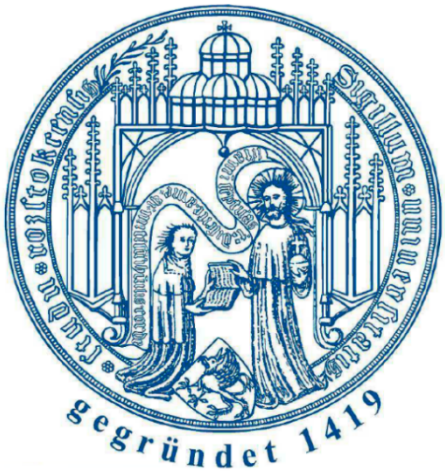
In vitro characterisation of factors controlling the innate immune response in Rainbow Trout (*Oncorhynchus mykiss*)



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Summary

The innate immune system is the first line of defence against infections, responding promptly to pathogen-associated molecular patterns (PAMPs). In terms of traits and components, the innate immune system of teleosts is similar to that of mammals. In addition, teleosts and other fish rely heavily on innate immune responses to fight infections. Despite the fact that gene or protein orthology and conservation allow for function similarity, the Whole Genome Duplication (WGD) that occurred early in the life of ray-finned fishes permitted the formation of paralog genes with novel functions in fish. Additionally, salmonids underwent an extra Whole Genome Duplication, which resulted in more gene copies. In Rainbow Trout (*Oncorhynchus mykiss*), around half of these genes have been preserved as probably functional duplicates, whereas 66 percent of the remaining singletons appear to have a pseudogenised duplicate. As a result of the complexity of their genome, functional concerns of trout genes must first be structural and functionally analysed.

Therefore, the aim of this thesis was focused towards the characterisation of selected factors able to regulate the innate immune response of rainbow trout and in salmonid cell models.

In vivo studies have been carried out on magnetically sorted living cells of myeloid and lymphoid origin from head kidney of naïve trout, upon stimulation with inactivated *Aeromonas salmonicida*.

A panel of 43 genes revealed a prominent reduction of cytokines and NF-kB signalling in the challenged groups, indicating the inhibitory role of *A. salmonicida* stimulation on the early innate immune response.For this reason, the NF-κB and the JAK/STAT pathway have been investigated and two families of factors, PIAS and NKIRAS proteins, have been studied in different tissues and cell populations, upon different stimuli.

In vitro overexpression experiments of *NKIRAS2a* and *NKIRAS2b* revealed a new sub-functionalisation of both transcript variants, apparently able to enhance the NF-κB activation and downstream gene expression. Knockout experiments with CRISPR/Cas9 method highlighted the essential role of PIAS factors in salmonid derived cell line, leading the cells to death. *Per contra,* overexpression experiments indicated a strong inhibitory effect on the NF-kB and on JAK/STAT pathways downstream gene.

Zusammenfassung

Das angeborene Immunsystem ist die erste Verteidigungslinie gegen Infektionen und reagiert schnell auf „pathogen-associated molecular patterns“ (PAMPs). In Bezug auf Eigenschaften und Komponenten ähnelt das angeborene Immunsystem der Knochenfische dem der Säugetiere. Darüber hinaus spielt die angeborene Immunantwort eine große Rolle bei der Bekämpfung von Infektionen bei Teleostier und anderen Fischen.

Trotz der Tatsache, dass Gen- oder Proteinorthologie eine Funktionsähnlichkeit ermöglichen, hat die vollständige Genomverdopplung (Whole Genome Duplication, WGD), die früh in der Evolution der Strahlenflosser auftritt, die Bildung zahlreicher paraloger Gene mit neuen Funktionen in Fischen ermöglicht. Zusätzlich trat bei den Salmoniden eine zweite vollständige Genomverdopplung auf, was zu weiteren Genkopien führte. Bei der Regenbogenforelle (*Oncorhynchus mykiss*) ist etwa die Hälfte dieser Gene als funktionelle Duplikate erhalten geblieben, während 66 Prozent der verbleibenden Singletons ein pseudogenisiertes Duplikat zu haben scheinen. Aufgrund der Komplexität ihres Genoms müssen die funktionellen Bestandteile der Forellengene zunächst strukturell und funktionsabhängig analysiert werden. Daher konzentrierte sich das Projekt auf die Charakterisierung ausgewählter Faktoren, die eine Rolle bei der Regulation der angeborene Immunantwort von Regenbogenforellen und Salmoniden Zellmodellen spielen.

In vivo-Studien wurden an mittels MACS separierten lebenden Zellen myeloischen und lymphoiden Ursprungs aus der Kopfniere naiver Forellen durchgeführt, nachdem diese mit inaktivierten *Aeromonas salmonicida* stimuliert worden waren.

Ein Gen-Panel von 43 ausgewählten Genen zeigte eine deutliche Verringerung von Zytokinen und NF-κB-Signalen in den stimulierten Gruppen, was auf eine hemmende Wirkung von *A. salmonicida* auf die frühe angeborene Immunantwort hinweist.

Aus diesem Grund wurden neben dem NF-κB- und dem JAK/STAT-Signalweg, auch zwei Proteinfamilien (PIAS- und NKIRAS-Proteine) in verschiedenen Geweben, Zellpopulationen und bei unterschiedlichen Stimuli untersucht. *In-vitro*-Überexpressionsversuche von *NKIRAS2a* und *NKIRAS2b* ergaben eine neue Teilfunktionalität beider Transkriptvarianten, die offenbar die NF-κB-Aktivierung und die nachgeschaltete Genexpression verstärken kann.

Knockout-Experimente mittels der CRISPR/Cas9-Methode unterstrichen die essentielle Rolle der PIAS-Faktoren in Salmoniden-Zelllinien, die letztendlich zum Zelltod führen. Im Gegensatz dazu wiesen Überexpressionsversuche jener Faktoren auf eine starke hemmende Wirkung auf den NF-κB-Signalweg und auf nachgeschaltete Gene des JAK/STAT-Signalwegs hin.

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Abbreviations

Aa, amino acid(s)

ANK, ankyrin

CDS, coding sequence

CHSE, Chinook salmon embryo

CIS, cytokine-inducible SH2 domain protein

CRISPR, clustered regularly interspaced short palindromic repeats

CXCL8, C-X-C motif chemokine ligand 8

DNA deoxy ribonucleic acid

ELAM, endothelial cell-leukocyte adhesion molecule

ERK extracellular signal-regulated protein kinases

GDP, guanosine diphosphate

GFP, green fluorescent protein

GTP, guanosine-triphosphate

hpi, hours post infection

IFN, interferon

IL, interleukin

IRAK4, IL-1 receptor associated kinase

JAK, Janus kinase

LG, linkage group

LPS, lipopolysaccharide

MAb, monoclonal antibody

MAPKs, mitogen-activated protein kinases

mEGFP, monomeric enhanced green fluorescence protein

MHC major histocompatibility complex

MKK, MAP kinase kinases

mPlum, mutant variant of red fluorescent protein

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NKIRAS, NF-κB-inhibitor-interacting Ras-like proteins

NLS, Nuclear localisation sequence

nt, nucleotide(s)

ORF, open-reading frame

PAMPs pathogen-associated molecular patterns

PEST, proline (P), glutamic acid (E), serine (S), and threonine (T)-rich domain

PRRs, pattern recognition receptors

PTPs, protein tyrosine phosphatases

qPCR, quantitative polymerase-chain reaction

RAGs, recombination-activating genes

Ras, Rat sarcoma

RHD Rel homology domain

RTG-2, Rainbow Trout Gonad-2

SAP, scaffold attachment factor A/B/acinus/PIAS

sgRNA, short guide RNA

SIM, SUMO-interacting motif

SNP, single-nucleotide polymorphism

SOCS, suppressor of cytokine signalling

SP-RING, Siz/PIAS RING finger

STAT, signal transducer and activator of transcription

SUMO, Small Ubiquitin-like Modifier

TGFB, transforming growth factor beta

TIR Toll/interleukin-1 (IL-1) receptor homology

TLR, Toll like receptor

TNF, tumour necrosis factor

TRAF, TNF receptor associated factor

WGD, whole-genome duplication

1. Introduction

Since the beginning of fish evolution, about 530 million years ago, these marine species have been able to respond to a large variety of environmental stimuli.

In this first fish lineage, known as Agnatha or jawless fish, innate immunity was the only defence system of these organisms.

Only 80 million years later, antigen-specific, adaptive immunity is thought to have emerged with the introduction of recombination-activating genes (RAGs) into the genomes of ancestral jawed cartilaginous fish (Agrawal et al., 1998).

Nevertheless, it is evident that many of the basic innate responses found in primitive jawless vertebrates are still crucial for survival of even the most developed mammals. Besides, it is clear that many of the invertebrate groups without any molecular trace of Major Histocompatibility Complex (MHC) still can recognise self from non-self by the adoption of other types of recognition molecules (Buchmann, 2014). Figure 1 schematise the different immune responses.

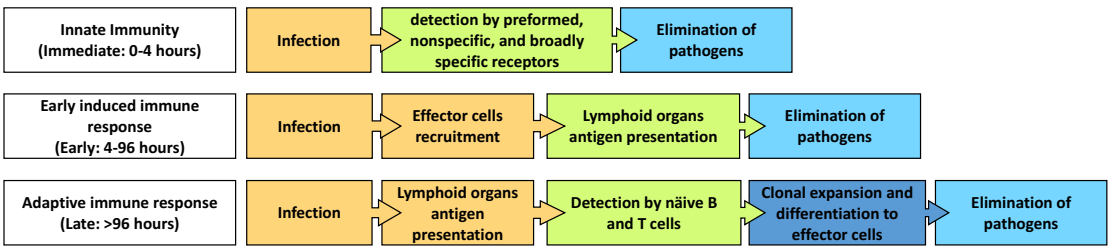

Figure 1: Differences between innate, early induced and adaptive immune response. Adapted from: Immunobiology, 7ed., (© Garland Science 2008, Figure 2-1)

Indeed, the ability to recognise self from non-self emerges as a fundamental function of any living organism (Zhu et al., 2013).

To compensate for their relatively basic and undifferentiated immune system, teleost fish evolved defensive mechanisms that vary from those of mammals by creating a variety of isotypes. Multiple genes have been found in a variety of studies, including cytokines such as TNF and IL1, lymphocyte cell surface markers such as CD4, CD8, and complement components such as C2 and C3 (Rebl & Goldammer, 2018). In

addition to two rounds of whole genome duplication early in vertebrate evolution, there is solid evidence that fish-specific whole genome duplication occurred in ray-finned fish about 320 million years ago (Tsukamoto et al., 2010). Furthermore, cyprinid and salmonid fishes are considered partially tetraploid (Edger & Pires, 2009; T. Wang, Huang, et al., 2011). Although distinct roles of the different gene copies have yet to be fully understood, the extra number of genes resulting from genome or chromosomal duplication may have driven speciation, adaptability, diversification, and promotion of novel activities across evolution (Sunyer, 2013). Therefore, teleost monocytes/macrophages, main player of phagocytosis process, have distinct activities and modulations than mammals due to entire genome duplication and environmental adaptability (Lu & Chen, 2019).

Phagocytosis is the primordial known mechanism of defence, discovered even in the Protozoa, the most primitive eukaryotic kingdom, in 1862 by Haeckel, but only fully understood 16 years later by Elie Metchnikoff (Köllner et al., 2002).

In vertebrate, the leading actors of this process are the macrophages, specialized cells able to recognise microorganisms. Macrophages detect microorganisms via the Pathogen-Associated Molecular Patterns (PAMPs) on their surface by Pattern Recognition Receptors (PRRs), facilitating the phagocytosis and initiating the killing mechanisms (Medzhitov & Janeway, 2000).

These innate immune receptors are a category of germ-line encoded receptors that have a genetically defined specificity and are strongly conserved across species. Pattern recognition receptors have distinct ligand-binding properties for unique types of PAMPs, but they are less discriminatory when it comes to fine molecular structure variations among those classes (Zhong et al., 2006).

Mannose-binding proteins, mannose receptors, scavenger receptors, Tool-like receptors and associated factors are some of the main proteins involved in microbial pattern recognition.

The TLR family is the most relevant and well-studied class in PRRs. TLRs were named and discovered based on their homology to the Drosophila melanogaster Toll protein (Medzhitov et al., 1997). TLRs are integral glycoproteins with an extracellular or luminal ligand-binding domain containing LRR motifs and a cytoplasmic signalling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain. Ligand binding to TLRs through PAMP-TLR interaction results in receptor oligomerization, which then initiates intracellular signal transduction (Dorrington & Fraser, 2019). Once TLRs are stimulated

by specific PAMPs, a variety of signalling pathways are induced. Signal transduction, as represented in figure 2, is initially regulated by a family of adaptor molecules, which determines the specificity of the reaction in part (Kawasaki & Kawai, 2014).

The binding of one or more adaptor molecules to a specific TLR is accompanied by activation of downstream signal transduction pathways involving phosphorylation, ubiquitination, or protein-protein interactions, resulting in the initiation of transcription factors that control the transcription of genes involved in inflammation and antimicrobial host defences (Paludan et al., 2020). TLR-induced signalling pathways can be generally defined based on their uses of various adaptor molecules, i.e., whether they are dependent on or independent of the adaptor MyD88 or TIR domain-containing adaptor causing IFN- (TRIF), as well as their activation of various kinases and transcription factors (Sahoo, 2020).

TLR-induced responses are regulated by three main signalling pathways: (i) NF-κB, (ii) mitogen-activated protein kinases (MAPKs), and (iii) IRF signalling pathway. Whereas NF-κB and MAPKs play critical roles in the induction of a proinflammatory response, IRF pathway regulate the expression of IFNs, which plays a central role on JAK-STAT signalling pathway activation (De Nardo, 2015).

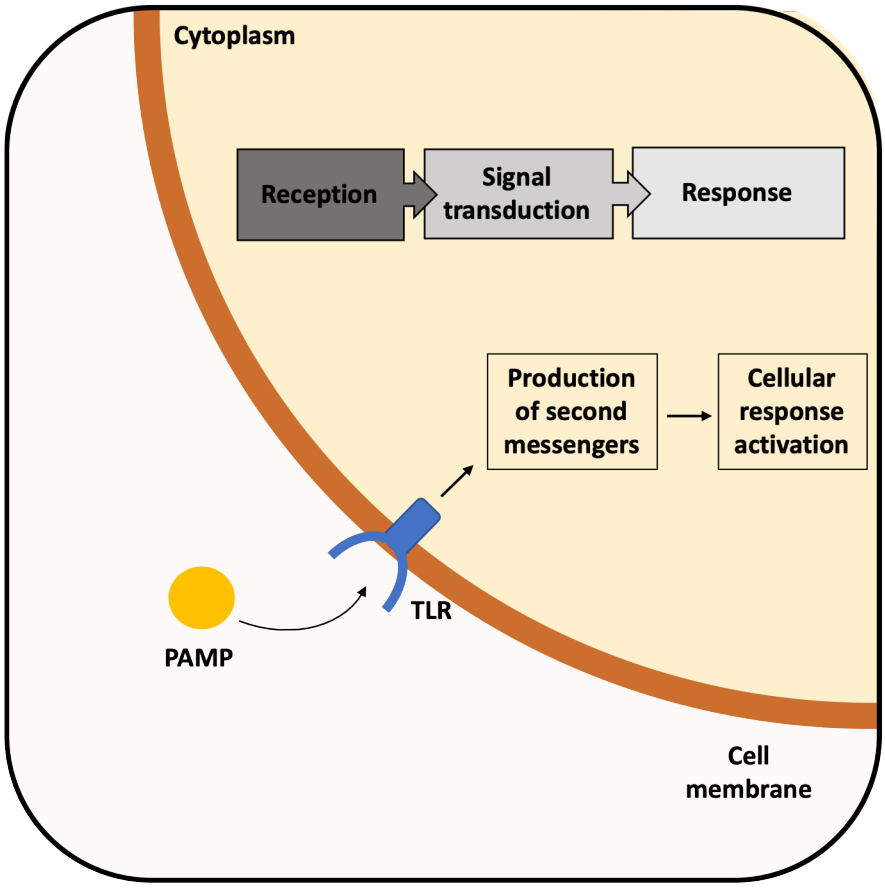

Figure 2: Schematic representation of signal transduction for TLRs. A specific stimulus activates production of second messengers through specific receptors activation and allows cellular response activation.

1.1 Modulation of signal transduction pathways downstream of TLRs

A robust immune response against pathogens requires activation of the signal transduction cascades. On the other hand, deactivating these pathways limits the potentially lethal actions of the immune system on the host and thus prevents it from self-destruction (X. Wang & Liu, 2007). Several systems are in place to precisely control the amount and duration of signals emerging from TLRs in order to avoid such issues.

