granulocytes (Fig. S7). The gill cell preparation contained a wider variety of cell types, most of which lymphoid cells (Fig. S7B upper left plot), and included some cell debris. Pronephrocytes were analyzed 4 h and 24 h after stimulation, while gill cells were only investigated after 4 h, because after 24 h their viability appeared importantly reduced (not shown). Experiments 1 and 2 were set up as independent repeats for confirmation of data, using three trout for the first and two trout for the second experiment. For unknown reason the absolute values of induced changes differed between the two sets of experiments, but both agreed that: (1) cytokine expression was much higher in non-stimulated gill cells than in pronephrocytes (Fig. S6), which agrees with the above described whole tissue analysis; (2) both *IFN-γ* and *IL-4/13A* were sensitive to PHA stimulation, while only *IFN-γ* was sensitive to poly I:C; (3) *IFN-γ* expression in gill cells was not enhanced by PHA or poly I:C, but these agents were able to counteract the "spontaneous" *IFN-γ* decrease observed in gill cells upon *in vitro* incubation; (4) gill cells exhibited a "spontaneous" increase in *IL-4/13A* expression upon *in vitro* incubation, which was not affected by addition of either PHA or poly I:C.

In summary, the experiments support Th2 function of fish IL-4/13A and Th2-bias of gill. Namely, trout *IL-4/13A* expression was not sensitive to poly I:C, which in mammals is a strong inducer of Th1 cytokines like IFN-γ but not of the Th2 cytokines IL-4 and IL-13

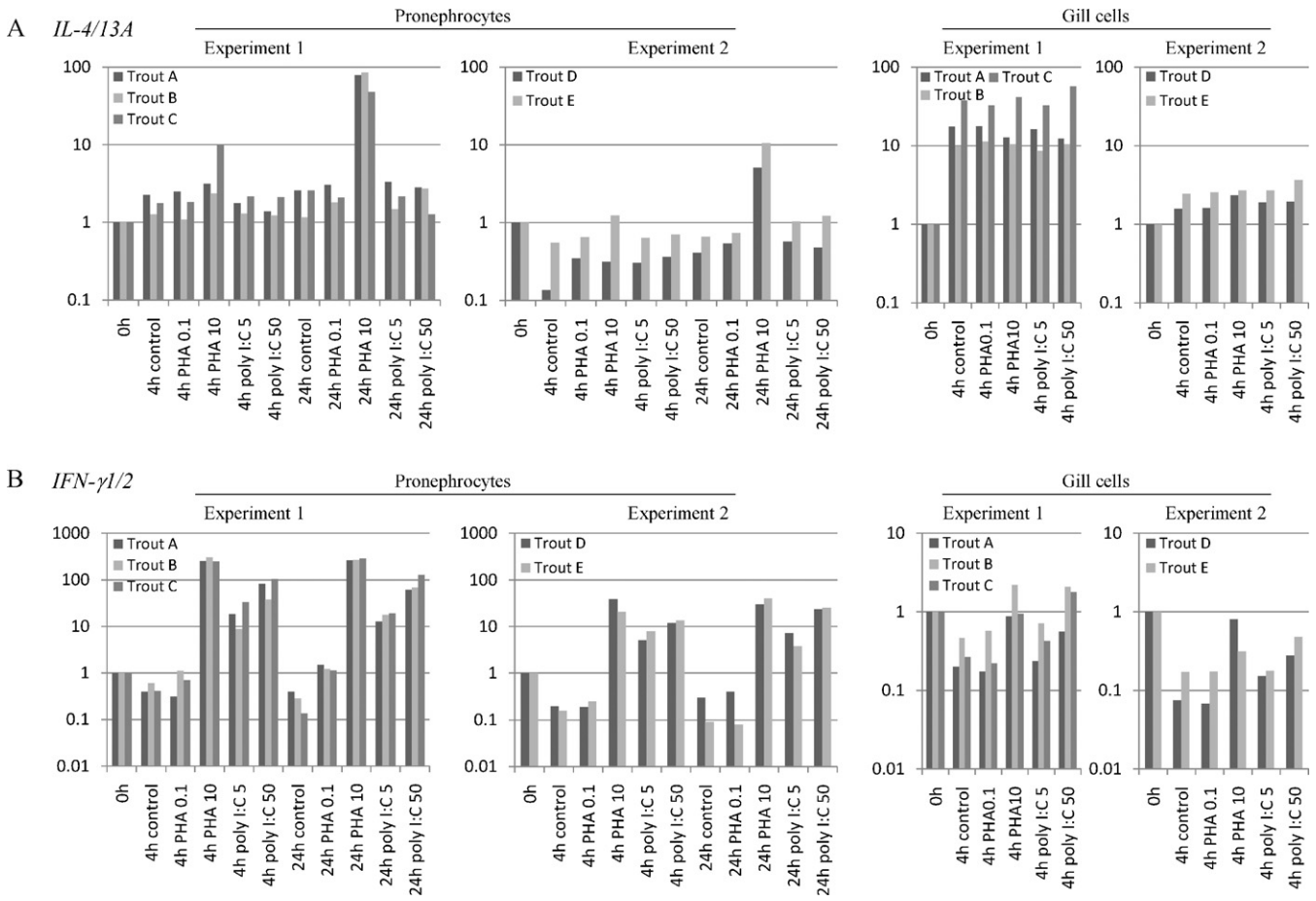

Fig. 5. *In vitro* expression of *IL-4/13A* and *IFN-γ* by isolated trout pronephrocytes and gill cells, analyzed by real-time PCR. The expression levels were determined by a relative standard curve method using *EF-1α* as endogenous reference, and are depicted in relation to the expression by the respective cell culture at $T = 0$ (set to 1).

(Victoratos et al., 1997). Furthermore, *IL-4/13A* and *IFN-γ* expression were enhanced and reduced in cultured gill cells, respectively, which agrees with mammalian Th1 and Th2 cytokines being stimulated under different (often opposing) conditions (Victoratos et al., 1997; Ansel et al., 2006). The readiness (no PHA or poly I:C necessary) with which gill cells enhanced *IL-4/13A* while reducing *IFN-γ* expression upon *in vitro* incubation supports that they are Th2-biassed. Lack of *IFN-γ* enhancement in gill cells after poly I:C treatment (which the immune system perceives as a double stranded RNA virus) agrees with a previous *in vivo* study showing that *IFN-γ* expression in salmon gill was not up-regulated by infection with virus, despite significant enhancement of other gene markers for virus infection (Jørgensen et al., 2007).