Upon TLRs stimulation, MYD88 is recruited to the Toll and IL-1 receptor (TIR) domain of the receptor and recruits itself IL-1 receptor associated kinase 4 (IRAK4).

Once activated, IRAK4 forms a complex with IRAK1 and IRAK2, the E3 ubiquitin ligase TNF receptor associated factor 6 (TRAF6) and the E2 ubiquitin conjugating enzyme 13 (UBC13) (Deliz-Aguirre et al., 2020).

Now UBC13 and TRAF6 promote the formation of K63-linked polyubiquitin chains on both TRAF6 and IRAK1, leading to the activation of NF-kB and MAPKK (Hayden et al., 2006) and regulating JAK-STAT dependent-gene expression (Wei et al., 2012).

Modulation at the receptor, adaptor, Myddosome, signalling, transcriptional, post-transcriptional (e.g., miRNAs), and post-translational levels are all critically necessary in order to regulate the response to a specific stimulus (De Nardo, 2015).

In vivo, inflammatory pathway suppression occurs over a time span ranging from seconds to years in wound healing and tissue repair, or is ongoing in chronic inflammation. The initial inflammatory stimulus must be sufficient to generate a response beyond the homeostatic anti-inflammatory threshold for efficient pathogen immunity to evolve (Magarian Blander & Sander, 2012). In the gut, for example, IL-10 inhibits TLR and NLR signalling from the gut flora to maintain normal intestinal function.

Infection with a pathogen invading the mucosal layers of salmonids, such as *Yersinia ruckeri*, linked to the enteric redmouth disease, generates a reaction that surpasses the homeostatic threshold that results in severe inflammation (Kumar et al., 2019).

Consequently, productive regulatory pathways are activated in proportion to the severity of the inflammatory insult and are susceptible to additional levels of control (Hu et al., 2008), as showed in figure 3a and 3b. It is reasonable to assume that there are at least as many anti-inflammatory pathways as proinflammatory pathways activated by microbe- and cell-damage-sensing systems. Anti-inflammatory pathways can be separated into cell-intrinsic and cell-extrinsic mechanisms, with many of them being discovered as a result of unexpected results from extensive examinations of genetically modified mice with inflammatory illnesses. Co-regulated inhibitors of TLR signalling, such as the (i) IL-1 receptor-associated kinase (IRAK-M), (ii) the suppressor of cytokine-signaling (SOCS)-1, (iii) the protein inhibitor of activated STATs (PIAS), (iv) the NF-κB-inhibitor-interacting Ras-like proteins' NKIRAS1 and NKIRAS2, (v) the NF-κB inhibitor (IκB), (vi) A20, and (vii) ABIN1, are examples of cell-intrinsic pathways (Kawasaki & Kawai, 2014). Deletion of any of these components results in complex inflammatory disorders due to a failure to suppress inflammatory signalling after it has been triggered by a microbial product or proinflammatory cytokine such as tumour necrosis factor (TNF). Cell-extrinsic mechanisms include anti-inflammatory cytokines such as TGF-beta, along with a plethora of substances that operate to inhibit inflammation via the aforementioned sequential repair process (Murray & Smale, 2012).

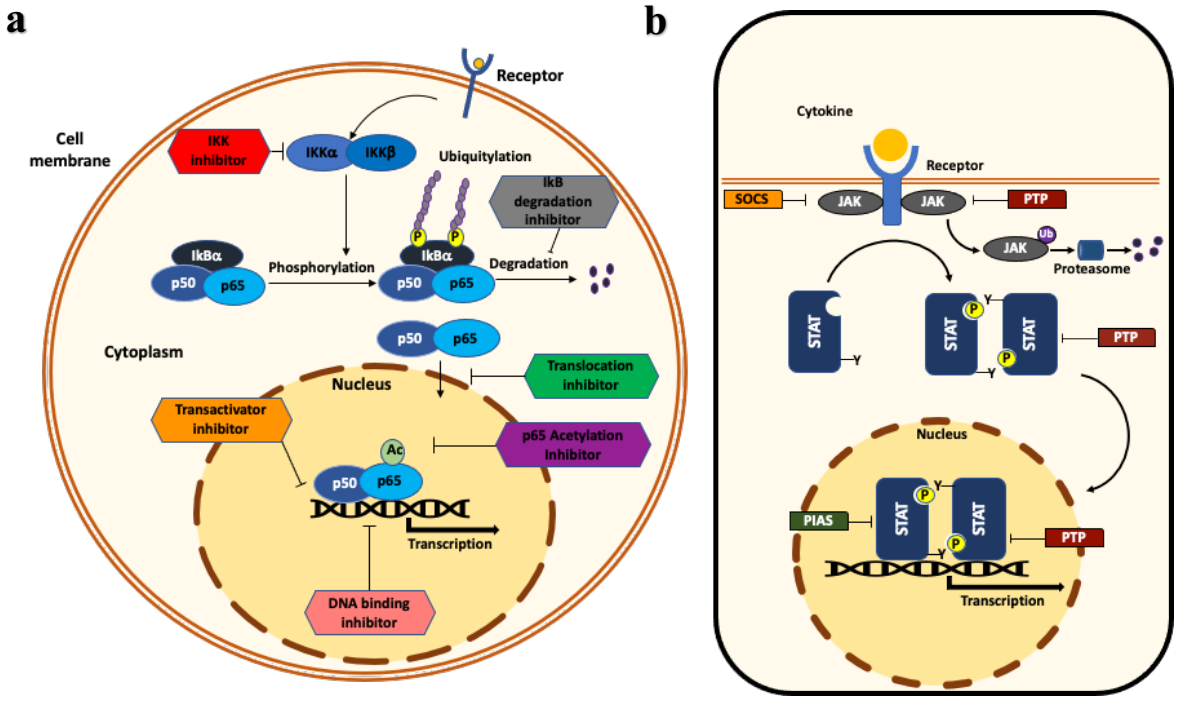

Figure 3: **a**) Inhibition of the NF-kB pathway at different points of the signalling cascade **b**) Inhibition of Jak-Stat Pathway by SOCSs, PIASs and PTP protein families.

Moreover, the control of the signal transduction downstream the TLRs is a specific target of several microorganisms, relying on escaping the immune system of the host their survival despite the fast-acting intracellular signalling pathways generated by PRRs (Reddick & Alto, 2014).

Other effector proteins such as double-stranded RNA-dependent protein kinase (PKR), 2', 5'-oligoadenylate synthetase (2'- 5' OAS), and adenosine deaminase acting on RNA (ADAR) support PRR activity. All of these proteins are involved in detecting viral components and triggering the production of proinflammatory cytokines or interferon (IFN) response factors. Viruses modify various cellular components in order to evade the innate immune response (Langevin et al., 2019).

Most host organisms have developed a second line of defence focused on microbial detection of PAMPs by PRRs and the subsequent generation of cell-intrinsic immune mechanisms and/or recruitment of immune cells if a bacterial pathogen is able to avoid death by antimicrobial peptides. Consequently, many bacterial pathogens have changed the molecular structure of their PAMPs in response to these difficulties, allowing them to escape immune identification through stealth and escape (Matsuura, 2013). Several Gram-negative bacteria use protein secretory mechanisms (such as type III, IV, and VI secretion systems) to transport virulence factors, also known as "effector" proteins, directly into the host cell (David, 2010). The type III translocon has now been proven to be a proinflammatory PAMP that can be identified by both the NLRP3 and NLRC4 inflammasomes (Brodsky et al., 2010). Because signal transduction is essential for relaying information from PRRs to nuclear transcription of proinflammatory modulators, bacterial pathogens primarily target its inhibition. Particularly the mitogen-activated protein kinase (MAPK) signalling axis and the NF-κB pathway, which are key players in innate immunity (Reddick & Alto, 2014). A collection of the most known bacterial effectors is reported on the following table (Table 1).

Table 1: Most known bacterial effectors.

Pathogen	Effector	Activity	Host targets	Pathway	Phenotype	Ref.
Porphyromonas gingivalis	Fimbriae	Binding	CXCR4/ TLR2	NF-κB inhibition	Reduced ROS production	(Hajishengallis et al., 2008)
Mycobacterium tuberculosis	?	Binding	CR-3	p38 MAPK activation	Reduced CD1 expression	(Gagliardi et al., 2009)
***Shigella* ssp.**	IpaH9.8	E3 ligase	NEMO/ ABIN-1	NF-κB inhibition	Inhibition of pro-inflammatory responses	(Ashida et al., 2010)
***Shigella* ssp.**	OspG	Kinase	UbcH5	NF-κB inhibition	Inhibition of pro-inflammatory responses	(D. W. Kim et al., 2005)
***Shigella* ssp.**	OspF	Phosphothreonine lyase	ERK1/2	ERK inhibition	Inhibition of pro-inflammatory responses	(Arbibe et al., 2007)
***Shigella* ssp.**	OspE	Binding	Focal adhesions	ILK activation	Stabilisation of intestinal lining	(M. Kim et al., 2009)
Aeromonas salmonicida	AopP	Acetyltransferase	?	NF-κB inhibition	Inhibition of pro-inflammatory responses	(Fehr et al., 2006)
Vibrio parahaemolyticus	VopA/P	Acetyltransferase	MKKs	MAPK inhibition	Growth arrest	(Trosky et al., 2007)
EHEC	EspG	Binding	ARFs/PAKs	Arf inhibition/PAK activation	Reprogramming of intracellular trafficking	(Selyunin et al., 2011)
EPEC/EHEC	NleH	Binding	RPS3	partial NF-κB inhibition	Increased bacterial colonisation, decreased mortality of host	(Wan et al., 2011)
EPEC	NleE	?	IKKβ	NF-κB inhibition	Inhibition of pro-inflammatory responses	(Vossenkämper et al., 2010)
EPEC	NleC/NleD	Proteases	RelA	NF-κB inhibition	Inhibition of IL-8 secretion	(Pearson et al., 2011)
Legionella pneumophila	LegK1	Kinase	IκB	NF-κB activation	Induction of pro-inflammatory responses	(Ge et al., 2009)
***Yersinia* ssp.**	YopJ	Acetyltransferase	MKKs	MAPK/ NF-κB inhibition	Induction of apoptosis/inhibition of pro-inflammatory responses	(Mukherjee et al., 2006)
***Yersinia* ssp.**	Invasin/YadA	Binding	β1-integrins	FAK activation	Actin rearrangements/bacterial uptake	(Uliczka et al., 2009)
***Yersinia* ssp.**	YopH	Phosphatase	Fyb	Fyn inhibition	Inhibition of phagocytosis	(Winter et al., 2010)
***Yersinia* ssp.**	YopH	Phosphatase	FAK/p130Cas	FAK inhibition	Disruption of focal adhesions/inhibition of phagocytosis	(Black & Bliska, 1997)
Bacillus anthracis	Anthrax toxin	Protease	MKKs	MAPK inhibition	Induction of apoptosis	(Ali et al., 2011)
Bacillus anthracis	LF	Protease	MEK1	MAPK inhibition	Induction of apoptosis	(Park et al., 2002)
Pseudomonas aeruginosa	ExoT	ADP-ribosyltransferase	Crk	Crk inhibition	Inhibition of invasion	(Pielage et al., 2008)

1.2 NF-kB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) signalling pathway

The NF-κB factors represent one of the best characterised families of immunorelevant transcription factors (Hayden & Ghosh, 2011), which comprises five members (p65/RelA, RelB, c-Rel and p50/NF-κB1, p52/NF-κB2) in most vertebrates (Gilmore & Wolenski, 2012).

The highly conserved N-terminal Rel homology domain (RHD), which hosts sequences essential for DNA binding, dimerization, and nuclear localisation, characterises the different proteins of this family.

Nevertheless, they can be divided into two subgroups on the basis of their sequence C-terminal to the RHD and on phylogenetic analysis of sequences in the RHD (Sullivan et al., 2007).

Proteins such as p100 and p105 in vertebrates and Relish in Drosophila are members of one subgroup known as the NF-κB proteins. Many NF-κB proteins have C-terminal IκB-like inhibitory domains composed of ankyrin (ANK) repeats that must be eliminated before the proteins can be activated (Liou & Ph, 2006).

The second subgroup, known as the 'Rel proteins,' is made up of proteins with C-terminal transactivation domains, such as c-Rel, RelA (p65), and RelB in vertebrates and Dorsal and Dif in Drosophila. Unlike NF-κB proteins, Rel proteins are not processed and generally act as transcriptional activators by virtue of their C-terminal domains (Hayden & Ghosh, 2008).

Their paired interaction induces a multitude of well-conserved signalling cascades (Ghosh et al., 1998), that play a central role in the activation, proliferation and apoptosis of vertebrate lymphocytes (Kawasaki & Kawai, 2014) and are thus of particular importance for both innate and adaptive immune responses (Beinke & Ley, 2004). The complex NF-κB-initiated programs of immune-gene expression should be directed against invasive pathogens, but they may exert their destructive potential also against the host itself (Rebl & Goldammer, 2018). Therefore, the activity of NF-κB is controlled by a range of different signal-responsive mechanisms (Dejardin et al., 2002).

Under homeostatic conditions, the 'inhibitors of NF-κB proteins' (IκBs) retain NF-κB factors in an inactive form in the cytosol (Bhatt & Ghosh, 2014). Eight IκB-like family members (IκBα, IκBβ, IκBγ/p105, IκBε, IκBζ, IκBNS/p100, IκBη, and Bcl-3) have been discovered in mammals (Gilmore & Wolenski, 2012).

Salmonids have only seven IκB-like family members, although no IκBβ proteins have been isolated from this fish family. Nevertheless, rainbow trout IκB has six ankyrin repeats, rather like human IκBα. As a result, some IκBα variants may be able to compensate for the loss of IκBβ in rainbow trout (Sarais et al., 2020).

The defining characteristic of IκB proteins is the presence of the ANK repeat, a prototypical 33-residue helix-turn-helix protein motif that is has been found in many proteins from bacteria to humans (Sullivan et al., 2007). They are classified into three subclasses: 1) the inhibitory ANK repeat domains in the C-terminal half of p105 and p100, which must be excluded for NF-κB activation (Hayden & Ghosh, 2008), 2) the independent IκB inhibitor proteins (IκBα, IκBβ, IκBε, IκBζ), which also undergo proteolysis when NF-κB is activated, and 3) the 'atypical' IκBs (Bcl-3 and IκBη) that can be either inhibitors or activators (Sullivan et al., 2007).

The alpha member of IκB proteins is a prototypic representative of the IκB family in mammals (Baeuerle & Baltimore, 1988) and in fish as well (Sangrador-Vegas et al., 2005; L. Wang et al., 2009; Y. Wang et al., 2015).

IκBα has been found to mask the nuclear localisation signals of NF-κB factors and thus prevents their access to NF-κB-specific response elements in the promoters of relevant genes (Henkel et al., 1992).

Many mammalian IκB proteins have additional regulatory sequences in addition to the ANK repeats. Some IκBs (IκBa, IκBb, IκBe, p100) have N-terminal serine residues that serve as IKK phosphorylation sites, and their phosphorylation serves as a signal for K48-linked polyubiquitination and proteasome degradation (Sullivan et al., 2007).

The IKK unit is also known as NEMO for NF-kB critical modulator. The enabled IKK complex catalyses the phosphorylation of IκBs, polyubiquitination, and subsequent degradation by the 26S proteasome, primarily via IKK in an IKK-dependent manner (Henkel et al., 1992).

Small guanosine triphosphatases are another family of proteins that play an important role in the transmission of cell surface signals. Ras, Rap, Ral, and Rho are examples of GTPases (Mayo et al., 2001).

Atypical Ras-like protein, known as κB-Ras, act a strong regulator of NF-κB activation. Indeed, κB-Ras proteins are able to mask the NLS sequences of the Rel-IκB complexes, retaining them in the cytoplasm (Fenwick, 2000).

Both, the canonical and non-canonical NF-κB pathways, represented in figure 4, lead to the activation of NF-κB, while the NKIRAS proteins stabilise the interaction of NF-κB and IκB and thus negatively interfere with the activity of NF-κB (Chen et al., 2003).
Subsequent to a challenging stimulation, the degradation of IκBα is promoted by pathogen-related receptors (PRRs) (DiDonato et al., 1997; Dobrovolskaia et al., 2003) and several pro-inflammatory mediators such as interleukin-1β (IL1B) or tumour necrosis factor α (TNF) (Mercurio et al., 1997).
An alternative NF-κB pathway, named the non-canonical NF-κB pathway, is centered on the processing of p100.
The non-canonical NF-κB activation is IKKβ- and IKKγ-independent, but dependent on IKKα dimmer alternatively (Sun, 2017).
The activation of the IKKα homodimers is regulated by NIK, also known as NF-κB-inducing kinase. NIK collaborates with IKK to cause processing of the p100 C terminus, which results in nuclear translocation of p52:RelB complexes. Finally, the dimers bind to DNA and activate gene transcription downstream. Moreover, while the canonical NF-κB pathway can be activated in minutes and is not dependent on new protein synthesis, the noncanonical NF-κB pathway takes several hours and necessitates the production of new protein (Zarnegar et al., 2008).
The LT-β or BAFF receptor play a crucial role, transferring the signal into the cytoplasm (Cildir et al., 2016).

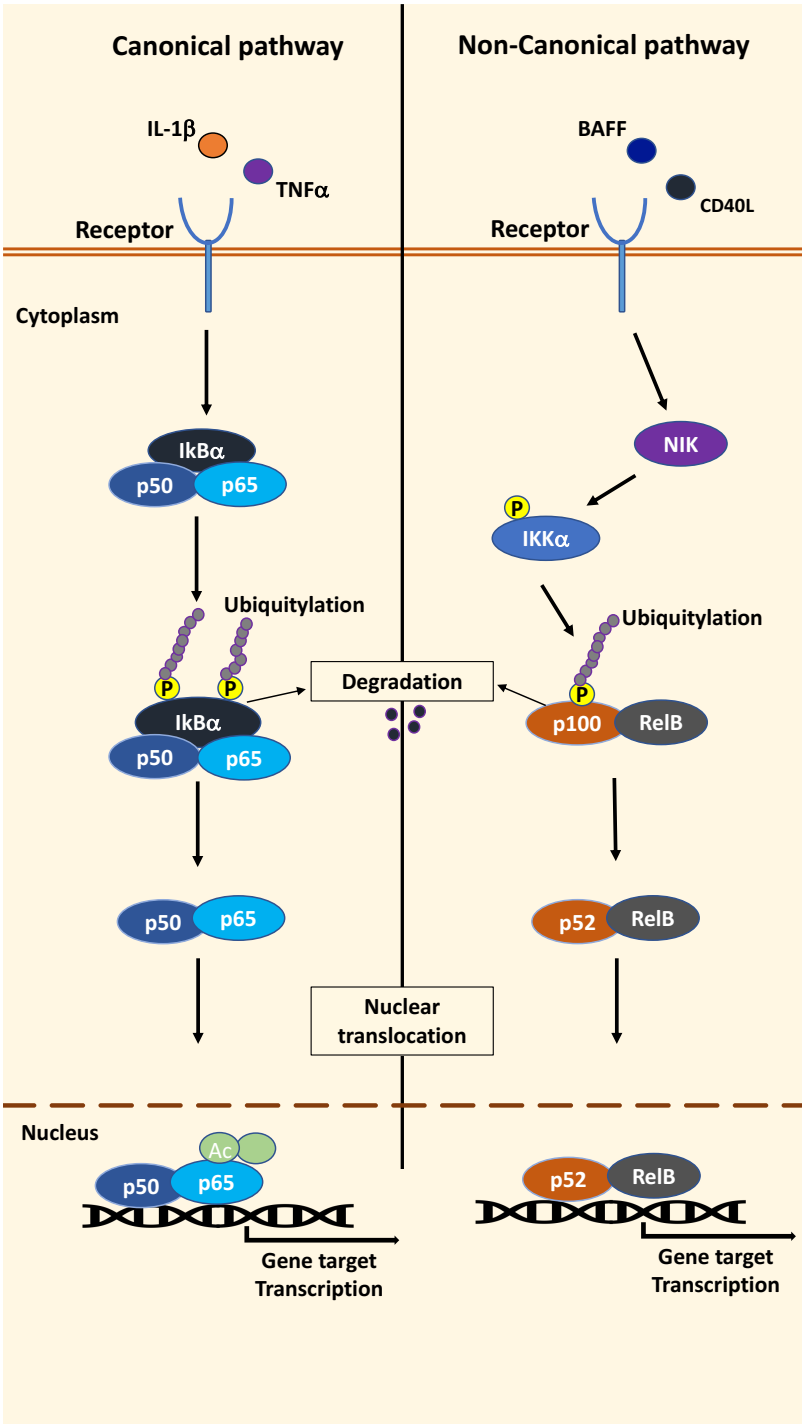

Figure 4: Representation of the canonical and non-canonical NF-κB pathway. Many signals, including those mediated by innate and adaptive immunological receptors, can activate the canonical route. It involves downstream signals activating the IKK complex, IKK-mediated IκB phosphorylation, and subsequent degradation, resulting in the archetypal NF-κB heterodimer RelA/p50 translocating to the nucleus in a fast and transitory manner. The non-canonical NF-κB pathway is dependent on phosphorylation-induced p100 processing, which is initiated by TNFR signalling. This route causes the sustained activation of RelB/p52 complex and is reliant on NIK and IKK, but not on the trimeric IKK complex.

1.3 The mitogen-activated kinase pathway

The MAP kinase cascade, schematically represented in figure 5, is involved in a variety of immune response processes, and it is one of the best conserved signalling pathways within the evolution.

MAP kinases can be divided in three main groups: (i) the extracellular signal-regulated protein kinases (ERK); (ii) the p38 MAP kinases and (iii); the c-Jun NH2-terminal kinases (JNK) (Zou & Secombes, 2011).

Nevertheless, all the MAP kinases share a tripeptide motif, Thr-X-Tyr, which can be dual phosphorylated for their activations.

The second aa residue characterise the three different groups, ERK (Thr-Glu-Tyr); p38 (Thr-Gly-Tyr); and JNK (Thr-Pro-Tyr). The phosphorylation of the Thr and Tyr aa residues is mediated by a set of seven MAP kinase kinases, specific for the different MAP groups (Arthur & Ley, 2013).

The MAP Kinase Kinases (MKK) MKK1 and MKK2 activate the ERK MAP kinases; the MKK3, MKK4, and MKK6 activate the p38 MAP kinases; and MKK4 and MKK7 activate the JNK pathway. Other MAP Kinase Kinase kinases (MKKK) activate the several different MAP kinase kinases. The MKKK are usually activated by G small protein as Ras and Rho family GTPases (Zou & Secombes, 2011).

MAP kinases are also required for the synthesis of various cytokines, chemokines, and other inflammatory mediators that mobilise the immune system to fight pathogenic infections in innate immune cells. MAP kinases have a role in the clonal expansion of effector T- and B-lymphocytes in adaptive immunity cells by modulating cytokine synthesis, cell proliferation, and survival (Dong et al., 2002).

Because MAP kinase pathways are activated by phosphorylation, phosphatase-mediated dephosphorylation of MAP kinases represents a highly efficient mode of kinase deactivation. MAP kinases are known to be deactivated by a variety of protein phosphatases, including tyrosine, serine/threonine, and dual-specificity phosphatases (Dong et al., 2002). In order to induce specific cellular responses to extracellular influences, the MAPK pathways must be both activated and deactivated.

Several serine/threonine phosphatases, including PP2A and PP2Ca, as well as tyrosine phosphatases, including PTP-SL and HePTP, have been shown ability to inactivate MAPKs. Furthermore, several MAPK phosphatases (MKPs) have dual specificity and can dephosphorylate both threonine and tyrosine residues in MAPKs (Keyse, 2000).

The binding of various MKPs to their substrate MAPK controls their catalytic activity. Individual MKPs differ in terms of expression pattern, subcellular localisation, and MAPK specificity, indicating the particular function of each MKP. Some MKPs are activated by their target MAPK pathways and function as negative feedback mechanisms. As a result, MKPs are engaged in MAPK inactivation to avoid excessive and incorrect signalling, which may result in a deficiency in normal cellular activities (Arthur & Ley, 2013).

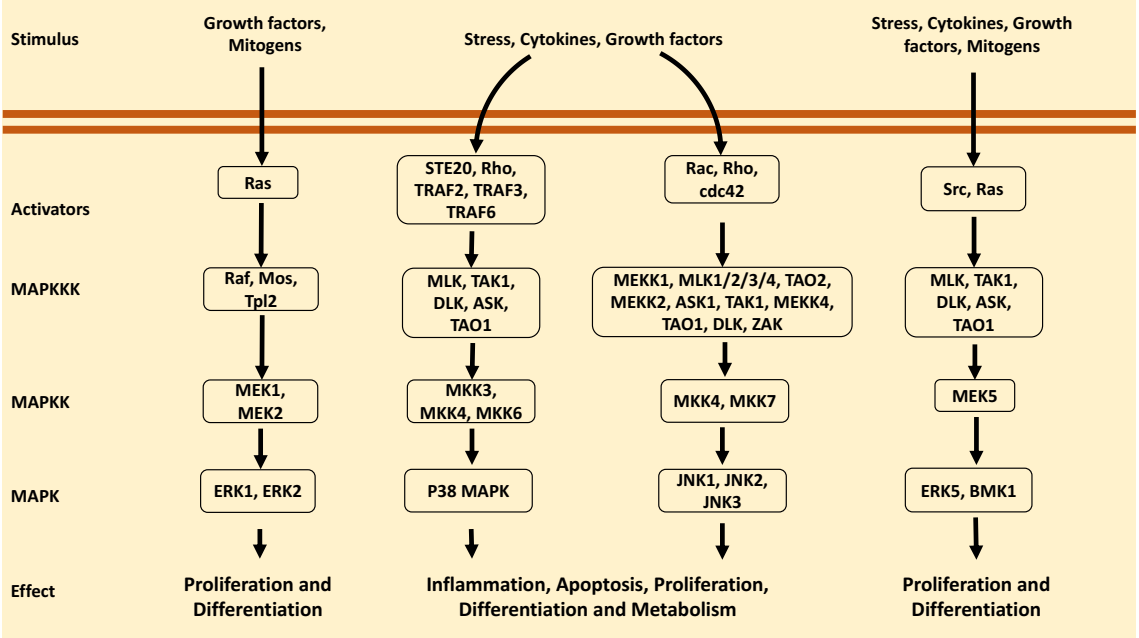

Figure 5: The four major MAPK cascades including their stimuli and substrates. The ERK pathway is usually activated by growth factors, while the JNK, p38 and ERK5 can be activated by stress, cytokines and growth factors.

1.4 JAK-STAT signalling pathway

The type I and type II cytokine-receptor superfamily include receptors for interferons, many interleukins, and colony-stimulating factors. These cytokines all follow the same signalling route: the JAK–STAT pathway (Fig. 6a).

The signalling through Janus kinases (JAK) and signal transducers and activators of transcription (STAT) (Rane et al., 2002; Wilks, 1989) transduces signals for development and homeostasis between the membrane and the nucleus (Bousoik & Montazeri Aliabadi, 2018; Rawlings et al., 2004). Upon the stimulus-dependent

activation of specific cytokine receptors, the four mammalian JAK proteins (JAK1, JAK2, JAK3 and TYK2) become activated and phosphorylate several other associated proteins, including themselves, other receptor chains and STAT factors (Laurence, 2012) (Table 1A). The seven mammalian STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) dimerise following phosphorylation and translocate into the nucleus, where they bind to specific DNA elements and regulate the transcription of target genes (Villarino et al., 2017).

The stat repertory in ray-finned fishes includes the same categories as in coelacanths and tetrapods, with stat1, stat2, stat3, stat4, stat5, and stat6 present in every teleost species. Two copies of each stat gene should have been produced when a WGD happened 350 million years ago during the early development of this group (Christoffels et al., 2006). This stat repertoire has been changed as a result of further duplication and gene loss. In Salmonids, a fish family tetraploidised by an additional WGD about 50–60 Myr ago, have been recognised two blocks *STAT3+5*, two blocks *STAT4+1*, four or five copies of *STAT1*, but only one *STAT2* and one *STAT6* gene, either as a result of an early total loss post-WGD or as a result of persistent selection pressures favouring a single copy (Boudinot et al., 2021).

The majority of STAT dimers identify an inverted repeat DNA motif with an 8–10 base pairs consensus sequence of 5'-TT($N_{4–6}$)AA-3'. Variations in the precise nucleotide sequence determine the differential binding affinity of a single activated STAT dimer for a single target DNA sequence.

This consensus DNA element is commonly known as a GAS element, referring to its initial identification as an interferon activation sequence detected by STAT1 homodimers. Cooperative dimer-dimer interactions mediated by NH2 -terminal amino acids influence the affinity of a STAT-DNA complex for a specific target gene promoter (Aaronson, 2002).

The various STAT proteins show different functions, although they are activated by overlapping sets of cytokines (O'Shea et al., 2013). The vertebrate STAT1 and -2 proteins mediate interferon (IFN) signalling, STAT4 and -6 mediate interleukin signalling and STAT3, -5A and -5B respond to a wide range of cytokines (Leonard & O'Shea, 1998).

Recent research has revealed that JAK–STATsignalling may be controlled at many stages via different methods. The suppressor of cytokine signalling (SOCS) proteins and

the recently identified protein inhibitor of activated STAT (PIAS) family, as well as other protein tyrosine phosphatases (PTPs), are important regulators.

JAK and STAT modulation by various protein changes, as well as crosstalk between distinct JAK–STAT pathways and other cellular signalling pathways, gives additional levels of control that may be critical (Fig. 6b).

The JAK–STAT signalling regulators SOCS proteins are important modulators in the immune system (Shuai & Liu, 2003).

All the eight members of the mammalian SOCS family have been found in fish, with several unique teleost members presumably resulting from fish-specific gene/genome duplication events (T. Wang, Gorgoglione, et al., 2011).

SOCS proteins have an SH2 domain flanked by a variable amino-terminal domain and a carboxy-terminal SOCS box. SOCS1, SOCS2, SOCS3, and CIS have all been extensively researched in the control of JAK–STAT signalling. These SOCS proteins are usually produced at low levels in unstimulated cells and are rapidly increased by cytokines, blocking JAK–STAT signalling and generating a classic negative-feedback loop (Yoshimura et al., 2007).

PIAS proteins (protein inhibitor of activated STAT) were first discovered as suppressors of STAT signalling (Liu et al., 1998). Four PIAS proteins have been so far identified in mammals (PIAS1, PIAS2, PIAS3 and PIAS4) and three in teleosts (pias1, pias2, pias4). The loss of PIAS3 gene in bony fish, probably as consequence of a WGD, has been compensated with the expansion of the gene pias gene family, by increasing the number of transcript factors. In trout, eight *Pias* genes encode at least 14 different pias transcripts (Sarais et al., 2021).

STAT signalling is regulated by all members of the PIAS family, according to recent data. PIAS1, PIAS2, and PIAS3 interact with STAT1, STAT3, and STAT4, respectively. In addition, PIAS4 also interacts with STAT1 (Shuai & Liu, 2005). PIAS1 and PIAS3 suppress STAT-mediated gene activation by inhibiting STATs-DNA binding activity. PIAS4 and PIASx, on the other hand, inhibit STAT1 and STAT4-mediated gene-activation without altering STATs-DNA binding activity. PIAS4 and PIAS2 suppress transcription by attracting additional co-repressor molecules such as HDACs (Niu et al., 2018). Besides, over 60 proteins, many of which are transcription factors involved in the immune system, are thought to interact with the protein inhibitor of activated STAT (PIAS) family (Shuai, 2006). The similarity between STATs and the Rel family proteins allows this proteins family to regulate the NF-kB pathway. PIAS

proteins can interact with NFKB1-p65 and repress the transcriptional activity (Liu et al., 2005).