3.8. IL-4/13A is predominantly expressed by surface IgM-negative gill cells

Trout gill cells were labelled with anti-IgM monoclonal antibodies, and surface IgM-positive (B cells) and -negative fractions of lymphoid cells were isolated by flow cytometry sorting (Fig. S7). Most *IL-4/13A* was found expressed by IgM-negative cells (Fig. 6), which may result from predominant expression of trout IL-4/13A by T cells as known for mammalian IL-4 and IL-13. Better identification of the *IL-4/13A* expressing lymphocytes has to await the establishment of antibodies against various cell surface markers in salmonid fish (e.g., against CD4 homologues).

3.9. Gill lymphoid tissue and thymus may be evolutionary related

During ontogeny of higher vertebrates the primordial thymus lobes migrate from the pharyngeal pouches on each side of the embryo to meet more central in the body, whereas in teleost fish the thymi remain situated at the roof of the gill cavities (Fig. S1). The epithelium covering the fish thymus directly lines the gill cavity (Chilmonczyk, 1992), and it has since long been speculated that in evolution the thymus derived from gill-associated lymphoid tissue (e.g., Matsunaga and Rahman, 2001). Our recent and present studies support this evolutionary model by finding that thymus and gill share unique immune features. Namely, (1) very recently we found that fish gill harbors an elongated intraepithelial organ along the apical edge of the interbranchial septa, which was designated interbranchial lymphoid tissue (ILT), and which like the thymus harbors vast amounts of T cells ($CD3\varepsilon^+$ cells) while B cells are very scarce (Haugarvoll et al., 2008; Koppang et al., 2010), and (2) in the present study we found that gill and fish/mammalian thymus immune milieus share high expression of IL-4/13-family and GATA-3 genes.

It is not known why fish thymus borders the outside world, while B cells develop in more internal tissues (in teleost fish in the pronephros). Maybe the B/T cell divide in evolution was related to some specialization in systemic versus mucosal immunity of B versus T cells. We speculate that, possibly only during some stage in juveniles, developing T cells in the fish thymus are tolerized against ubiquitous waterborne antigens. This hypothesis requires future

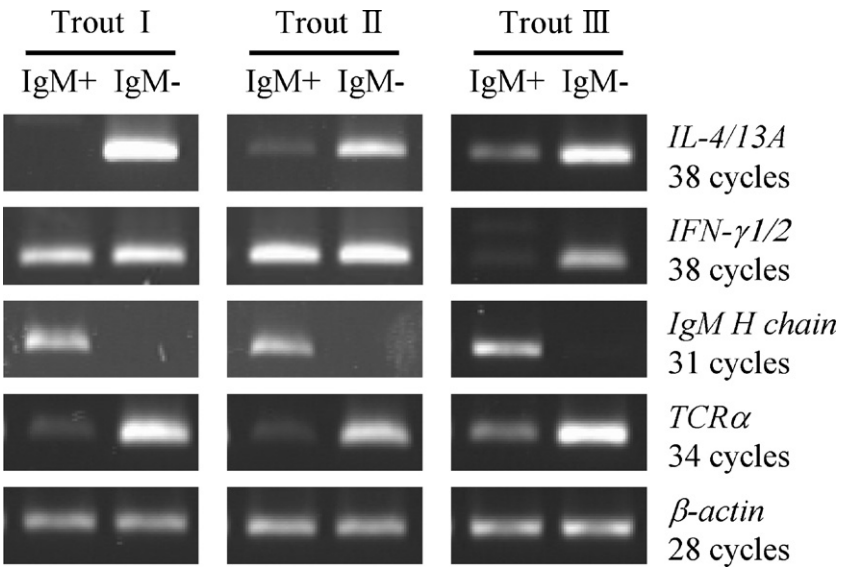

Fig. 6. *IL-4/13A* is predominantly expressed by surface IgM-negative gill lymphocytes. IgM-positive and -negative lymphoid gill cell fractions were isolated by FACS (Fig. S7), followed by RNA isolation and expression analysis by conventional semi-quantitative RT-PCR. *IgM H* and *TCRα* were analyzed as a measure for B cell separation; *β-actin* was analyzed as a measure for RNA quality.

investigation of possible uptake of waterborne antigen through the epithelial layer that separates the thymus interior from the gill cavity.

4. Conclusion

The present study identified rainbow trout and Atlantic salmon *IL-4/13A*, which is a homologue of the mammalian Th2 cytokine genes *IL-4* and *IL-13*. Salmonid *IL-4/13A* and *GATA-3* were found highly expressed in thymus, gill, and skin. The high expression in thymus is probably associated with early T cell development as known in mammals. The high expression in gill may be associated with a Th2/Treg environment that protects from parasites as well as from inflammatory Th1 and Th17 responses. The high expression in skin may have a similar function as in gill, although it is puzzling that in skin the high amount of *IL-4/13A* is not matched by high expression of genes indicative for immunosuppression. Th2 identity of trout *IL-4/13A* was supported by *in vitro* studies showing lack of sensitivity to poly I:C and differences in expression patterns compared with the pivotal Th1 cytokine gene *IFN-γ*.

Neither Th1, Th2, Th17, nor Treg cells have been properly identified in fish yet, and for terminology we borrow from studies in mammals. However, fragmentary evidence, including the present study, suggests the conservation of all these T cell types throughout vertebrate species including fish (e.g., Mitra et al., 2010).

From practical point of view the immune milieus of fish skin and gill are very important with regard to fish vaccination strategies. The Th2-bias of these tissues suggests that generally they are not the optimal sites for vaccine administration against viral disease. From fundamental science point of view the gill immune environment is interesting because in evolution it probably was here that the thymus originated. The high expression of *GATA-3* and *IL-4/13A* in both gill and thymus supports that these tissues are related.

We hope that the identification of salmonid *IL-4/13A* genes and the realization that fish thymus, gill, and skin are *IL-4/13A*-rich milieus, will prove beneficial to fish immunology and vaccinology research. Furthermore, we hope that this study may help unravel the evolutionary origin of the T cell system and lead to an increased awareness that Th1/Th2 balances can differ per tissue.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2011.02.014.