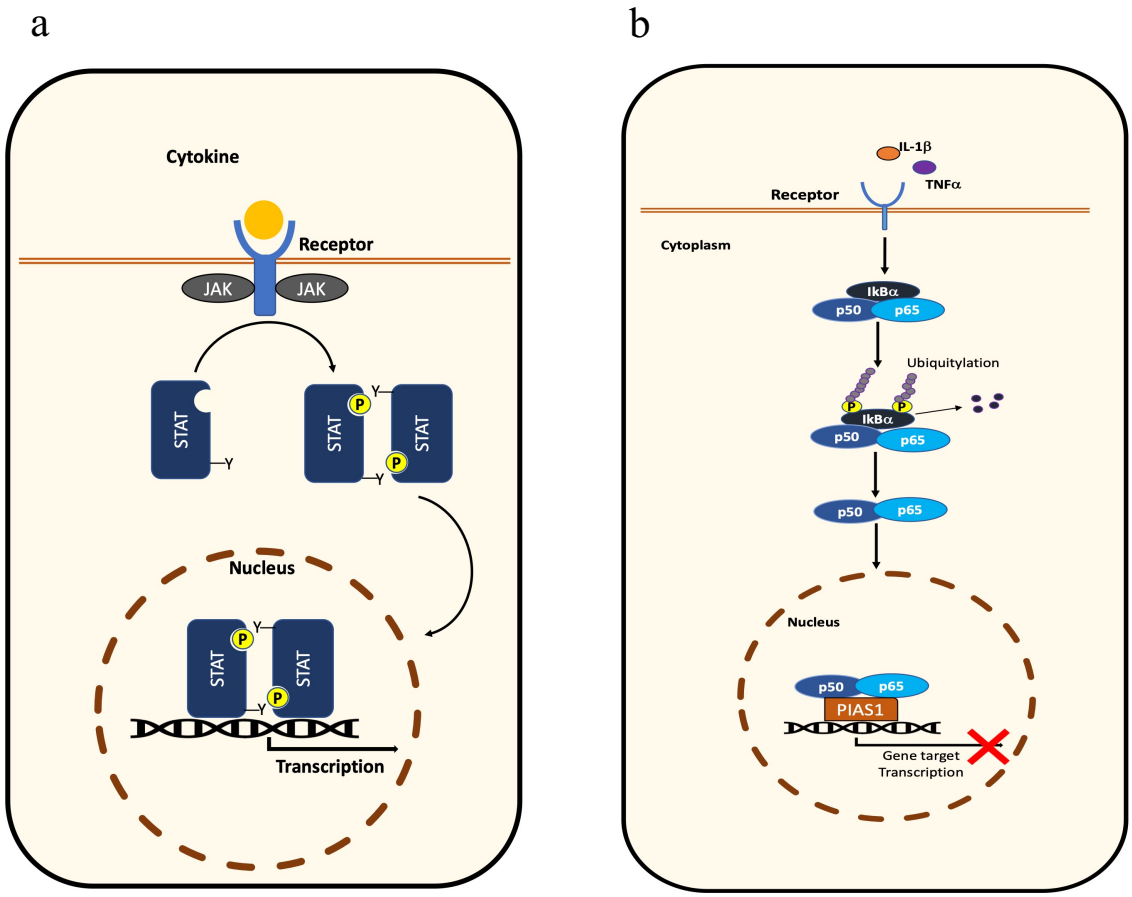

Figure 6: a) A wide range of cytokines and growth factors activate the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway. JAK proteins are activated when a ligand binds to its receptor. Activated JAKs self-phosphorylate and phosphorylate (P) the receptor with which they are connected. This activates the STAT transcription factor family, which is phosphorylated by JAKs, resulting in STAT protein homo- or heterodimerization and translocation to the nucleus, where they bind to response sites on DNA and regulate transcription of downstream target genes. b) PIAS1 inhibits NF-κB target genes expression blocking the accession to promoter sequences.

1.5 CHSE cells and alternative cell models from fish

The literature on fish cell and tissue culture is substantial, and it contains reports on fish viruses and viral illnesses. The necessities of fish researchers significantly encouraged

the development of fish cell culture to the current level of routine application in research and diagnosis. Fish cell cultures are promising in vitro techniques for investigating the host defence mechanism and, as a result, aiding in the utilisation of immunological information for the health protection of aquacultured fish and shellfish. Additionally, piscine cell culture has significant benefits over mammalian cell culture in terms of thermal flexibility, stronger tolerance to hypoxia, and easier cell culture preservation for extended periods (Goswami et al., 2022) .

Since the first establishment of the RTG-2 cell line in 1962 (Wolf & Quimby, 1962), several fish cell lines have been isolated from various tissues, including the ovary, fin, swim bladder, heart, spleen, liver, eye muscle, vertebrae, brain, and skin (Fig. 7). In 1994, 124 novel fish cell lines from various fish species have been reported. By 2010, around 283 cell lines had been created from finfish all over the world (Pandey, 2013).

Recent reviews indicate that in 2020, 783 cells line derived from different tissues of finfish has been so far isolated (Thangaraj et al., 2021).

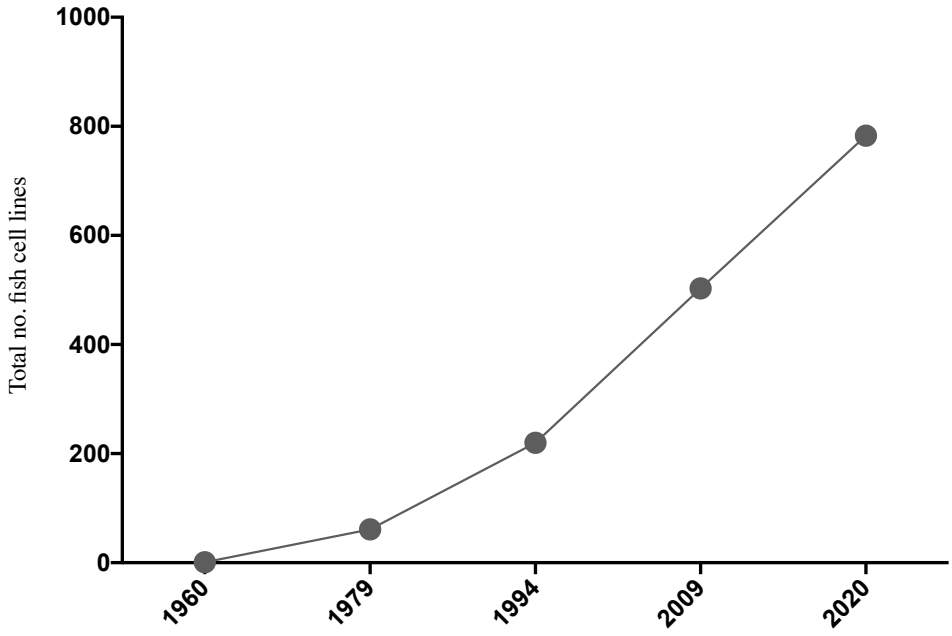

Figure 7: Number of finfish derived cell lines isolated since 1962 according to recent reviews.

CHSE-214 is an epithelial cell line that was created in 1964 from Chinook salmon embryonic tissue (McCain, 1970).

Thenceforward, numerous research on this cell line led to intriguing discoveries on the development of an antiviral response to extracellular dsRNA (Lannan et al., 1984), transfection investigations (Hansen & Jørgensen, 2007), fish virus proliferation (Song et al., 2005) and cytokine signalling due to viral infections (Fig. 8) (Jensen et al., 2002).

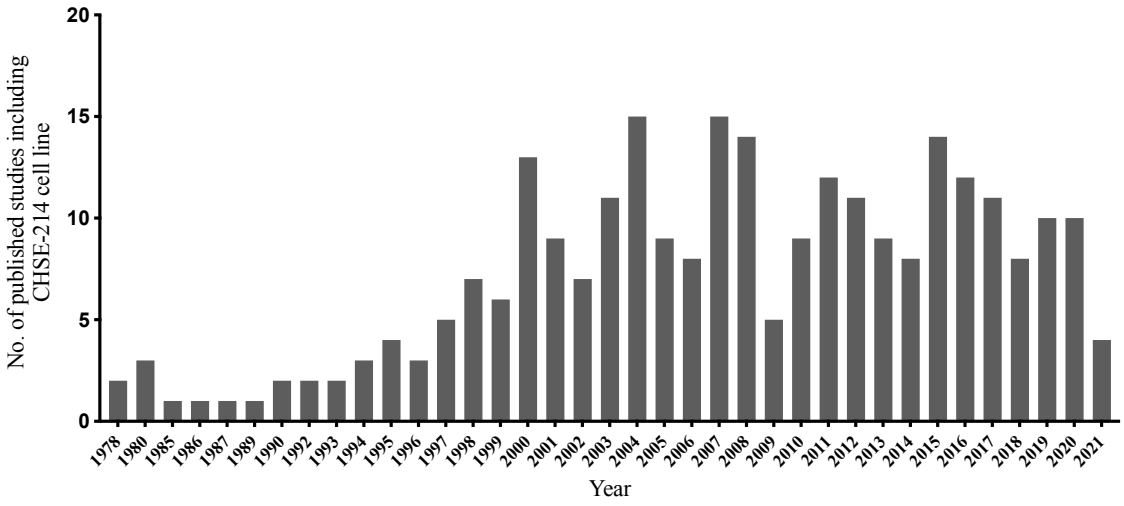

Figure 8: Number of research articles per years on PubMed Database (https://pubmed.ncbi.nlm.nih.gov/) including CHSE cells since 1978.

Noteworthy, even though the use of CRISPR-Cas9-based gene editing methods in fish has previously been reported, for the first time, an efficient approach for gene editing was created in a fish cell line CHSE derived from Chinook salmon *Oncorhynchus tshawytscha* (Dehler et al., 2016). CRISPR/Cas9 genome-editing technologies offer the ability to significantly accelerate genetic gain for production attributes. Infectious illness, for instance, is a severe barrier to aquaculture productivity and hence a key target for selective breeding and genome-editing methods. Due to the difficulty of nondestructive measurement of the trait in breeding candidates, the feasibility of utilising cell culture genome-wide pooled CRISPR screens, and the frequent availability of early life in vivo-established challenge models, host resistance to certain pathogens is a suitable trait for the use of genome-editing technologies (Gratacap et al., 2019).

1.6 Hypothesis and aim of the study

This work of the Campus bioFISCH M−V was financed by the European Maritime and Fisheries Fund (EMFF) and the Ministry of Agriculture and the Environment of Mecklenburg-Western Pomerania, Germany (Grant #: MV-II.1-LM-004).

Fish diseases endanger the aquaculture industry, but only little is known about the concerted interaction of immune-relevant genes rainbow trout, mostly about inhibitors, during host-pathogen interaction. Several immune genes inhibitors have been studied in this thesis to provide the basic knowledge of more effective preventive measures against diseases.

Thereby, the overarching hypothesis are:

i) Rainbow trout inhibitors of the main innate immune pathways developed different functions compared to their mammalian counterparts.

ii) Inhibitors play a key role during pathogen challenges, and they may serve as valuable indicative parameters to evaluate the immune status activation and response.

Genome duplication has a long history in salmonids (e.g., Atlantic salmon, Pacific salmon, and trout). Salmonids have had one additional genome duplication, in addition to the three ancient genome duplications that all teleosts are thought to share.

Thus, due to the complexity of their genome, functional considerations of trout genes therefore first require their structural and functional analysis.

The main objectives of the present work are consequently:

i) Expression analysis of innate immune response during inactivated Gram-negative (*A. salmonicida* ssp. *salmonicida*) stimulation on relevant immune cell fractions derived from head kidney of rainbow Trout. **(Study III)**

ii) Characterisation of candidate genes involved in the inhibition of innate immune pathways of rainbow Trout. **(Study I and II)**

2. General discussion

The initial defence mechanism against infections is the innate immune system, which responds quickly to pathogen-associated molecular patterns (PAMPs). Teleosts have a similar innate immune system than mammals, in terms of characteristics and components.

In teleost fish, there are evident orthologous cytokines, including of the Class I, Class II, chemokines, TNF superfamily, and IL1 families, which are similar to PRRs.

This evidence, when combined with the PRR orthologues, implies that the innate immune system in teleost fish uses the same general recognition and cascade mechanism as mammals.

Although gene or protein orthology and conservation allow for the possibility of function similarity, the Whole Genome Duplication (WGD) that happened early in the life of ray-finned fishes, allowed the creation of numerous novel or semi-novel genes with novel functions in fish (Tsukamoto et al., 2010).

It is possible that evolution pursued different roads to the "economics of genomics" by finding different ways to regulate genes (Wittbrodt et al., 1998).

About half of these genes have been maintained as presumably functioning duplicates in rainbow trout, whereas 66% of the remaining singletons appear to have a pseudogenised duplicate (Kassahn et al., 2009). Additionally, the collinearity and gene order of trout homoeologous chromosomes are still noteworthy, suggesting that the salmonid WGD was caused by an auto-tetraploidisation event (Lien et al., 2016).

Although it has been proposed that more ancient duplications at the base of the vertebrates and early in teleost evolution were a source of extra genetic material, leading to diversification, innovation, and eventually speciation, the functional and evolutionary consequences of genome duplication are still unknown (Uribe et al., 2011).

2.1 In vivo studies, expression profiling of relevant innate immune genes upon inactivated A. salmonicida stimulation

The goal of this research **(Study III)** was to explore the gene expression profile of the early immune response in myeloid (Mab21P) and lymphoid (Mab21N) cells from the rainbow trout head kidney and to identify potential regulatory factors to further

investigate. To determine the immunological state following intraperitoneal (i.p.) stimulation with the extremely pathogenic strain JF5505 of inactivated *A. salmonicida* ssp. *salmonicida*, genes specific for innate and adaptive immunity were chosen.

In salmonids and other fish species, the head kidney resembles the shape and function of mammalian bone marrow, and it is one of the most significant hematopoietic organs. In the head kidney, macrophages and other myeloid cells differentiate (Ellis, 2001).

These cells play a crucial role in the innate immunity defence against a wide range of infections (Weiskopf et al., 2016). Our findings revealed a general downregulation of the genes associated with macrophages and dendritic cells, with the exception of *LYG*, *SPIC*, and *CD209*, which became progressively more expressed at the later time points investigated.

CD209 is an important mediator of dendritic cell/T-cell clustering and T-cell activation (Lin et al., 2009). These data suggest that the early immune response has been effectively activated, which is supported by the increased expression of the inflammation marker *IL1B*.

The downregulation of *CD83*, *CD80/86*, and *DAA* levels in myeloid cells may be caused by reduced NF-κB factor activation. Concurrently, decreasing levels of *CD83*, *CD80/86*, and *DAA* may imply a reduced ability of T cells to activate, as well as decreased lymphocyte proliferation and cytokine release in the head kidney (Axelsson et al., 2020; Schwenteit et al., 2013).

The first downregulated and then elevated gene expression level of T-cell receptor gamma (*TARP*) in the fraction enriched in lymphocytes and thrombocytes might be explained by a mechanism known as state-dependent inactivation: The T-cell receptor complex adapts to continued stimulation after complete downregulation (Trendel et al., 2019).

The registered *CD36* decreased expression may be due to a particular signalling mechanism that activates platelets (Fink et al., 2015). Fish thrombocytes, unlike human thrombocytes, are nucleated cells that may phagocyte and control the immune response. Rainbow trout thrombocytes express genes encoding proteins involved in activation and aggregation, antigen presentation, and immunological regulation (Köllner et al., 2004).

Previous research has shown that toll-like receptor (TLR) ligands can downregulate *CD36* via TLR-induced cytokines (Sánchez-Gurmaches et al., 2012; Triantafilou et al., 2006).

Upon stimulation with inactivated *A. salmonicida*, a profile of the complement characteristic genes, revealed an overall downregulation of their expression. For several Gram-negative bacteria, complement avoidance methods have already been described (Heesterbeek et al., 2018). In the first 12 hours post-stimulation (p.s.), the gene expression of complement components in myeloid cells was severely reduced if compared to control groups. The downregulation of *CFH* and *CFI* at each time point examined suggests that *A. salmonicida* suppression of the complement system is not limited to the early stages of infection. Bacteria, including *A. salmonicida*, have been shown to have and change surface antigens such outer membrane proteins, capsules, and lipopolysaccharide (LPS) in order to suppress the complement system (Lambris et al., 2008). Membrane antigens most likely interfered with the pro-inflammatory response, as evidenced by a decrease in *IL6* and a slight rise in *IL1B* levels at early time points. These pro-inflammatory cytokines stimulate the transcription of complement-related genes, which were therefore low in abundance (Volanakis, 1995). In the first two time periods examined, we found no evidence of important regulation in the Mab21N cells.

This lack of response could be explained by the influx of activated cells to the peritoneum, leaving only a remaining part of inactivated cells on the head kidney, which did not activate any of the immune related genes.

Earlier stimulation reduced the production of a variety of proinflammatory factors in myeloid and lymphoid cells. Another sign of *Aeromonas* inhibitory function was the downregulation of immune system regulators (*IL10*, *SERPING1*, *NKIRAS2a*, *RIP2K*, *PIAS*) in both cell fractions.

The expression profiles of *PIAS* and *NKIRAS* gene variants on the two analysed immune relevant cell populations, Mab21N and Mab21P, demonstrated a noteworthy expression difference, providing more evidence of sub-functionalisation.

The gene profiling of the five *NKIRAS* transcripts in rainbow trout lymphoid and myeloid cells indicated that all factors are higher expressed on myeloid derived cell rather than lymphoid cells, except *NKIRAS1*, highlighting the importance of *NKIRAS1* to maintain a homeostatic level of NF-κB activation (**Study I**).

Meanwhile, lymphocytes and platelets-enriched cell fraction contained predominantly pias2a2 transcripts, which are highly homologous to pias1a1 transcripts, the others Pias transcripts were expressed at the greatest levels in the cell fraction enriched with myeloid cells, indicating the relevance of Pias factors in immunophysiology (**Study II**).

Additional transcript profiles on other immune relevant organs in teleosts as peritoneum and spleen upon the same stimulation previously described, revealed an interesting regulation of the *PIAS* and *NKIRAS* factors. *NKIRAS2a* transcripts were downregulated during all the considered timepoints, supporting a reduction of the pro-inflammatory factors.

PIAS1 and *PIAS2* genes expression profiles followed the same downregulatory effect as observed for *NKIRAS2a*. Nevertheless, expression analysis of sorted B IgM+ cells from head kidney and spleen showed a mild upregulation.

Furthermore, the gene analysis of the five *NKIRAS* transcripts in rainbow trout lymphoid and myeloid cells **(Study I)** indicated that *NKIRAS* expression remained at a usually low transcript level or was only minimally changed following bacterial infection. A review of 50 transcriptome investigations on salmonid fish following diverse immunological stimuli indicated that the copy counts of *NKIRAS1* and *NKIRAS2* seldom surpass those reported in control fish. This suggests that the transcription of *NKIRAS* genes is subject to low variation.

Because mammalian *NKIRAS* has been shown to govern NF-κB and, by extension, multiple immune-gene programmes, the presence of NKIRAS appears to be a critical mechanism for maintaining homeostasis (Chen et al., 2003; Gilmore, 2006)

2.2 Gene analysis of NKIRAS and PIAS factors in rainbow trout

Recognising the centrality of the immune system in avoiding infection and disease establishment, there is a significant selection pressure working on immune genes. In response to these stimuli, immune genes are often developing quickly (Kosiol et al., 2008; Shultz & Sackton, 2019). Multiple rounds of WGD events, for instance, are thought to have led to the evolution of the adaptive immune system in vertebrates. WGD occurrences, in fact, result in duplicated copies of all genes, on which selection can affect, resulting in the retention or elimination of one or both copies (Colgan et al., 2021). Following WGD, ohnologs undergo one of four basic configurations: (i) nonfunctionalisation, in which the new duplicate loses functionality due to mutation; (ii) subfunctionalisation, under which the duplicates preserve relevant features of the original gene function; (iii) neofunctionalisation, in which a gene copy takes on a new

function; and (iv) conservation of the same function by both copies of the gene (Campbell et al., 2019).

The genes encoding *NKIRAS1* and *NKIRAS2* are most likely paralogues that originated from a whole-genome duplication (WGD) that occurred in the ancestral vertebrate in all extant vertebrate species (Tago et al., 2010).

NKIRAS1 and *NKIRAS2* appear to be present as just one gene copy in salmonid fishes, even though they underwent two more WGDs compared to mammals. Nonetheless, variable splicing of the original rainbow trout NKIRAS2 mRNAs most likely permits the production of at least four alternative NKIRAS2 variants with different 5′-ends. This splicing method does not modify exon order or length, as previously found in rainbow trout, but it does lengthen the N-terminus of NKIRAS2 variants b, c, and d in comparison to their paralogue NKIRAS2a in rainbow trout and orthologues in all other vertebrate species (**Study I**).

In human, four distinct Pias proteins are encoded, while PIAS3 has been lost in fish. We discovered eight Pias-encoding genes in *Oncorhynchus sp*. in our work, which are most likely the product of two more genome duplications in teleosts and salmonids (**Study II**). At least 14 distinct pias transcripts are encoded by these eight genes. It is possible that the *PIAS* gene family in fishes was extended by new members as a result of the fish-specific and salmonid-specific duplications, which compensated for the loss of *PIAS3*.

Synteny analysis of the both trout family factors revealed a well conserved gene localisation for all *NKIRAS1*, *NKIRAS2* and *PIAS4*. Nevertheless, *PIAS2* demonstrated a marked difference between teleosts and mammals.

2.3 Proteins structural analysis confirmed high conservation levels.

From a structural point of view, both protein families maintained the prototypical structure retrieved also in mammals and other vertebrates.

Since we observed (**Study I**) that the key aa residues and motifs of the NKIRAS orthologues were largely maintained during vertebrate evolution, our structural study of rainbow trout NKIRAS sequences revealed that all variants may operate similarly to their mammalian counterparts.

Nevertheless, the shortest variation appears to represent the prototypic NKIRAS2 form from a structural standpoint. *NKIRAS1* (but not *NKIRAS2*) is found in the Atlantic salmon *S. salar* as two distinct coding sequences (*XM_014177875* and *XM_014177876*), one of which extends beyond the 5′ end, comparable to the longer *NKIRAS2* variations seen in rainbow trout.

The three rainbow trout Pias1 proteins have a PIAS architecture that is quite similar to that of their human ortholog (**Study II**).

This structural similarity implies that the three Pias1 proteins found in rainbow trout are functionally equivalent to their vertebrate counterparts. One of the two Pias2 proteins found in trout is similar to human PIAS1, but its paralog and one of the Pias4 paralogs do not include the SAF-A/B, Acinus and PIAS (SAP) domain or one nuclear localisation sequence (NLS).

Similarly, unlike their mammalian counterparts, all Pias4 proteins from trout lack the ST-rich region, which is required for SUMO-associated pleiotropic interactions.

Confocal microscopy of both protein families demonstrated a conserved subcellular localisation of all the proteins characterised in our studies compared to mammal orthologs.

2.4 In vitro functional studies revealed new insights

The performed *in vitro* studies gave important information about functionality and impact on two different pathways, such as NF-κB and JAK-STAT.

In CHSE-214 cells, we found that gene expression of rainbow trout *NKIRAS1*, *NKIRAS2c*, *PIAS1* and *PIAS2* inhibited NF-κB activity. These findings are consistent with prior results on mammalian NKIRAS and PIAS proteins (Andrea Oeckinghaus et al., 2014; Liu et al., 2005), and this mechanism appears to be retained in fish.

Overexpression experiments showed that the aforementioned factors from rainbow trout lowered the baseline activity of NF-κB in unstimulated cells to a similar amount and in a dose-dependent manner.

Additionally, in **Study II** we created a trunked version of Pias1 protein, in order to test the effective necessity of the N- termini to regulate the NF-κB activity. Our results determinate not a significative difference between the full and the trunked Pias1 protein,

apparently demonstrating that the activity of Pias1 protein on the regulation of NF-κB is independent from this protein region.

Overall, PIAS2 demonstrated to be the most effective on inhibiting the activation of NF-κB.

Per contra, *NKIRAS2a* and *NKIRAS2b* gene expression showed an inducing impact on NF-κB activity. The selectivity of the NKIRAS variations for the diverse combinations of NF-κB/IκB complexes might explain these opposing NF-κB regulatory mechanisms.

As a preliminary stage, the five NKIRAS variations must precisely interact with the various IκB proteins in rainbow trout that have been found independently from the prototype.

The difference in the expression of the reporter construct and the inflammatory genes following overexpression of the distinct NKIRAS variants might be explained by the diverse NKIRAS-mediated mechanisms regulating the activity of particular NF-κB/Rel factors.

Furthermore, we discovered evidence for regulation in the transcript quantity of pro-inflammatory cytokine genes (*il10*, *tgfb*, *il14/13* and *cxcl8*) in response to overexpression of factors from both protein families (**Study I and Study II**).

In general, PIAS1 and NKIRAS factors showed a similar mild up-regulatory effect on the considered pro-inflammatory cytokine genes. Nevertheless, NKIRAS factors demonstrate to be more effective on upregulation of *tgfb* and *cxcl8,* particularly for NKIRAS2a and NKIRAS2c.

As counterpart, the overexpression of Pias2 showed an overall downregulation of the analysed cytokines, playing an opposite role if compared to the other factors considered in this work. Interestingly, both factors were equally effective at decreasing the amount of activated NF-κB, despite the fact that Pias2 lacks the SAP domain with the intrinsic LxxLL motif, unlike Pias1 who does not lack this domain.

In this respect, it is worth mentioning that the expression of a mammalian PIAS3 mutant with point mutations in the LxxLL motif did not interfere with NF-κB activity (Chung et al., 1997).

The PIAS factors are mostly known for their regulatory activity on STATs proteins and consequently on the JAK-STAT pathway activation and the expression of the target genes. Against this background, we further analysed the effect after overexpression of Pias1 and Pias2 on a panel of downstream genes. In order to induce an artificial viral response, we co-transfected our cell with a *ifnγ*–expressing plasmid.

Pias1 showed a strong downregulation of *il6* transcript levels, giving additional evidence of its regulatory function of the JAK-STAT pathway, also known as the IL6 pathway. As counterpart, *pias2* lowered the transcript levels of numerous additional NF-κB-dependent genes (*ifna3, stat4,* and *il10*), as well as the STAT-dependent genes *socs1*, *gata3*, and *mmp9* (**Study II**).

Furthermore, we investigated the transcript level of *mx*, which encoded protein is activated by type I and type II interferons and inhibits the replication of various RNA and DNA viruses.

Expression analysis on *ifnγ*–expressing cell demonstrated that overexpression of *pias1*, *pias2* and truncated *pias1* robustly decreased the transcript level of *mx.*

Remarkably, the truncated version of *pias1* modulated only the expression levels of *mx,* contrasting with *pias1* and *pias2* both able to regulate various of the selected immune relevant genes.

Because all transcript variations of pias1 and pias2 were abundantly detectable in our expression investigations on salmonid fish cell models, we decided to use CRISPR/Cas9 technology to knock out both components.

Unfortunately, because Pias factors are required for the survival of CHSE-derived fish cells (Munarriz et al., 2004), this strategy proved unsuccessful.

A resume of the results on **Study I and II** is reported in the following heatmap. (Fig. 6)

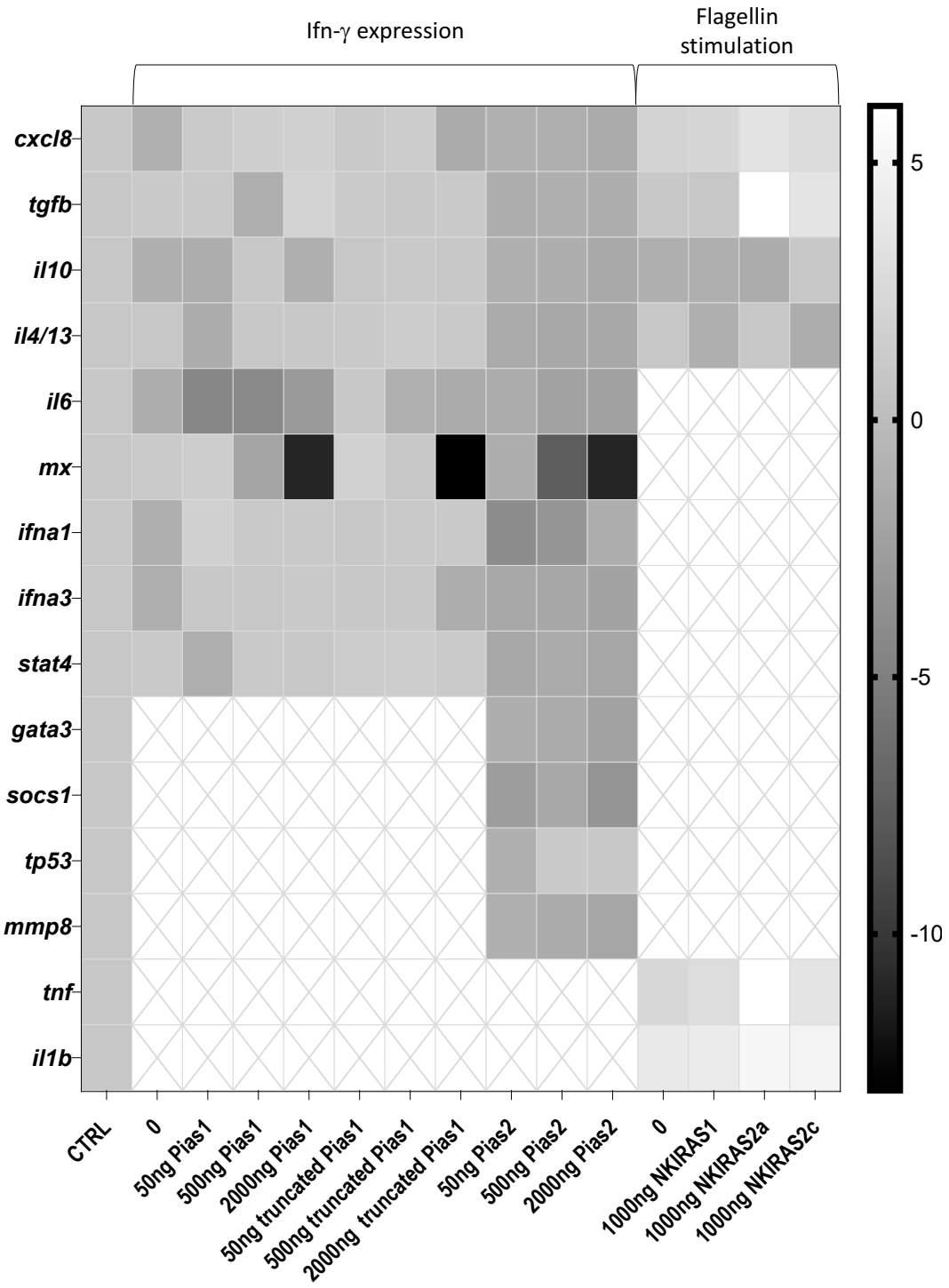

Figure 6: The heatmap resumes the results expressed in fold-change obtained after stimulation of CHSE-214 cells with ifn-γ expression and flagellin stimulation with different quantities of transfected plasmid expressing the candidate genes in **Study I and Study II.** Increased and decreased transcript levels in the samples of the stimulated group compared with the controls are indicated by white and black fields, respectively, according to the legend on the right. Crossed are reported the genes with not available data.

3. Conclusions and future perspectives

The innate immune genes and their regulators play an important role in the host-defence methods.

The focus of this project was directed on the characterisation and functional analysis of different immune-relevant candidate genes, their role and expression regulation upon different stimuli in salmonid cell models and *in vivo* on specific cell populations from head kidney of rainbow trout.

We describe here the structure and functionalities of five *NKIRAS* transcript variants encoded by two genes, which have demonstrated to differently modulate the activation of the NF-kB pathway than in mammals.

For the first time, an extended characterisation of the Pias factors family of rainbow trout has been performed, revealing interesting insights about the activity, function, specific tissue expression and subcellular localisation of the different proteins. Application of the most recent genome editing methods, as CRISPR-Cas9, aiming a deletion of the *PIAS1* gene of CHSE-derived cell models, led the cells to death; raising the hypothesis of its crucial role in regulation of NF-kB and IFN pathways.

At the same time, we established a panel of genes, comprising the most important immune pathways, which can be suitable to evaluate the effective inflammatory response upon different stimuli, and be helpful for future vaccination trials.

Regulation of immune genes is often the aim of invasive pathogen like bacteria and virus. Therefore, greater knowledge of the molecular host-pathogen interactions over the course of an infection is critical for the development of molecular tools and effective disease treatments.

Future studies should address on the interaction of NF-κB and its associated factors *in vivo*, and may investigate the influence of Pias proteins from trout on different immune cell subsets. Additionally, supplementary studies about the expression profile of the early phase of the innate immune response during inactivated bacterial stimulation on cells derived from different organs and different time points, could be helpful in order to improve knowledge of *A. salmonicida* infection and contrast future harmful outbreaks.

Noteworthy, the application of genome editing technologies, as CRISPR-Cas9, is increasingly becoming more important over years, and it drastically improved and revolutionised many aspects of host-pathogens, stress signalling and immune-related

genes researches in different fish species. Even though, the bioethical implications of CRISPR-Cas9 technology for the environment, agriculture, and livestock must not be underestimated.

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5. Publications

5.1 Statement of contributions

I hereby declare that my share of the publications summarised in this paper is as follows:

Publication I

Characterisation of the teleostean κB-Ras family: The two members NKIRAS1 and NKIRAS2 from rainbow trout influence the activity of NF-κB in opposite ways.
Fabio Sarais, Henrike Rebl, Marieke Verleih, Sven Ostermann, Aleksei Krasnov, Bernd Kollner, Tom Goldammer , AlexanderRebl.
Fish & Shellfish Immunology Volume 106, November 2020, Pages 1004-1013
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- Cloning
- Cell culture
- Reporter-gene analyses
- qPCR
- Data analysis
- Visualisation
- Writing- Original draft preparation

Publication II

PIAS Factors from Rainbow Trout Control NF-κB- and 2 STAT-dependent Gene Expression.
Fabio Sarais, Sophia Kummerow, Ruth Montero, Henrike Rebl, Bernd Köllner, Tom Goldammer, Bertrand Collet and Alexander Rebl.
International journal of molecular science
DOI: https://doi.org/10.3390/ijms222312815

- sgRNA design
- CRISPR/Cas9
- Cloning
- Cell culture
- Reporter-gene analyses
- qPCR
- Data analysis
- Visualisation
- Writing- Original draft preparation

Publication III

The Early Immune Response of Lymphoid and Myeloid Head-Kidney Cells of Rainbow Trout (*Oncorhynchus mykiss*) Stimulated with *Aeromonas salmonicida*.

Fabio Sarais, Ruth Montero, Sven Ostermann, Alexander Rebl, Bernd Köllner and Tom Goldammer.