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DNA vaccination against a fish rhabdovirus promotes an early chemokine-related recruitment of B cells to the muscle

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ABSTRACT

In fish, intramuscular (i.m) injection of plasmid DNA encoding viral proteins has proved a highly effective vaccination strategy against some viral pathogens. The efficacy of DNA vaccination in teleost fish is based on the high level of viral antigen expression in muscle cells inducing a strong and long-lasting protection. However, the mechanisms through which this protection is established and effectuated in fish are still not fully understood. Moreover, similarities to mammalian models cannot be established since DNA vaccination in mammals usually induces much weaker responses. In this work, we have focused on the characterization of the immune cells that infiltrate the muscle at the site of DNA injection in vaccinated fish and the chemokines and chemokine receptors that may be involved in their infiltration. We have demonstrated through diverse techniques that B lymphocytes, both IgM^+ and IgT^+ cells, represented a major infiltrating cell type in fish vaccinated with a viral haemorrhagic septicaemia virus (VHSV) glycoprotein-encoding DNA vaccine, whereas in control fish injected with an oil adjuvant mainly granulocyte/monocyte-type cells were attracted. Among twelve chemokine genes studied, only CXCL11_L1, CK5B and CK6 mRNA levels were up-regulated in DNA vaccinated fish compared to fish injected with the corresponding vector backbone. Furthermore, the transcription of CXCR3B, a possible receptor for CXCL11_L1 was also significantly up-regulated in vaccinated fish. Finally, experiments performed with recombinant trout CK5B and CK6 and chemokine expression plasmids revealed that these chemokines have chemotactic capacities which might explain the recruitment of B cells to the site of DNA injection. Altogether, our results reveal that there is an early chemokine-related B cell recruitment triggered by i.m. DNA vaccination against VHSV which might play an important role in the initial phase of the immune response.

1. Introduction

DNA vaccination has proved as an effective method for inducing a potent and long-lasting protection against different fish viruses, especially rhabdoviruses such as infectious hematopoietic necrosis virus (IHNV) or viral haemorrhagic septicaemia virus (VHSV) [1–4]. Significant protection levels have also been achieved with DNA vaccines constructed for other fish viruses [5–7]. Concerning IHNV and VHSV vaccination in rainbow trout (*Oncorhynchus mykiss*), a single intramuscular (i.m.) injection of a naked DNA plasmid coding for the glycoprotein G is capable of providing fish with full long-lasting protection [8,9] and in consequence, one of the very few antiviral vaccines licensed for fish is an IHNV DNA vaccine [10]. Although a non-specific protection state elicited at early times post-vaccination seems to correlate with the induction of type I interferon (IFN) [9], the mechanisms through which these vaccines confer specific long-term protection are still not clear. Both humoral [9] and cellular [11] responses are triggered, but are not clearly correlated with protection.

Many studies have addressed the transcriptional response to vaccination in the spleen or head kidney [12,13], but not many have focused on studying what happens at the injection site. This early local response should dictate all the downstream mechanisms elicited, being likely key for the outcome of the later systemic

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response. The genes modulated in the muscle after injection were studied for an IHNV DNA vaccine [14], demonstrating up-regulation of genes predominantly related to IFN. Additionally, in the context of an extensive characterization of the kinetics of VHSV G-protein expression in the membrane of myocytes in response to DNA vaccination, a significant infiltration of leukocytes around the injection site was described [15]. However, the nature of immune cells was not clearly established.

In rainbow trout, three different immunoglobulins (Igs) have been reported [16–18], which render two main subpopulations of central B cells, IgM^+ cells and IgT^+ cells. Although it seems that IgT^+ cells play an important role in mucosal responses [19], recent evidence implicates IgT in systemic responses as well [20]. Our results demonstrate that both IgM^+ and IgT^+ cells are recruited to the muscle upon DNA vaccination, in contrast to reports in mammals, in which macrophages, T cells and neutrophils are the predominant recruited cell types [21,22]. To gain an insight into the mechanisms responsible for this B cell mobilization, we have also studied the transcription of a wide range of chemokine genes and all chemokine receptors known for rainbow trout. These data contribute to a better understanding of the early immune mechanisms elicited by DNA vaccination in fish.

2. Materials and methods

2.1. VHSV DNA vaccine

The VHSV DNA vaccine used in this study, designated pVHSV, has been previously described [15,23]. The empty pcDNA3 plasmid was used as a control.

2.2. Fish

Rainbow trout (*O. mykiss*) were maintained at 11–16 °C in a re-circulating water system and fed with a commercial diet (Skretting). Prior to any experimental procedures, fish were acclimatized to laboratory conditions. All the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and have been approved by the corresponding local Ethics Committees.

2.3. Transcriptional studies in muscle

For the transcriptional studies, fish were injected i.m. with 1 μg of pVHSV plasmid, 1 μg of pcDNA3 in PBS (50 μl) or the same volume of PBS in the epaxial muscle below the dorsal fin by use of a 27 G needle. In some experiments, VHSV virus, propagated as previously described [24], was heat-inactivated (56 °C for 30 min) and injected i.m. in parallel to the DNA vaccine (1×10^6 $TCID_{50}$ per fish). At days 3, 6 and 14 post-vaccination, six fish from the different groups were killed and the muscle area surrounding the injection site sampled and placed in Trizol (Invitrogen) for RNA isolation following the manufacturer's instructions.

RNAs were treated with DNAse I to remove genomic DNA that might interfere with the PCR reactions. An amount of 1 μg of RNA was used to obtain cDNA in each sample using the Superscript III reverse transcriptase (Invitrogen) and oligo $(dT)_{12-18}$ following the manufacturer's instructions.

To evaluate the levels of transcription of different immune genes, real-time PCR was performed with a LightCycler® 480 System (Roche) using FastStart SYBR Green Master mix (Roche) following the manufacturer's instructions. Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 amplification cycles (30 s at 95 °C and 1 min at 60 °C). For each gene, expression was normalized to that of elongation factor 1α (EF-1α) expression in each sample and expressed as $2^{-\Delta Ct}$ as described previously [25]. Primers used were already optimized [25,26] and are shown in Supplementary Fig. 1.

To verify that IgM and IgT transcripts quantified were not sterile Ig transcripts, PCRs were performed using a forward primer specific for a subgroup or a set of IGHV genes in combination with a reverse primer Cμ or Cτ specific for IGHM or IGHT genes, in a pooled cDNA sample from each experimental group as previously described [20] (Supplementary Fig. 1).

2.4. Immunocytochemistry

To evaluate the infiltration through immunohistochemistry, fingerlings of 3–4 g were anaesthetised in 0.01% benzocaine and injected with 20 μg of pVHSV or empty plasmid dissolved in 0.9% NaCl or with the same volume of 0.9% NaCl in the muscle. Muscle sections obtained from control and vaccinated fish ($N = 3$) at day 7 post-immunization were subjected to an indirect immunocytochemical method for detection of trout IgM and IgT using monoclonal antibodies F1-18 and F1-15 kindly donated by Dr. Kurt Buchmann and Dr. Karsten Skjoedt as previously described [27,28]. The specificity of the reactions was determined by omitting the primary antibodies.