Fishes

DOI: https://doi.org/10.3390/fishes7010012

- Conceptualisation
- Methodology
- Software
- Validation
- Formal analysis
- Investigation
- Data curation
- Writing-original draft preparation
- Visualisation

5.2 Publications

Study I: Characterisation of the teleostean κB-Ras family: The two members NKIRAS1 and NKIRAS2 influence the activity of NF-κB in opposite ways

Control and modulation of the NF-κB activity is a key factor that regulates immune response and protects it from overreactions. We identified the orthologous sequences of *NKIRAS1* and NKIRAS2 from the rainbow trout *Oncorhynchus mykiss*.
The structure of five NKIRAS variants in rainbow trout modulate the activity of NF-κB and, thus, the pro-inflammatory immune responses in a teleostean model cell. Beyond NF-κB signal transduction, NKIRAS proteins may conceivably control further signalling cascades. Interestingly, two variants NKIRAS2a and NKIRAS2b enhance the activation of NF-κB and expression of pro-inflammatory genes.

Highlights

- We identified one *NKIRAS1* and four *NKIRAS2* variants in rainbow trout.
- NKIRAS1 and NKIRAS2 are localised together with NF-κB p65 in CHSE-214 cells.
- The investigated NKIRAS variants modulate the NF-κB activity in different ways.
- The shortest NKIRAS2 variant induced a stronger expression of cytokines in vitro.

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Full length article

Characterisation of the teleostean κB-Ras family: The two members NKIRAS1 and NKIRAS2 from rainbow trout influence the activity of NF-κB in opposite ways

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ABSTRACT

Two structurally similar NF-κB-inhibitor-interacting Ras-like proteins (NKIRAS) regulate the activity of the transcription factor NF-κB and thereby control several early immune mechanisms in mammals. We identified the orthologous sequences of *NKIRAS1* and *NKIRAS2* from the rainbow trout *Oncorhynchus mykiss*. The level of sequence identity was similarly high (≥68%) between the two and in comparison to their mammalian counterparts. Strikingly, *NKIRAS2* was present as four transcript variants. These variants differed only in length and in the nucleotide composition of their 5' termini and were most likely generated by splicing along unconventional splice sites. The shortest *NKIRAS2* variant was most strongly expressed in a lymphocyte-enriched population, while *NKIRAS1* was most strongly expressed in cells of myeloid origin. Fluorescent-labelled NKIRAS1 and NKIRAS2 proteins from rainbow trout were detected in close association with the p65 subunit of NF-κB in the nucleus and cytoplasm of CHSE-214 cells. Subsequent reporter-gene experiments revealed that NKIRAS1 and a longer NKIRAS2 variant in rainbow trout decreased the level of activated NF-κB, while the two shortest NKIRAS2 variants increased the NF-κB activity. In addition, the overexpression of the shortest NKIRAS2 variant in CHSE-214 cells induced a stronger transcription of the genes encoding the pro-inflammatory cytokines TNF, CXCL8, and IL1B compared to non-transfected control cells. This is the first characterisation of NKIRAS orthologues in bony fish and provides additional information to the as yet underexplored inhibition pathways of NF-κB in lower vertebrates.

1. Introduction

The 'NF-κB-inhibitor-interacting Ras-like proteins' NKIRAS1 and NKIRAS2 (also known as κB-Ras 1 and κB-Ras 2 [1]) are members of the hyperfamily of Ras proteins accounting for more than 150 GTP-binding proteins largely involved in cell-signal-transduction networks [2]. NKIRAS1 and NKIRAS2 contribute to the maintenance of homeostasis in mammalian cells by controlling the activity of NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B cells) factors [3].

The NF-κB/Rel factors represent one of the best characterised families of immunorelevant transcription factors [4], which are of particular importance for both innate and adaptive immune responses [5]. Overshooting of immune reactions and pathological responses is prevented by controlling the activity of NF-κB by a range of different

Abbreviations: aa, amino acid; CDS, coding sequence; CXCL8, C-X-C motif chemokine ligand 8; GDP, guanosine diphosphate; GFP, green fluorescent protein; GTP, guanosine-triphosphate; hpi, hours post infection; IL, interleukin; MAb, monoclonal antibody; mPlum, mutant variant of red fluorescent protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NKIRAS, NF-κB-inhibitor-interacting Ras-like proteins; nt, nucleotide(s); ORF, open-reading frame; PEST, proline (P), glutamic acid (E), serine (S), and threonine (T)-rich domain; Ras, Rat sarcoma; SNP, single-nucleotide polymorphism; TGFB, transforming growth factor beta; TNF, tumour necrosis factor; qPCR, quantitative polymerase-chain reaction; WGD, whole-genome duplication.

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signal-responsive mechanisms [6]. Under homeostatic conditions, the 'inhibitors of NF-κB proteins' (IκBs) maintain NF-κB factors in an inactive form in the cytosol [7]. Eight IκB-like family members have been discovered in mammals [8,9], and the alpha member is a prototypic representative in mammals [10] and fish [11]. IκB masks the nuclear localisation signals of NF-κB factors [12], thereby preventing their access to NF-κB-specific response elements in the promoters of relevant genes. NKIRAS proteins interact with the PEST domains of IκB proteins, mainly of the beta type [1,13], to impede their degradation and to restrain the inhibitory complex and its substrate NF-κB within the cytoplasm [1,14]. This inhibition can be removed through activated pattern-recognition receptors and several pro-inflammatory mediators that promote the degradation of IκBα [15].

The NKIRAS proteins are activated through the binding of GTP and Mg^{2+} to the 'switch I' and 'switch II' domains [16]. The subsequent conformational rearrangement of NKIRAS1 and NKIRAS2 allows recruitment of downstream effector proteins [17,18]. NKIRAS2 bound to GDP has been reported to suppress the phosphorylation of the NF-κB p65 subunit, thereby interfering with its interaction with the transcriptional coactivator p300 [19].

Comprehensive phylogenetic comparisons of teleostean Ras factors have been published so far [20,21], but functional studies on the role of Ras proteins in fish immunity are rare [22] and nothing is known about the involvement of NKIRAS during the early and presumably NF-κB-driven immune processes in fish. Here, we characterised the two NKIRAS factors in the rainbow trout *Oncorhynchus mykiss* and investigated their structural and functional properties in terms of interference with the early immune-activating mechanisms of salmonid fish against *Aeromonas salmonicida* infections.

2. Material and methods

2.1. Fish

Rainbow trout (*O. mykiss*) were obtained from a local commercial fish farm (Forellenzucht Uthoff GmbH, Neubrandenburg, Germany) and kept at 15 °C in 1000 L tanks in partially recirculating water systems and a light period of 12 h per day. For the stimulation trial, fish (30–50 g) were intraperitoneally injected with 5×10^5 colony-forming units (CFU) of the highly virulent JF5505 strain of *A. salmonicida* ssp. *salmonicida* (diluted in 200 μL phosphate buffered saline [PBS]) using a 20G needle. Control fish were injected with 200 μL PBS. At 6, 12 and 24 h post stimulation, four fish per treatment group were euthanised using an overdose of benzocaine (100 mg/L). All experimental procedures complied with the relevant European guidelines on animal welfare (Directive 2010/63/EU on the protection of animals used for scientific purposes) and were approved by the institute's ethics board (approval ID: FLI 28/17).

2.2. Cell sorting

Cells were isolated from individual head kidneys, and 4×10^4 cells were used for labelling with 50 ng MAb21 [23] per 2×10^5 cells at 4 °C for 30 min. The cells were then washed and centrifuged (515×*g*, 4 °C, 5 min). The cell pellet was resuspended in 180 μL autoMACS Running Buffer (Miltenyi Biotec) before adding 20 μL of anti-mouse secondary antibody conjugated to magnetic beads (Miltenyi Biotec) for each 1×10^7 cells. After incubation for 20 min at 4 °C, the cells were washed with 700 μL autoMACS Running Buffer and centrifuged for 10 min at 300×*g*. Cells were resuspended in 500 μL autoMACS Running Buffer and sorted using a single magnetic column and the posselS program on the AutoMACS Pro Separator (Miltenyi Biotec). The separated positive and negative fractions were centrifuged for 7 min at 515×*g* and 4 °C and the cell pellets were resuspended in 350 μL RNA RLT lysis buffer (Qiagen) and stored at −70 °C. RNA extraction and cDNA synthesis were conducted as described previously [24]. Subsequent expression analysis was conducted on MAb21-negative cells (a lymphocyte-enriched population containing mainly B cells, T cells and thrombocytes) and MAb21-positive cells (a population enriched with cells of myeloid origin).

2.3. Construction of NKIRAS-expression constructs

We amplified the open-reading frames (ORFs) of *NKIRAS1* and three *NKIRAS2* transcript variants using the oligonucleotide primers listed in Table 1 and the Platinum Taq High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Amplicons were subcloned in pGEM-T Easy (Promega), retrieved by digestion with the restriction enzymes *Hind*III and *Eco*RI and inserted 'in frame' into the mammalian expression vector v280 [25,26] (previously double-digested with the above restriction enzymes). The resulting plasmids (v280_NKIRAS1 and v280_NKIRAS2) were utilised for functional analyses.

We identified the subcellular localisation of the NKIRAS1 (XM_021560247) and NKIRAS2 (XM_021557708) variants in salmonid model cells by inserting a fragment coding for a derivative of the red fluorescent protein (mPlum) and a green fluorescent protein (GFP) at the 3' end of the CDS of our v280_NKIRAS1 and v280_NKIRAS2 plasmids. The mPlum and GFP fragments had previously been amplified from commercial vectors (mPlum: pmPlum, ClonTech/Takara; GFP: pAM 505, NCBI-nucleotide accession code: AF140578) and inserted into the v280 clones using the restriction sites *Cla*I and *Hind*III or *Hind*III and *Eco*RI, respectively.

2.4. Cell culture, transfections and luciferase assay

The CHSE-214 cell line derived from *Oncorhynchus tshawytscha* was cultured as described previously [25]. Endotoxin-free preparations (ZymoPure II Plasmid Maxi Prep Kit, ZymoResearch) of the expression constructs were transfected into CHSE-214 cells using the X-tremeGENE HP DNA Transfection Reagent (Roche). For co-transfection assays in six-well plates, we used 50 ng of the modified NF-κB-responsive promoter of the endothelial-leukocyte adhesion molecule gene (ELAM-1--luc) [27] and increasing concentrations (from 100 ng to 750 ng) of the v280_NKIRAS1 and v280_NKIRAS2 plasmids.

Induction experiments were carried out in 24-well plates. Three wells of each row were left as unstimulated controls, while the other three were stimulated for 3 h or 24 h with 100 ng/mL flagellin isolated from the Gram-negative bacterium *Salmonella typhimurium* (tlrl-stfla; Invivogen), which has been proven to induce inflammatory responses in CHSE-214 cells [25,26,28]. Three hours after incubation, the cells were harvested in ice-cold PBS for RNA isolation and the subsequent profiling of immune-gene expression. Twenty-four hours after stimulation, the luciferase activity of the cell lysates was measured with the Dual-Luciferase Reporter Assay System (Promega) in a Lumat LB9501 luminometer (Berthold). The values were normalised against the protein concentration of the CHSE-214 cell extracts. Each transfection was assayed in triplicate; each transfection experiment was repeated three times.

2.5. Confocal microscopy

The CHSE-214 cells were transfected with vectors expressing either mPlum-tagged NKIRAS1 or GFP-tagged NKIRAS2a from rainbow trout to monitor the transfection efficacy and the intracellular localisation of both factors. Live-cell imaging was performed as described previously [25]. Hoechst 33342 dye (1 mg/mL; Sigma Aldrich/Merck) was used to visualize the nuclei. For co-staining with the NF-κB subunit p65, the CHSE-214 cells were fixed (10 min, 4% paraformaldehyde, Merck), permeabilised (5 min, 0.1% Triton X-100, Sigma-Aldrich/Merck) and incubated in the dark with monoclonal anti-p65 (D14E12) antibody from rabbit (1:200, 30 min, Cell Signalling) and donkey anti-rabbit Alexa 488 secondary antibody (1:100, 30 min, Sigma-Aldrich/Merck).

Table 1
Primers used in this study.

Gene symbol	NCBI accession code	Primer sequence 5'→3' (sense, antisense)	Fragment length [bp]
Construction of NKIRAS-expression constructs:			
NKIRAS1	XM_021560247	ATGCAAGCTTATGGGGAAAGGTTGCAAAGT, TTAACGACATCCACATCCAAGG	576
NKIRAS2a	XM_021557708	ATGCAAGCTTATGGGCAAGAGTTGTAAAGTGGTGG, ATGCGAATTCGCTATCGACAGAGCCACTCCCC	576
NKIRAS2b	XM_021557707	ATGCAAGCTTATGACGTCACTCAGCGAGGACG, ATGCGAATTCGCTATCGACAGAGCCACTCCCC	669
NKIRAS2c	XM_021557706	ATGCAAGCTTATGTTCTGGAATGACGTCACTCAG, ATGCGAATTCGCTATCGACAGAGCCACTCCCC	675
Quantitative PCR analysis:			
NKIRAS1	XM_021560247	GATCGTGGTGTAAAGGAACAGTT, TCCACACTGTAGACCAGCACAA	119
NKKIRAS2a	XM_021557708	CATCATCATGGGCAAGAGTTGTA, CTGAGCCCGCAATATGATTGG	108
NKIRAS2b	XM_021557707	TTTTCTGGCTCGTGCAGTTACC, AATATGATTGGCATACAGCAGTTG	130
NKIRAS2c	XM_021557706	CGAGGACGAGTGGGCAGAGT, GCCCGCAATATGATTGGCATAC	171
NKIRAS2d	XM_021557705	TGCATGTCTGCCTGTCTCTTTTT, TGAGCCCGCAATATGATTGGCA	201
TNF-1	NM_001124357	AACGATGCAGGATGAAATTGAGC, GGCCGTCATCCTTTCTCCACT	161
IL10	NM_001245099	TGCCCAGTGCAGACGTGTACC, CGGGGCTCTTCAAGTGGTGTA	179

2.6. Quantitative PCR (qPCR) analysis

We profiled the quantity of the various *NKIRAS* transcripts in rainbow trout leucocytes by establishing a panel of discriminating oligonucleotide primers that allowed a selective quantification of the *NKIRAS1* and the four *NKIRAS2* transcript variants (Table 1). The qPCR primers were designed (using Pyrosequencing Assay Design software v.1.0.6; Biotage) to amplify fragments between 108 nt and 201 nt. We further profiled the expression of cytokine-encoding genes in CHSE-214 cells. The *Oncorhynchus*-specific primer pairs for *IL1B*, *CXCL8*, *TGFB-2*, and *IL4/13* were published previously [25]; the primer pairs for *TNF-1* and *IL10* were optimised for *O. tshawytscha* (Table 1). *EEF1A1* [29] and *RPS5* [24] were chosen as reference genes for normalisation of the qPCR data. Quantitative PCR analysis was performed on the LightCycler-96 system (Roche), essentially as previously described [25]. The cDNA input into the individual RT-qPCR assays was equivalent to 2.5 ng total RNA isolated from cells.

2.7. Data analysis

A parametric *t*-test or nonparametric Mann-Whitney *U* test provided by GraphPad Prim 8 software was used to evaluate the statistical significance of the qPCR data. In all these tests, a two-tailed p value of <0.05 was considered statistically significant. The ClustalW and T-Coffee expresso alignment tools were used to compare multiple NKIRAS nucleotide and amino acid (aa) sequences.

The Molecular Evolutionary Genetics Analysis package MEGA7 [30] was used to construct the phylogenetic tree using the Neighbour-Joining method with a bootstrap test based on 1000 replications. Three-dimensional structures were obtained using PHYRE2 (Protein Homology/analogY Recognition Engine V 2.0) [31]. Signal peptides and disordered protein regions were predicted using the online tools SignaIP-5.0. and PrDOS [32].

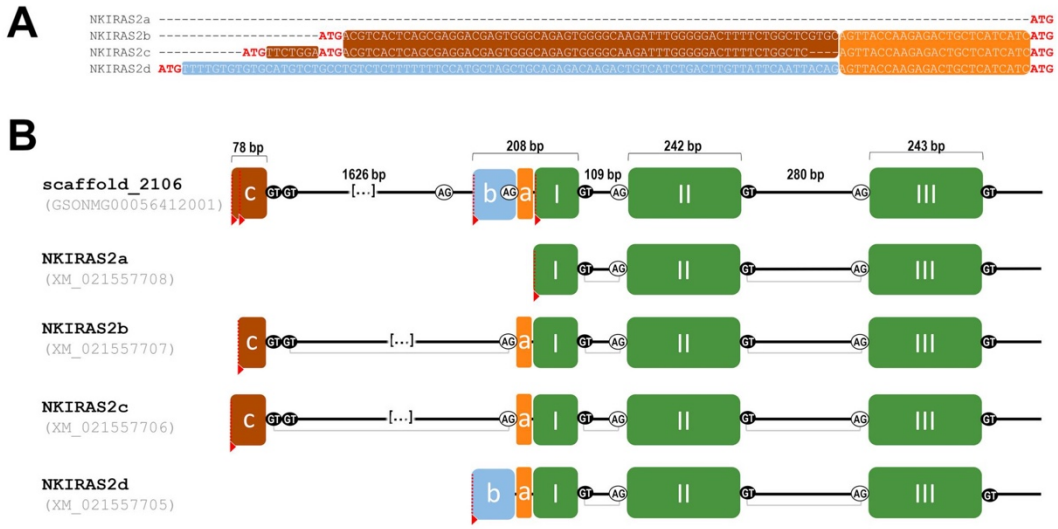

Fig. 1. Structural comparison of the four NKIRAS2 transcript variants from rainbow trout. (**A**) Only the coding sections were aligned, in which the four variants a to d differ from each other. Identical sections are colour coded. Potential start codons are marked in red. (**B**) Schematic representation of the NKIRAS2 scaffold 2106 (Genoscope *O. mykiss* genome; ID: GSONMG00056412001) in rainbow trout and the four resulting transcript variants (along with NCBI nucleotide IDs). The coding exons conserved in extant vertebrates are indicated by green boxes and Roman numerals. Other sequence segments are coloured according to (A). The lengths of the exons/coding sequence fragments and introns are given above the illustrations. The (possible) transcription start is indicated by a red arrowed line. Splice donor (GT) and acceptor sites (AG) are indicated by filled and open circles, respectively. For each transcript, the used splice sites are linked by grey brackets. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Differential splicing generates four NKIRAS2 variants alongside one NKIRAS1 transcript in rainbow trout

Our search of the NCBI-gene database for NKIRAS-like sequences from rainbow trout revealed one coding sequence (CDS) (accession code: XM_021560247) orthologous to the human *NKIRAS1* gene and four transcript variants X1 to X4 (XM_021557705 to XM_021557708) orthologous to the human *NKIRAS2* gene (Suppl. Fig. 1). The CDS of *NKIRAS1* from rainbow trout comprises 579 nt and shares the same length with the shortest *NKIRAS2* variant (X4), which is henceforth referred to as *NKIRAS2a*. The 579-nt long sequence of *NKIRAS2a* is also included in the variants X1 to X3, which also bear specific 5' termini of different lengths and nucleotide compositions. The longest *NKIRAS2* variant (X1) comprises 693 nt and is henceforth termed *NKIRAS2d*, followed by *NKIRAS2c* (X2, 678 nt) and *NKIRAS2b* (X3, 672 nt). The coding sequences of all five *NKIRAS* transcripts share an identity of >69% with the human *NKIRAS1* and an identity of >77% with the human *NKIRAS2*. The coding sequences of *NKIRAS1* and *NKIRAS2a* from rainbow trout share an identity of 68% due to 192 exchanged nucleotides (Suppl. Fig. 1). We verified the lengths and nucleotide compositions of *NKIRAS1* and *NKIRAS2a* to *NKIRAS2c* by cloning and repeated sequencing.

The individual 5' regions of the *NKIRAS2* variants *b*, *c* and *d* share a 25-nt sequence immediately upstream of the common start codon (Fig. 1A, orange box). Interestingly, a ≥96% identical sequence fragment resides in other coding gene sections from bacteria (e.g. sequence ID: SIT85884), protozoans (e.g. CDW78563) and vertebrates (e.g. XM_028038946), but they are translated into completely different amino acid (aa) sequences. In variants *b* and *c*, a 64-nt long sequence (Fig. 1A, brown box) is located upstream of this 25-nt motif. The 25-nt and 64-nt segments are separated by 4 additional nt in the case of variant *b*, whereas variant *c* contains an additional 10-nt long sequence at its 5' end. In variant *d*, a different 89-nt long sequence is located upstream of the 25-nt motif (Fig. 1A, blue box).

A BLAST search of these three different 5′-UTR segments against the rainbow trout genome [33] identified an approximately 3030-nt long genomic sequence on scaffold 2106 (Fig. 1B). The ORF of *NKIRAS2a* is distributed across three exons (I, 94 nt; II, 242 nt; III, 243 nt) and has the same organisation seen in mammals. The additional 25-nt, 64-nt and 89-nt long segments, which characterise the individual *NKIRAS2* versions *–b*, *–c* and *–d*, respectively, are all encoded in the genomic region upstream of the conventional three *NKIRAS2* exons and allow the generation of four different transcript variants via differential splicing.

We also investigated whether undiscovered transcript variants of *NKIRAS* might exist by screening an RNA-sequence read collection of two rainbow trout strains [34]. We did not identify any further transcript variants of *NKIRAS1* and *NKIRAS2*; however, we observed that both the *NKIRAS1* and *NKIRAS2* transcripts bore a certain degree of sequence variability. The CDS of *NKIRAS1* contained 11 single-nucleotide changes, contributing to a 1.9% variability (Fig. 2A),

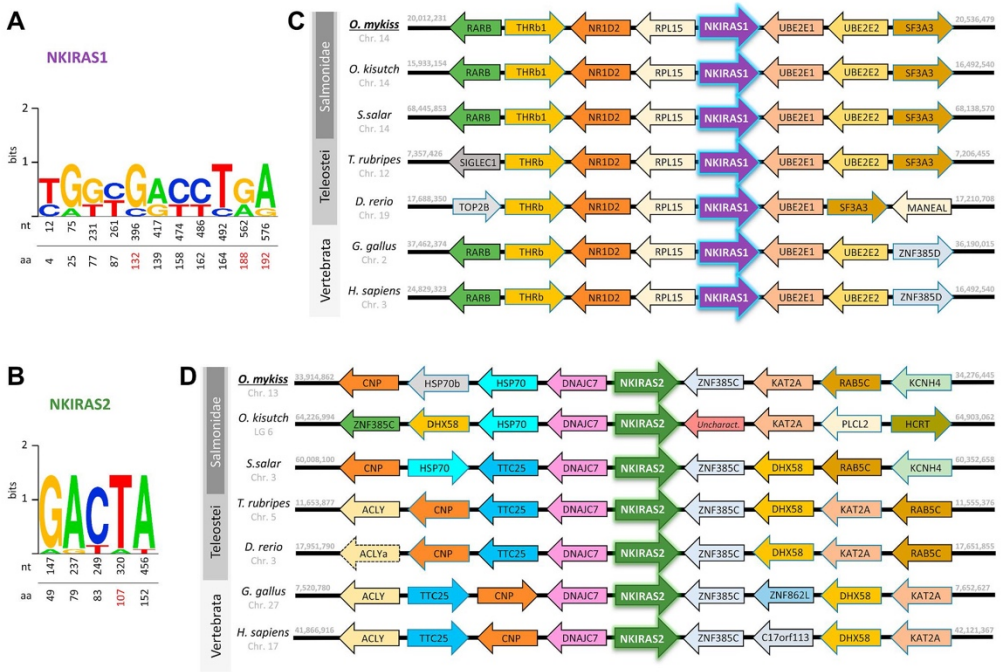

Fig. 2. Sequence conservation of (A) *NKIRAS1* and (B) *NKIRAS2* from rainbow trout. The identified single-nucleotide changes in the coding sequences of both genes are represented as sequence logos. The frequency of a nt variation at its specific sequence position is given below the drawing, alongside the position of the affected aa. Red numbers represent conservative mutations; black numbers represent silent mutations. **Synteny between (C) *NKIRAS1* and (D) *NKIRAS2* loci from different vertebrate species.** The gene order was identified using Genomicus v1.01. Species name and chromosomal location (in nt) are listed on the left. Arrows represent genes found in synteny; the colour indicates orthologous genes and their direction indicates the gene orientation. The figure is not scaled; the position of the depicted genes is given in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

which corresponds approximately to the average variability assessed for the rainbow trout genome [35]. The variability of *NKIRAS2* was comparably lower (five single nucleotide changes, 0.9% variability; Fig. 2B).

As a last test, we checked the number of *NKIRAS* transcripts in other salmonid species. Our search in the NCBI gene database revealed only one entry each for the genomic sequences of both *NKIRAS1* and *NKIRAS2* in salmonid fishes (*O. mykiss*, *Salmo salar* and *Salvelinus alpinus*). The genes flanking *NKIRAS1* (Fig. 2C) and *NKIRAS2* (Fig. 2D) are similarly arranged across different vertebrate genomes, although a few of the neighbouring genes are reversed in their arrangement.

3.2. NKIRAS1 and NKIRAS2 from rainbow trout share well-conserved structural motifs

The five *NKIRAS* sequences from trout encode proteins with lengths ranging from 192 to 230 aa. The aa sequences among the vertebrate NKIRAS orthologues are relatively well conserved (>60%; Table 2). In line with this observation, the encoded NKIRAS proteins from trout contain the same motifs and domains that have been characterised in their mammalian NKIRAS counterparts (Fig. 3A). We also found two and three SxDx motifs, which are recognised and modified by *A. salmonicida* to impede the catalytic activity of Ras proteins from the infected host and control the activity of leukocytes [36].

Despite the numerous structural similarities, 64 aa residues still allowed discrimination between NKIRAS1 and NKIRAS2 (Fig. 3A). Notably, a signal peptide of 17 aa has been predicted only in *NKIRAS2d*. In our phylogenetic tree, both genes separate, despite the high degree of sequence identity, due to these differing aa residues (Fig. 3B1). The three-dimensional reconstructions of NKIRAS1 (Fig. 3B2) and NKIRAS2a (Fig. 3B3) from rainbow trout illustrate the well-conserved quaternary structure of particular domains. The characteristics of the active domains, which are critical for the GTPase activity, are located in a similar configuration as observed in their human counterparts [37].

3.3. NKIRAS copy number is slightly modulated after infection with A. salmonicida

The human and murine *NKIRAS1* and *NKIRAS2* genes are widely expressed in various tissues, including immune cells [3]. Our qPCR measurements (Fig. 4A) revealed that *NKIRAS2a* (~3000 copies/μg RNA) was the most strongly expressed *NKIRAS* gene in the MAb 21-negative cell population from rainbow trout enriched with lymphocytes, whereas *NKIRAS2b* was the least expressed variant (~200 copies/μg RNA). In the MAb21-positive cells of myeloid origin, *NKIRAS1* was most strongly expressed (~670 copies/μg RNA), whereas all other *NKIRAS* transcripts were expressed at a similarly low level (<100 copies/μg RNA).

Infection with a highly virulent strain of *A. salmonicida* significantly upregulated the levels of the *NKIRAS2* variants *a*, *b*, and *c* ($p < 0.05$) in the MAb 21-negative cell population by about twofold at 12 or 24 h post infection (hpi) compared to PBS controls (Fig. 4B). In the MAb21-positive cell population, the *NKIRAS1* level was slightly downregulated within 24 h, while the *NKIRAS2a* level was 2.7-fold and 4.0-fold upregulated after 12 h and 7 d, respectively (Fig. 4C). However, the fourfold increase resulted only in about 230 copies/μg RNA. The concentrations of the other *NKIRAS* transcripts at 7 days post infection returned to values similar to those measured in the cells of naïve trout.

3.4. Overexpressed NKIRAS1 and NKIRAS2 are localised in the cytoplasm and nucleus of salmonid cells

We overexpressed fluorescent-labelled *NKIRAS1* and *NKIRAS2a* from rainbow trout in CHSE-214 cells to investigate the localisation of the salmonid NKIRAS factors. Confocal microscopy revealed that NKIRAS1 (flagged with mPlum) and NKIRAS2 (flagged with GFP) apparently shared the same subcellular localisation in the nucleus and cytoplasm (Fig. 5A1, A2) as is observed in humans [38]. We also stained native NF-κB p65 factors using a monoclonal anti-RELA antibody from rabbit. In unstimulated cells, NKIRAS1 and NKIRAS2 seem to restrain p65 within the cytoplasm (Fig. 5B1, B2), but we could not detect transfer of p65 to the nucleus after stimulation with flagellin for 1 h (data not shown).

3.5. NKIRAS1 and NKIRAS2a modulate the activity of NF-κB in opposite directions in CHSE-214 cells

Stimulation of CHSE-214 cells with flagellin increased the level of active NF-κB by an average of 2.8-fold (Fig. 5C1-C4, 2nd column). We transfected the CHSE-214 cells with increasing concentrations (250 ng to 750 ng) of the plasmids expressing *NKIRAS1* to drop the flagellin-induced NF-κB activation down to the level of the control cells (Fig. 5C1). Transfection with the same amounts of the NKIRAS2c-encoding plasmid had a similar dampening effect on the level of activated NF-κB, even though the lowest concentration (250 ng) of transfected *NKIRAS2c* had a less pronounced effect compared with the NKIRAS1-encoding plasmid (Fig. 5C2). By contrast, increasing concentrations of NKIRAS2a (Fig. 5C3) and NKIRAS2b (Fig. 5C4) raised the level of activated NF-κB by 21-fold (with a quantity of 750 ng of transfected plasmid).

3.6. Overexpression of NKIRAS1 and NKIRAS2 increases the in vitro expression of specific cytokines

We also investigated the influence of the three NKIRAS factors NKIRAS1, NKIRAS2a and NKIRAS2c, which had modulated the NF-κB level in different directions, on the expression of cytokine-encoding genes in CHSE-214 cells. These genes included those for the pro-

Table 2
Amino-acid sequence identity of NKIRAS1 and NKIRAS2a from rainbow trout with orthologous sequences from selected vertebrates.

Species	Accession Number		Sequence identity [%]	
	NKIRAS1	*NKIRAS2*	*NKIRAS1*	*NKIRAS2a*
O. kisutch	XP_020355835[a]	XP_020342081[a]	99.48	98.96
S. salar	XP_013997908[a]	XP_014048269[a]	99.48	97.40
T. rubripes	XP_003969113[a]	XP_003964849[a]	90.62	89.06
P. reticulata	XP_008419502[a]	XP_008401777[a]	89.58	86.98
G. aculeatus	ENSGACP00000010534[b]	ENSGACP00000011568[b]	92.71	88.02
D. rerio	NP_001093546[a]	NP_001003433[a]	84.90	85.94
A. sinensis	XP_006021262[a]	XP_006032823[a]	61.46	78.65
P. vitticeps	XP_020663250[a]	XP_020667364[a]	70.83	79.69
G. gallus	XP_004939460[a]	NP_001006333[a]	73.44	79.69
H. sapiens	NP_065,078[a]	NP_001001349[a]	73.96	77.60

[a] NCBI accession code.
[b] ENSEMBL accession code.

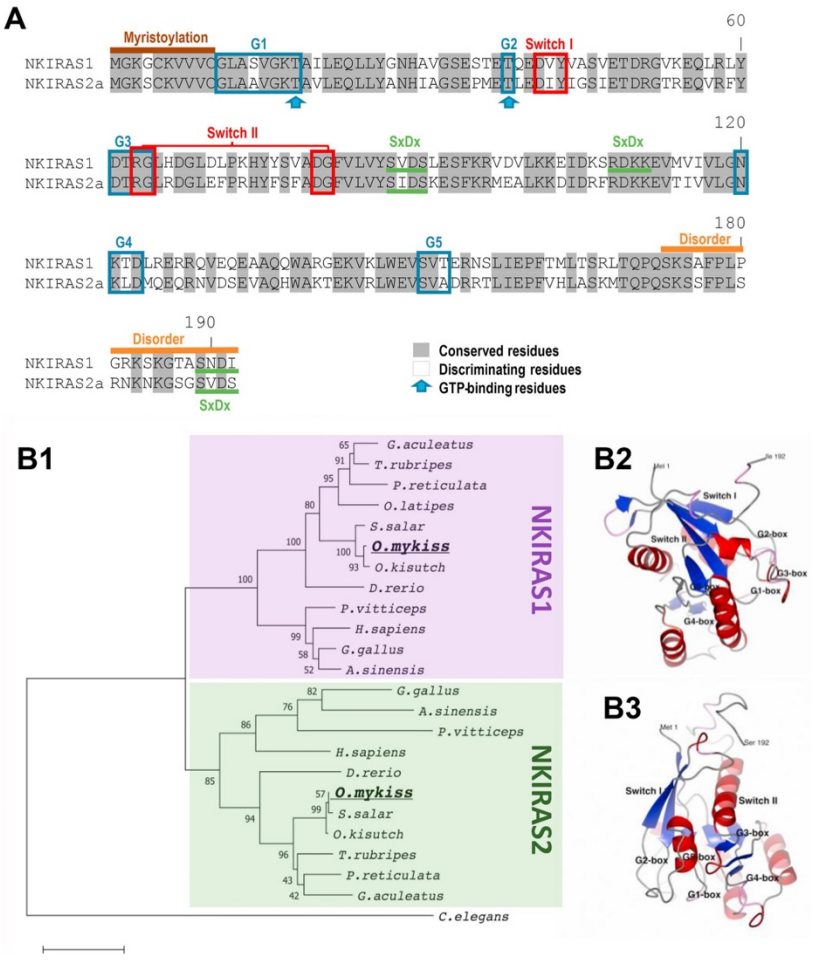

Fig. 3. Comparison of the protein sequences of NKIRAS1 and NKIRAS2a. (**A**) The primary sequences of the proteins are expressed in one-letter codes. Characteristic motifs are framed and labelled: a myristoylation signal MGxxCxxxxC (position 1–10); G1-box GxxxxGKS/T (11–18); G3-box DxxG (61–64); G4-box N/TKXD (121–123); G5-box/SAK motif (150–152); switch I (41–43) and switch II region (63–64 and 80–81); disorder region (173–192); and the *A. salmonicida*-responsive SxDx motifs (87–90, 107–110, 189–192). Conserved aa residues are highlighted in grey. (**B1**) Phylogenetic tree constructed with NKIRAS1 and NKIRAS2 protein sequences of rainbow trout *O. mykiss* (underlined) and its orthologue sequences from other vertebrates. NKIRAS from *Caenorhabditis elegans* was used as an outgroup. The bootstrap values are given at the node of each clade. The scale bar represents a genetic distance of 0.1 aa substitutions per site. The tertiary structures of (**B2**) NKIRAS1 and (**B3**) NKIRAS2 from rainbow trout are drawn to the right side of the respective phylogenetic cluster. β-sheets and α-helices are highlighted in blue and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inflammatory cytokines *IL1B*, *TNF* and *CXCL8* and the anti-inflammatory cytokines *TGFB2*, *IL10* and *IL4/13* (Fig. 6).