2.5. Isolation and characterization of infiltrated B cells from muscle after DNA injection

To isolate and further characterize the cells infiltrated in the muscle after DNA vaccination, fish weighing approx. 150 g received six i.m. injections of 40 μl (1 mg/ml) of the VHSV DNA vaccine or the empty vector as control. Another group was injected with the same volume of complete Freund's adjuvant (Sigma-Aldrich). Injection needles were placed approx. 1 cm deep into the dorsal muscle. At the day of sampling, fish were killed and muscle tissue around the injection site (approx. $1 \times 1 \times 1$ cm) excised into ice cold medium (Iscove's DMEM/Ham's F12; Gibco). The tissue was cut into small pieces and trypsin solution added at a final ratio of 10% (v/v). After 1 h of incubation at room temperature, the suspension was filtered through a nylon mesh (100 μm), layered onto a two-step Percoll gradient (1.035 g/ml and 1.08 g/ml) and centrifuged for 40 min at $650 \times g$ at 4 °C. The interphase was collected and washed twice.

Infiltrating cells (1×10^6 cells/ml) were stained using primary antibodies against either IgM (mab 4C10; [29]), CD8α [30] and IgT [19]. IgM and CD8α staining was carried out simultaneously using TRICOLOR anti-mouse Ig (Caltag) and FITC anti-rat IgG (H + L) (Jackson ImmunoResearch) conjugates, respectively, while IgT^+ cells were single-stained using an Alexa488 anti-mouse-IgG (Caltag) conjugate.

2.6. Production of recombinant CK5B and CK6

To produce recombinant trout CK5B and CK6 in *Escherichia coli*, the cDNA sequences encoding the mature chemokines were amplified and cloned into a modified pET vector (Novagen) using specific primers (Supplementary Fig. S1). After sequence confirmation, the chemokine expressing plasmids were transformed into *E. coli* strain BL21 Star (DE3)pLysS (Invitrogen). The recombinant proteins were produced as described previously [31,32].

2.7. Chemotactic assays with recombinant CK5B and CK6

The capacity of rainbow trout recombinant CK5B and CK6 to attract leukocytes from different sources was evaluated. For this, leukocytes from spleen, blood and head kidney were isolated from naïve fish following the method previously described [33]. Cells

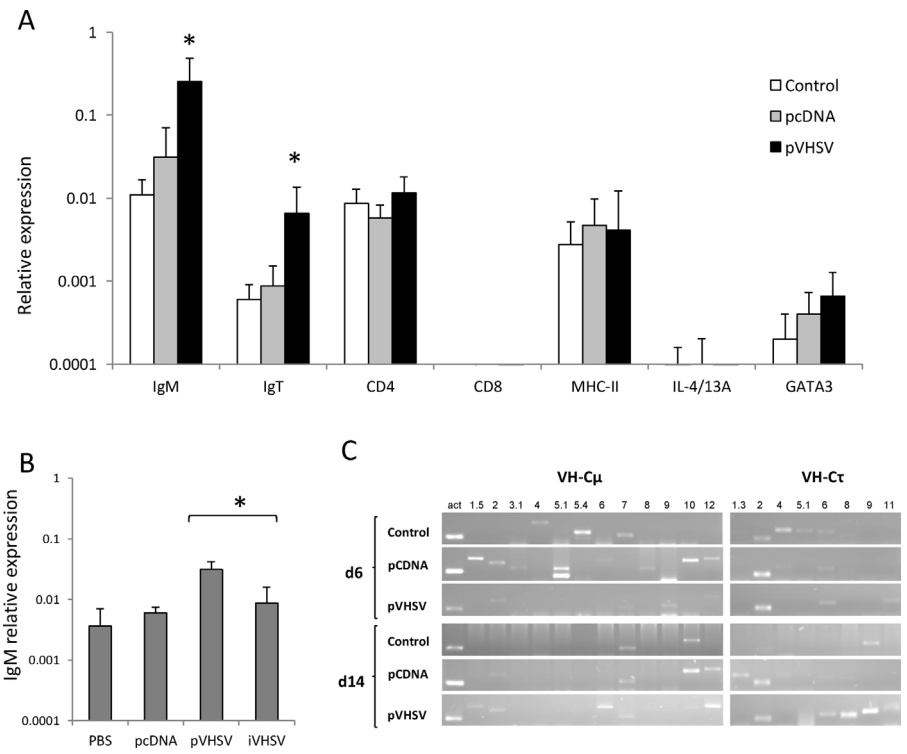

Fig. 1. (A) Levels of transcription of immune genes specific for lymphocyte subpopulations in the muscle of DNA vaccinated fish. Rainbow trout were i.m. vaccinated with 50 μl of PBS containing 1 μg of the pVHSV vaccine, 1 μg of the empty plasmid (pcDNA) or PBS only as a control. At day 6 post-injection six trout from each group were killed and the muscle area around the injection site sampled to determine the levels of expression of immune genes specific for lymphocyte subpopulations by real-time PCR. Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± *SD*. * Levels of expression significantly different than those observed in fish injected with the empty plasmid ($p < 0.05$). (B) Levels of IgM transcription in fish injected with inactivated VHSV (iVHSV) in comparison to fish injected with the DNA vaccine or corresponding controls at day 6 post-injection. Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± *SD*. * Levels of expression significantly different between experimental groups ($p < 0.05$). (C) Amplification by PCR of the different rearranged VH-Cμ and VH-Cτ combinations of the BcR in muscle at 6 and 14 days after vaccination. PCR were performed (40 cycles) combining a VH family specific forward primer (VH1 to VH13) with a C specific reverse primer (Cμ or Cτ). Only combinations amplified in at least one group are shown. β-actin (act, 30 cycles) was used as housekeeping gene.

were resuspended in L-15 with 2% FCS at a concentration of 1×10^6 cells/ml.

The chemotaxis assays were performed in chemotaxis chambers introduced in 24-well plates (Costar-Corning Life Sciences). Six hundred μl of different dilutions of CK5B or CK6 in culture medium were placed at the bottom of the wells, while 100 μl of the different leukocyte cell suspensions were loaded to the upper chamber. After 2 h at 20 °C, the number of cells that had migrated to the bottom of the wells was quantified by flow cytometry as previously described [25].

2.8. In vivo chemotactic assays using CK5B and CK6 expression plasmids

Plasmids coding for rainbow trout CK5B and CK6 previously obtained [34] were used to confirm the chemotactic capacity of these chemokines. Fish were injected i.m. with 1 μg of either pCK5B, pCK6 or the pcDNA3.1 control plasmid (in 50 μl of PBS). At day 6 post-injection, three fish per group were sampled to evaluate the levels of infiltration through histological techniques.

2.9. Statistics

Data handling, analyses and graphic representation was performed using Microsoft Office Excel 2010. Statistically significant differences were determined using a Mann–Whitney *U* test or a one-way ANOVA ($p < 0.05$).

3. Results

3.1. B cells are a major leukocyte population infiltrated in the muscle of vaccinated fish

Real time PCR analysis revealed a significant increase in transcription levels of both IGHM (IgM) and IGHT (IgT) in vaccinated compared to mock-vaccinated fish from day 6 post-vaccination (Fig. 1A). Although CD4, MHC-II and GATA-3 transcription was detected in the muscle, the levels of transcription were not significantly different between the different experimental groups. CD8 and IL-4/13A transcription remained mostly undetected in all groups. The up-regulation of IGHM transcription in response to pVHSV was not observed when the inactivated virus was i.m. injected (Fig. 1B). The levels of IGHM and IGHT remained elevated at day 14 post-vaccination (data not shown). Several different VH-Cμ and VH-Cτ transcripts were retrieved by PCR from the muscle at days 6 and 14 (Fig. 1C), indicating the presence of rearranged B cell receptor mRNAs in the infiltrated cells. We found a higher number of different VH-Cμ and VH-Cτ amplifications for pVHSV samples at day 14 in comparison to control and pcDNA samples.

Immunohistochemistry analyses were also conducted to detect IgM^+ and IgT^+ cells in the muscle. In concordance with the transcriptional studies and in contrast to the situation in higher vertebrates [21,22], high levels of both IgM and IgT were found at the injection site (Fig. 2).

To further verify that B cells were actually present in the muscle, we optimized a protocol for the isolation of infiltrating cells.

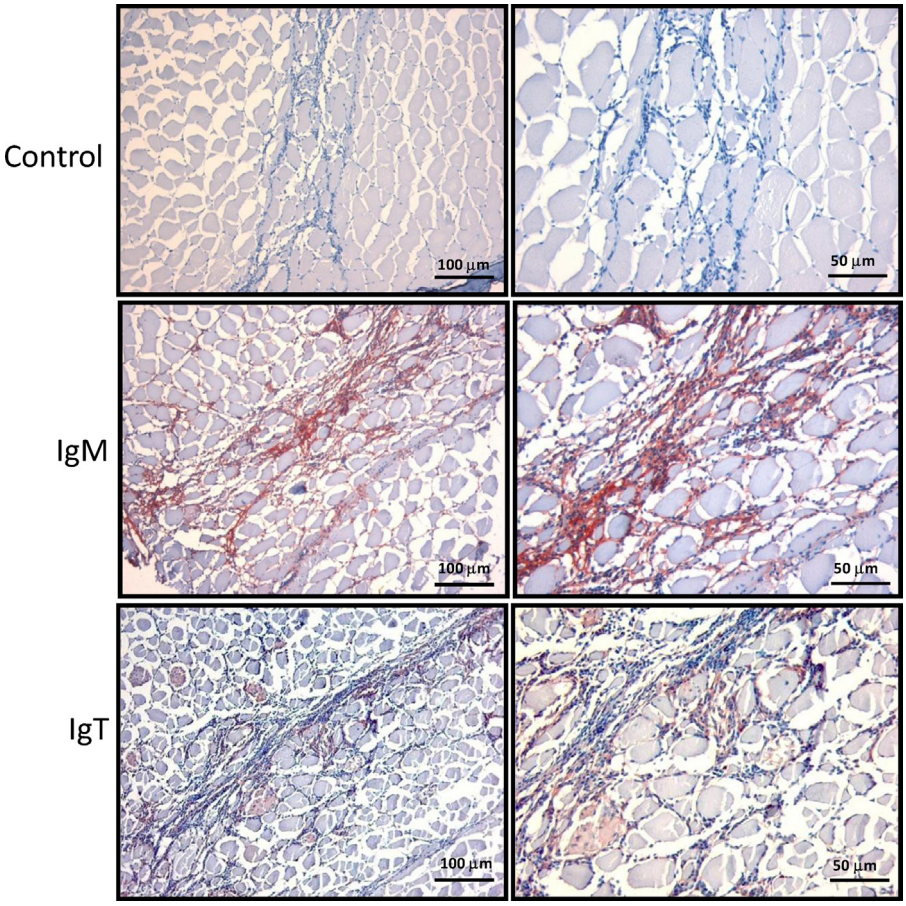

Fig. 2. Immunohistochemical detection of B cells in the muscle of DNA vaccinated fish. IgM and IgT were detected at day 7 post-vaccination using specific monoclonal antibodies in muscle sections from fish vaccinated with 20 μg of a VHSV DNA vaccine. The specificity of the reactions was determined by omitting the primary antibodies (isotype control). Two micrographs are shown in each case with different magnifications (20× and 40x). Bars correspond to 100 μm and 50 μm, respectively.

Although some infiltrating cells were detected in response to the empty plasmid, the number of cells in vaccinated fish was higher. Infiltrated cells collected 6 days after i.m. VHSV DNA vaccination mostly had a profile of lymphocyte-type cells, with small size and low complexity (Fig. 3A). Within this population, IgM^+ cells were a predominant cell type (Fig. 3A), with a lower percentage of IgT^+ cells and $CD8^+$ cells present. When CFA was injected instead of the DNA vaccine, the profile of the infiltrated cells was more heterogeneous, including a large number of granulocyte/monocyte-type cells with bigger size and higher granularity (Fig. 3B). Among the gated lymphocyte-type cells, we again detected a high percentage of IgM^+ cells in addition to IgT^+ and $CD8^+$ cells. When fish were sampled at a later time post-vaccination (day 17), infiltrating cells could not be recovered from the group injected with the empty plasmid but only from the groups injected with the VHSV vaccine or CFA. The experiment was repeated once and similar results were observed. These results confirm that rainbow trout B cells are recruited to the vaccine injection site and reveal that both IgM^+ and IgT^+ cells have the capacity to infiltrate the muscle.

3.2. Chemokine and chemokine receptor transcription at the injection site

We evaluated the levels of transcription of different chemokines in vaccinated and mock-vaccinated fish, studying a wide selection of rainbow trout chemokines that include representatives of the chemokine clades previously established [35,36]. At day 3 post-vaccination, CK1, CK3, CK5B, CK6, CK9, CK10, CK11, CK12, CXCL8_L1, CXCL_F1 and CXCL11_L1 were transcribed in the muscle (Fig. 4A), but CXCL11_L1 was the only chemokine for which transcription was significantly higher in vaccinated animals. At day 6 post-vaccination, CXCL11_L1 levels were similar in all groups, whereas CK5B and CK6 mRNA levels were significantly elevated (Fig. 4B). At day 14 post-vaccination, no significant differences were apparent between the groups (Fig. 4C).

The levels of transcription of trout chemokine receptors in the muscle of vaccinated and mock-vaccinated fish were studied at day 6 post-injection. Although CCR6, CCR9B and CXCR3A transcription was almost undetected in all samples, transcription of CCR7, CCR9, CCR13, CXCR1, CXCR3B and CXCR4 was found in muscle (Fig. 5). However, only the transcription of CXCR3B was significantly higher in vaccinated fish.

3.3. Trout CK5B and CK6 exhibit chemoattractant capacity

Since the chemoattractant capacity of trout CK5B and CK6 had not been studied, we produced these chemokines as recombinant proteins and purified them under denaturing conditions (Supplementary Fig. 2). Recombinant CK5B and CK6 were used in chemotaxis assays in vitro using blood, spleen and head kidney leukocytes. Blood and head kidney leukocytes were significantly attracted by recombinant CK5B at a concentration of 1 ng/ml, while splenocytes did not respond to CK5B (Fig. 6A). In the case of CK6, only head kidney leukocytes were significantly attracted by this chemokine (Fig. 6B). These results confirm that CK5B and CK6 both have chemotactic capacities.

To further support the hypothesis that the up-regulation of CK5B and CK6 transcription observed at day 6 post-vaccination

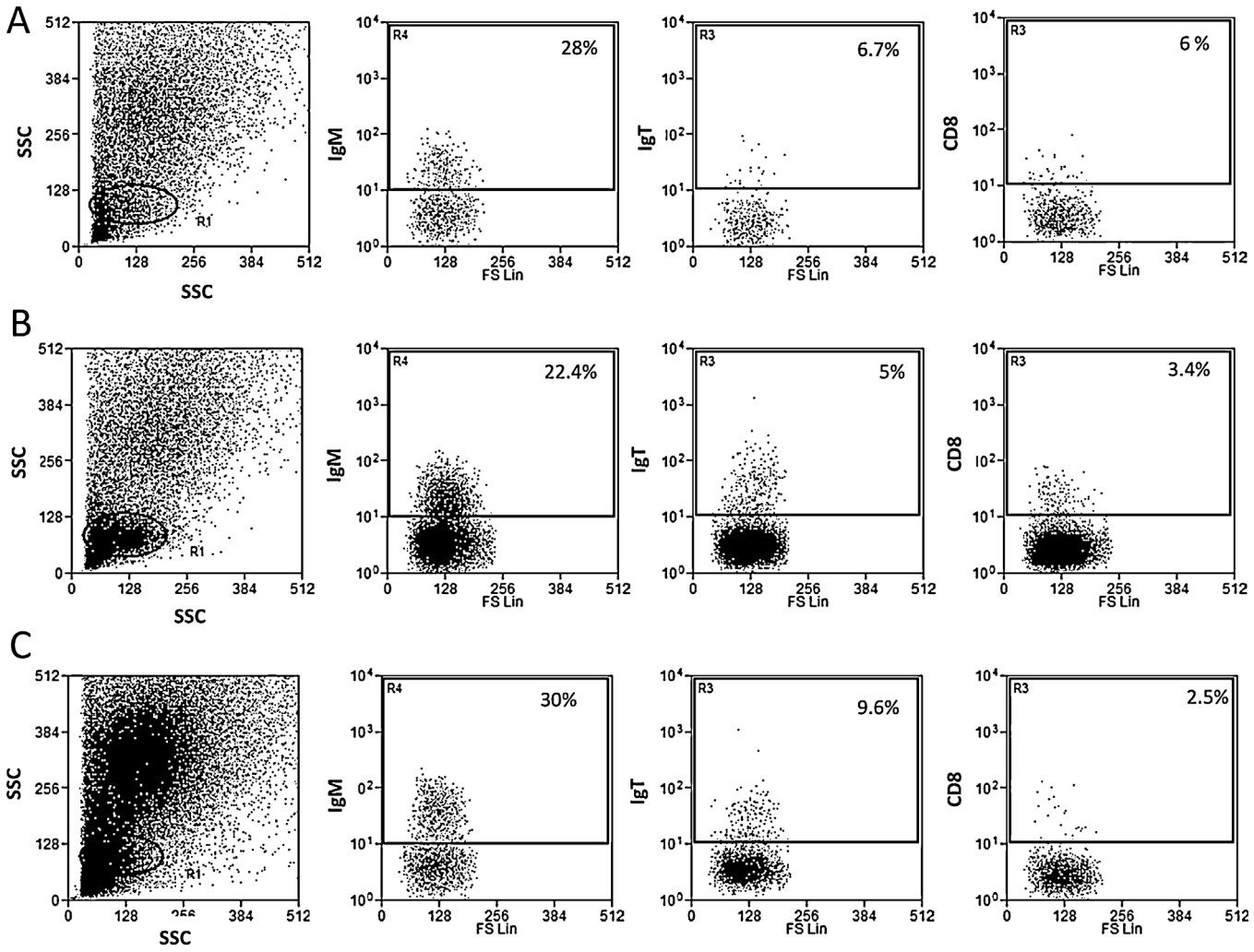

Fig. 3. Flow cytometry dot plots showing IgM$^+$, IgT$^+$ and CD8$^+$ cells isolated at day 7 from the muscle of fish injected with the empty pcDNA plasmid (A), with the VHSV DNA vaccine (B) or with CFA (C). Percentages of specific cell types within the lymphocyte population (gated region R1) are included in the FSC/FL1 or FSC/FL4 plots, respectively. The data shown correspond to the pooled leukocytes isolated from four fish each. Percentages refer to the positive cells within the lymphocyte region.

was contributing to the recruitment of B cells to the muscle, CK5B and CK6 coding plasmids were injected i.m. and the infiltration of IgM$^+$ and IgT$^+$ cells studied through immunohistochemistry. The infiltration of IgM$^+$ cells in response to both pCK5B and pCK6 was visible through immunohistochemistry (Fig. 6C and D). Conversely, we could never detect a significant infiltration of IgT$^+$ cells.

4. Discussion

Although different studies have analysed different aspects of the immunogenicity of DNA vaccines in fish, none have addressed the early recruitment of leukocytes to the muscle, or analysed which chemokines and chemokine receptors could be responsible for such a mobilization. In mammals, it is well known that myocytes do not express MHC class II nor co-stimulatory molecules and therefore the priming of T cells occurs through the recruitment and activation of professional antigen presenting cells (APCs), mainly dendritic cells (DCs) [37,38] and not B cells, which are only marginally activated in response to vaccination [38]. In the current work, we have established that both IgM$^+$ and IgT$^+$ B lymphocytes migrate in large numbers to the area of antigen expression after injection of a VHSV DNA vaccine. In most teleost species, the presence of professional APCs has not been clearly established [39] and no surface markers are available for fish DCs. In addition to DCs, B cells are also considered highly efficient APCs in mammals [40], and therefore it is possible that fish B cells mobilized to the muscle after DNA vaccination could play a role in antigen presentation locally in addition to other APCs, taking into account the absence of lymph nodes in teleost fish. Furthermore, the very high constitutive levels of CD80/86 in trout IgM$^+$ cells reported by Zhang et al. [41], led the authors to speculate that fish IgM$^+$ B cells could be major APCs in fish. We also isolated some CD8$^+$ cells from the vaccinated muscle, even though no CD8 transcripts were detected. On the other hand, we detected CD4 transcripts but we could not analyse the presence of CD4$^+$ cells through flow cytometry because there is no antibody available to date. Interestingly, our results reveal a novel non-mucosal role for IgT$^+$ cells, as also observed by Castro et al. [20]. In any case, more work should be performed to establish the role of all these cells mobilized to the muscle in the immunity to DNA vaccination.

Notably, this massive B infiltration into the muscle was not observed when fish were injected with inactivated virus instead of the DNA vaccine. Although this could suggest that the expression of the VHSV glycoprotein in the cellular membrane of myocytes contributes to a greater immune response, it might be possible that it is a consequence of a higher amount of G protein available due to the strong and prolonged production of G protein observed after DNA vaccination [15]. This higher amount of G protein may trigger

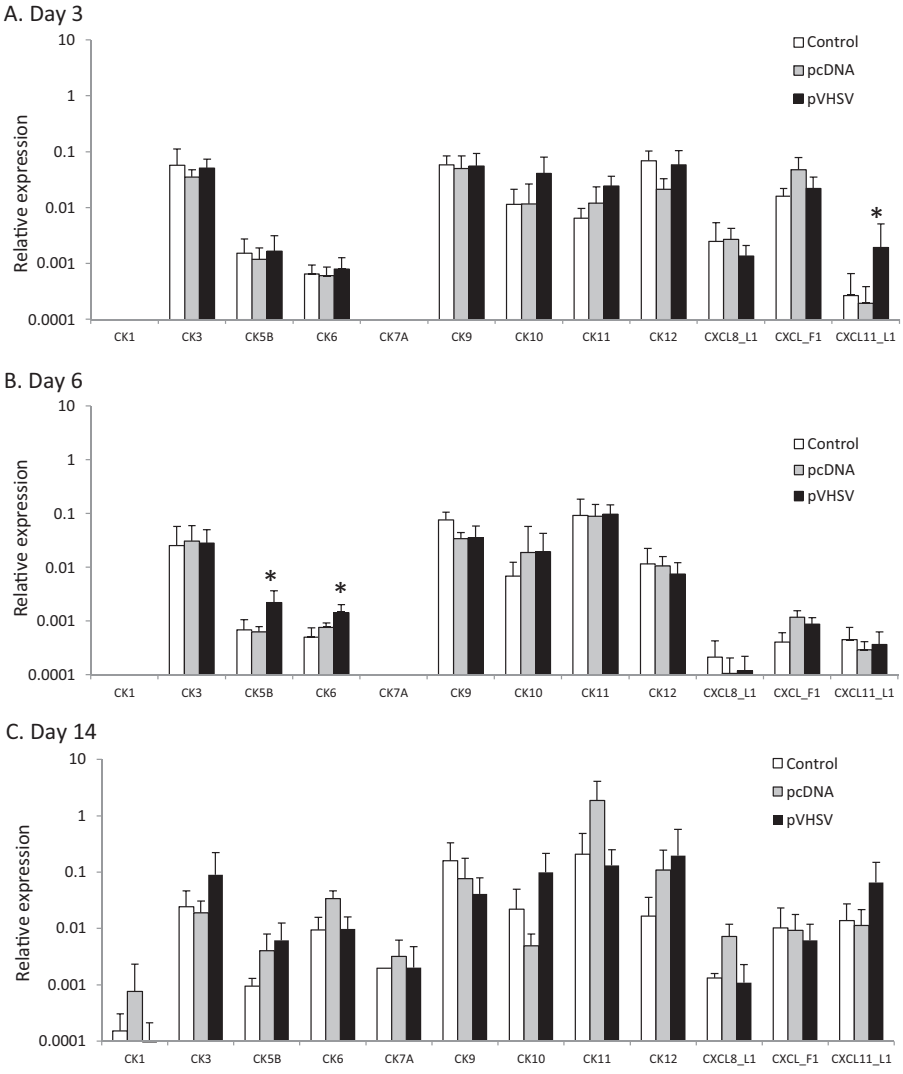

Fig. 4. Chemokine transcription at the muscle injection site. Rainbow trout were i.m. vaccinated with 50 μl of PBS containing 1 μg of the pVHSV vaccine, 1 μg of the empty plasmid (pcDNA) or PBS only as a control. At days 3 (A), 6 (B) and 14 (C) post-injection six trout from each group were killed and the muscle area around the injection site sampled to determine the levels of expression of a selection of chemokine genes by real-time PCR. Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± *SD*. * Levels of expression significantly different to those observed in fish injected with the empty plasmid ($p < 0.05$).

not only a quantitatively different response but also one of a different nature. Previous reports have shown that the G protein from the rhabdovirus vesicular stomatitis virus (VSV) induces a T-dependent response when inoculated at low doses, but a T-independent response when higher doses are administered [42]. In fact, the VSV G protein has been shown to be an excellent T-independent antigen [43], as occurs with other viral proteins [44]. Interestingly, one of the most effective DNA vaccines tested in mice has been a glycoprotein-encoding DNA vaccine against rabies, another rhabdovirus [45]. Thus, it might be relevant to evaluate whether the responses elicited by all rhabdoviral DNA vaccines have important T-independent components that render them especially effective.

To understand the mechanism by which leukocytes are recruited to the muscle, we studied the levels of transcription of trout chemokines and chemokine receptors. The transcription of CXCL11_L1, a chemokine closely related to mammalian CXCL10 [36], was up-regulated in the muscle of vaccinated fish at day 3 post-injection. Although at this early stage, there is still a considerable infiltration of leukocytes as a result of tissue damage, it might be interesting in the future to study whether CXCL11_L1 is also capable of recruiting B cells as reported in some mammalian species [46], despite the fact that previous evidence in trout suggests that

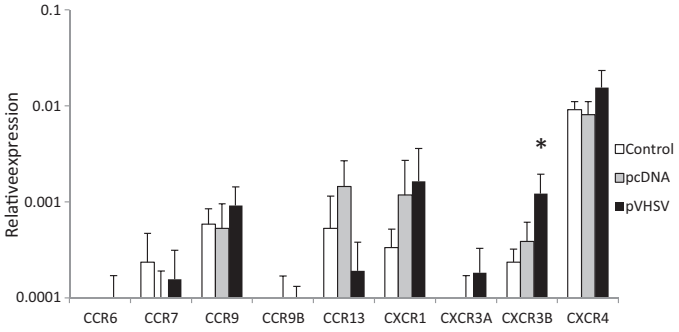

Fig. 5. Transcription of chemokine receptors at the muscle injection site. Rainbow trout were i.m. vaccinated as described in the Fig. 4 legend. At day 6 post-injection six trout from each group were killed and the muscle area around the injection site sampled to determine the levels of expression of rainbow trout chemokine receptor genes by real-time PCR. Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± *SD*. * Levels of expression significantly different to those observed in fish injected with the empty plasmid ($p < 0.05$).

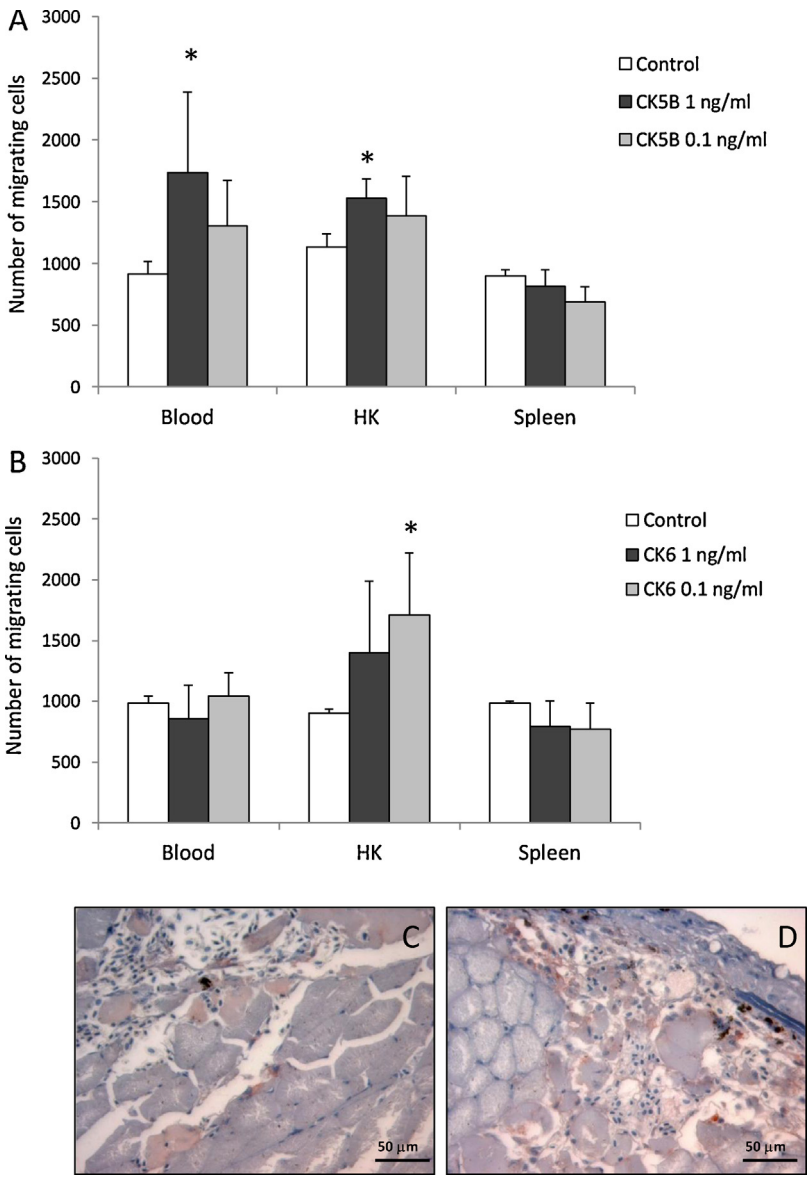

Fig. 6. Chemotactic capacity of rainbow trout recombinant CK5B and CK6 chemokines. The chemotaxis assays were performed in chemotaxis chambers introduced into 24-well plates. Six hundred μl of different dilutions of chemokines in culture medium were placed at the bottom of the wells. Controls with media alone were also included. One hundred μl of the different leukocyte cell suspensions (blood, head kidney, HK and spleen) were loaded into the upper chamber. The upper and lower chambers were separated by a 3 μm pore-sized polycarbonate filter. After 2 h of incubation at 20 °C, the number of cells that had migrated to the bottom of the wells was quantified by flow cytometry at a constant flow time (1 min) of the medium in the lower chamber. The migrating cells were analysed based on side and forward light scatter parameters. Data are shown as the mean number of migrated cells ± *SD* ($N = 6$). * = Number of migrated cells significantly higher than those observed in corresponding controls ($p < 0.05$). C. Detection of IgM in muscle of trout injected with pCK5B. D. Detection of IgM in muscle of trout injected with pCK6.

CXCL11_L1 is mainly chemoattractant for macrophages and $CD4^+$ cells [36]. CXCL10 is a gamma interferon inducible CXC chemokine, as is CXCL9 and CXCL11, all of them sharing the same receptor, CXCR3, which in trout has two different isoforms [47]. Interestingly, we have observed a significant up-regulation of CXCR3B in vaccinated animals that suggests that this chemokine–chemokine receptor association is maintained in teleost. On the other hand, transfection of human primary muscle cells with a DNA vaccine provoked an increased CXCL10 transcription [22]. At day 6 post-vaccination, a significant up-regulation of CK5B and CK6 was found in the vaccinated muscle. According to the classification of CC chemokines established by Peatman and Liu [35], rainbow trout CK5B groups with mammalian CCL5 genes. Although CCL5 is associated mostly with the recruitment of T helper cells [48], it was also up-regulated in the central nervous system together with a significant B cell infiltration [46] and CCR3, one of its possible receptors, has been detected on bone marrow plasma cells [49]. Surprisingly, CCL5 was also up-regulated in human primary muscle cells transfected with a DNA vaccine, suggesting some conservation in the elicited mechanisms in mammals and fish [22]. On the other hand, CK6 has been ascribed to the CCL17/22 group. In mammals, CD40-stimulated B cells secrete CCL17 and CCL22, attracting memory $CD4^+$ T cells to an antigen rich environment [50]. In this study, we have demonstrated the chemoattractant capacity of CK5B and CK6. Furthermore, plasmids encoding trout chemokines provoked an infiltration of IgM^+ cells when injected into the muscle of rainbow trout. Taken together, these data suggest that CK5B and CK6 contribute to the recruitment of B cells into the muscle.

In conclusion, DNA vaccination against VHSV in rainbow trout induced a large infiltration of both IgM^+ and IgT^+ cells B cells. This infiltration was a characteristic feature in response to DNA vaccination which was neither observed after i.m. injection of inactivated

virus nor of CFA and was accompanied with an up-regulation of CXCL11_L1, CK5B, CK6 and CXCR3B genes. We have also established the chemotactic capacity of CK5B and CK6, strongly suggesting that the induced chemokines contribute to the observed leukocyte recruitment to the muscle.

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Conflict of interest statement: Authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.11.062.

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