Stimulation of the non-transfected control cells with flagellin for 3 h significantly increased the copy numbers of the pro-inflammatory cytokine genes *IL1B* by fourfold, and *CXCL8* and *TNF* by approximately twofold, whereas the copy numbers of the genes *TGFB2*, *IL10* and *IL4/13* remained almost at the same levels under all the examined conditions. We detected almost no changes in cells that were stimulated and transfected with *NKIRAS1* versus stimulated non-transfected cells. The stimulation and transfection with *NKIRAS2c* increased the level of *TNF* and *IL1B* by 125–150%. The stimulation and transfection with *NKIRAS2a* provoked an increase in the transcript abundance of *TNF* (254%; $p = 0.03$), *CXCL8* (162%; $p = 0.09$), and *IL1B* (133%; $p = 0.74$) over the stimulated non-transfected cells.

4. Discussion

NKIRAS1 and NKIRAS2 constitute a small Ras subfamily which has been widely investigated with regard to the regulation of NF-κB in mammals [3]. In all extant vertebrate species, the genes encoding NKIRAS1 and NKIRAS2 are most likely paralogues [39] that descended from a whole-genome duplication (WGD) occurring in the ancestral

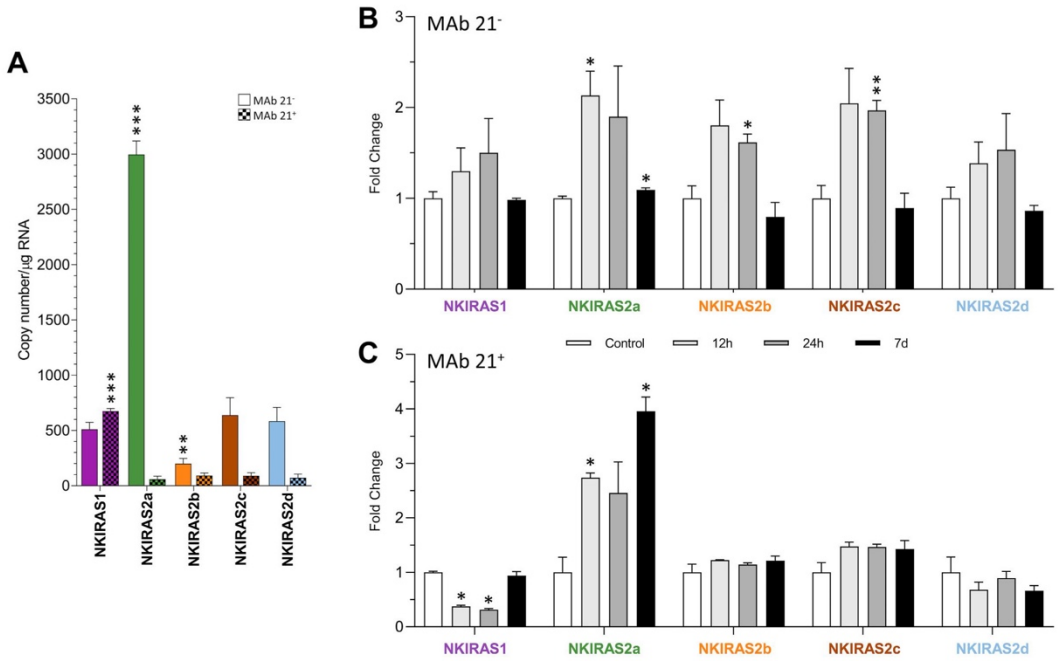

Fig. 4. Expression of NKIRAS genes in lymphoid cells of rainbow trout before and after stimulation. (**A**) Transcript levels of NKIRAS1 (purple bars), NKIRAS2a (green), NKIRAS2b (orange), NKIRAS2c (brown), and NKIRAS2d (light blue) were determined in MAb 21-negative lymphoid cells (non-patterned columns) and MAb21-positive cells of myeloid origin (chequered columns) from healthy rainbow trout. Asterisks represent a significantly different transcript level compared with the NKIRAS1 transcript level (**$p < 0.01$; ***$p < 0.001$; *t*-test). After stimulation with *A. salmonicida*, the aforementioned genes were profiled at 0 h (white bars), 12 h (light grey), 24 h (dark grey) and 7 days (black) post challenge in (**B**) MAb21-negative and (**C**) MAb21-positive cells. Bars represent the averaged copy numbers (n = 3) normalised against two reference genes; error bars represent the standard deviation. Asterisks represent a significantly different post-stimulation transcript level compared with the respective 0 h control value (*, $p < 0.05$; **$p < 0.01$, one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

vertebrate [40]. Although salmonid fishes underwent two additional WGDs compared with mammals [33,41,42], *NKIRAS1* and *NKIRAS2* each appear to be present as only one gene copy. Nevertheless, differential splicing of the original NKIRAS2 mRNAs from rainbow trout most likely allows the generation of at least four different *NKIRAS2* variants with differentially long 5′-ends. This splicing mechanism does not affect the succession of the exon order or the length of particular exons, as observed previously in rainbow trout [24,25], but it elongates the N-terminus of the *NKIRAS2* variants *b*, *c*, and *d* compared to their paralogue *NKIRAS2a* in rainbow trout and their orthologues in all other vertebrate species. From a structural point of view, the shortest variant *a* therefore seems to be the prototypic *NKIRAS2* version. In the Atlantic salmon *S. salar*, *NKIRAS1* (but not *NKIRAS2*) is present as two different coding sequences (XM_014177875 and XM_014177876), one of which extends beyond the 5′-end, similar to the longer *NKIRAS2* variants from rainbow trout. Accordingly, the mechanism of the N-terminal NKIRAS-protein extension does not seem to be restricted to rainbow trout; however, the functional consequence associated with this structural change remains unknown.

The present gene profiling of the five *NKIRAS* transcripts in the lymphoid and myeloid cells of rainbow trout confirmed that the expression of *NKIRAS* either remained at a generally low transcript level or was only slightly altered after bacterial infection. A revision of ~50 transcriptome studies on salmonid fish after exposure to various immune stimuli [43] confirmed that the copy numbers of *NKIRAS1* and *NKIRAS2* rarely exceed those of the transcript level detected in the control fish. This indicates that the transcription of *NKIRAS* genes is subject to low variability. The permanent presence of NKIRAS seems to be a crucial mechanism for maintaining homeostasis, as mammalian NKIRAS has been proven to control NF-κB [13] and, by extension, numerous immune-gene programs [5,6]. Our structural analysis of the NKIRAS sequences from rainbow trout suggested that all variants may function similarly to their mammalian counterpart because the essential aa residues and motifs of the NKIRAS orthologues were well preserved during vertebrate evolution.

The microscopy examination of *NKIRAS*-overexpressing CHSE cells did not provide evidence of a direct influence of NKIRAS on the translocation of NF-κB. This may reflect the artificial overexpression of NKIRAS or the stress to which the cells were exposed by the previous transfection. Our reporter-gene experiments in CHSE-214 cells revealed that expression of rainbow trout NKIRAS1 and NKIRAS2c reduced the activity of NF-κB, in agreement with previous reports on mammalian NKIRAS proteins [1]. By contrast, expression of NKIRAS2a and NKIRAS2b had an inducing effect on the activity of NF-κB. We found evidence to support an increase in the transcript amount of pro-inflammatory cytokine genes, at least in response to NKIRAS2a expression, in stimulated CHSE-214 cells compared to cells without overexpressed NKIRAS2a.

The reason for these contrary NF-κB regulation mechanisms may be the specificity of the NKIRAS variants for the various combinations of

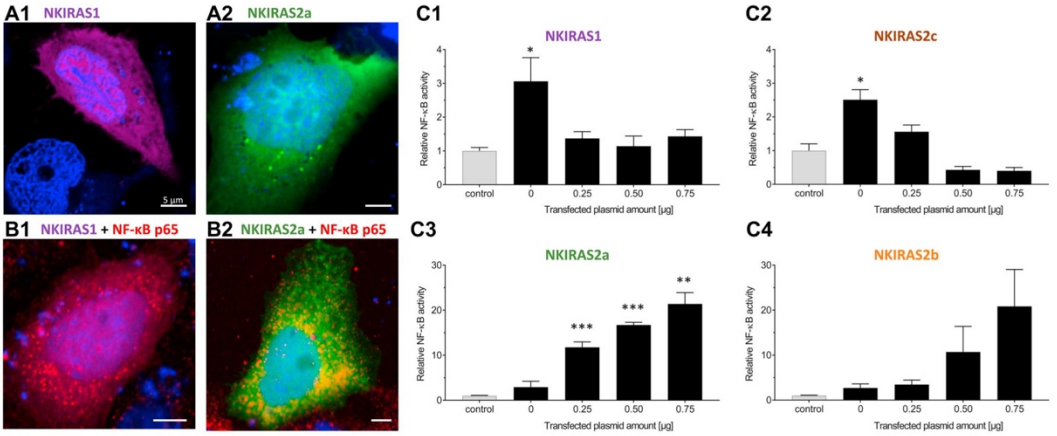

Fig. 5. Impact of overexpressed NKIRAS on the activation of NF-κB in salmonid cells. Confocal analysis of (**A1**) mPlum-tagged NKIRAS1 (purple) and (**A2**) GFP-tagged NKIRAS2a (green) in CHSE-214 cells, 48 h after transfection. Nuclei were stained with Hoechst 33342 dye (blue). The co-localisation of (**B1**) NKIRAS1 and (**B2**) NKIRAS2 with NF-κB was displayed by labelling CHSE-214 cells with anti-RELA antibodies (red) 24 h after transfection with NKIRAS-expressing plasmids. White scale bar represents 5 µm in all images. The luciferase activity of CHSE-214 cells overexpressing (**C1**) NKIRAS1, (**C2**) NKIRAS2c, (**C3**) NKIRAS2a, and (**C4**) NKIRAS2b was determined after a 24 h stimulation with 100 ng/mL flagellin and compared to the non-transfected, unstimulated control cells (set 1.0). The concentrations of the plasmids used for the transfection of the cells are indicated on the abscissa. Statistical significance compared with the control group was assessed using one-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Standard error of the mean (SEM) is indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

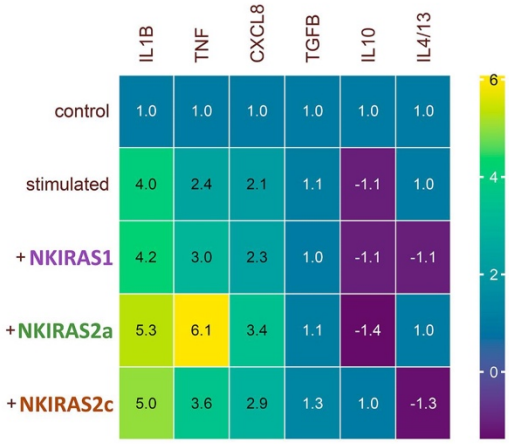

Fig. 6. Expression profiling of stimulated NKIRAS-overexpressing CHSE-214 cells. The HeatMap illustrates the averaged fold-change values (according to the legend on the right) of the mRNA concentrations measured in non-transfected cells (2nd row) and transfected cells (either with *NKIRAS1*-, *NKIRAS2a*- or *NKIRAS2c*-expression plasmids, 3rd to 5th row) after a 3 h stimulation with 100 ng/mL flagellin and compared to unstimulated control cells (set as 1.0, 1st row). The quantified transcripts are listed as gene symbols above the scheme. All expression values were normalised against the geometric mean of two reference genes.

NF-κB/IκB complexes. This would require, as a first step, that the five NKIRAS variants interact specifically with the multiple IκBα proteins in rainbow trout that have been identified apart from the prototype [11]. No IκBβ proteins have yet been isolated from salmonid fish; however, IκBα from rainbow trout contains six ankyrin repeats, as occurs in the human IκBβ, whereas the human IκBα has only five ankyrin domains [44]. Particular IκBα variants may therefore compensate for the loss of IκBβ in rainbow trout. A second point is that these IκB proteins, in turn, interact intimately with the NF-κB/Rel factors. However, few of those factors contain a transcription activation domain, such as p65 (RelA); others such as p50 (NFKB1) or p52 (NFKB2) lack this domain entirely and require interactions with further transcription factors to induce transcription [5,6]. The different NKIRAS-mediated mechanisms controlling the activity of certain NF-κB/Rel factors could explain the difference in the expression of the reporter construct and the inflammatory genes after overexpression of the individual NKIRAS variants.

The finding that transcript variants of NKIRAS2 exert different impacts on the activity of NF-κB could be viewed as unexpected, especially in light of the fact that these variants differ only in their N-termini, which account for only about one sixth to one seventh of the shortest variant 2a. Nevertheless, the N-terminus has been reported to determine several key properties of a protein [45], such as the half-life. In this context, we refer to the predicted 17 aa signal peptide of variant d of NKIRAS2. This signal peptide could guide the insertion of NKIRAS2d into cellular membranes along the secretory pathway, and its cleavage would ensure that the mature NKIRAS2 variant d factor starts with a glutamic acid residue at the N-terminus. The presence of this residue decreases the half-life of a protein to 1 h [46]. NKIRAS2d would therefore be a rather fragile factor compared to its NKIRAS2 isoforms that start with a methionine residue and have an expected half-life of 30 h [46]. Alternatively, the preservation of signal peptides in the mature protein could provide additional features, such as selective coupling to G proteins or regulative functions [47]. Regardless of the presence of signal peptides, the N-terminus is well established to determine the affinity of a protein for involvement in different cell signalling cascades [48]. The different N-termini probably also affect the interaction of NKIRAS with PEST domain-containing factors other than IκB, such as hormone receptors. This hypothesis might be addressed in future analyses.

5. Conclusions

We describe here the structure of five NKIRAS variants in rainbow trout which also modulate the activity of NF-κB and, thus, the pro-inflammatory immune responses in a teleostean model cell. Beyond NF-κB signal transduction, NKIRAS proteins may conceivably control further signalling cascades. Unfortunately, insufficient knowledge is available regarding the NF-κB network in fish; therefore, the obtained data cannot be integrated and adequately evaluated. Our future research will address the interaction of NF-κB and its associated factors in bony fish.

CRediT authorship contribution statement

Fabio Sarais: Cloning, Cell culture, Reporter-gene analyses, qPCR, Data analysis, Visualization, Writing- Original draft preparation.
Henrike Rebl: Confocal microscopy.
Marieke Verleih: SNP analysis.
Sven Ostermann: Cell sorting.
Aleksei Krasnov: Data analysis.
Bernd Köllner: Acquisition of funding.
Tom Goldammer: Supervision, Acquisition of funding.
Alexander Rebl: Conceptualization, Supervision, Data Validation, Visualization, Writing- Reviewing and Editing.
All authors discussed the results and commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.08.052.

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Study II: PIAS Factors from Rainbow Trout Control NF-κB- and STAT-Dependent Gene Expression

Signalling via Janus Kinases (JAK) and Signal Transducers and Activators of Transcription (STAT) allow eukaryotic cells to send a wide range of information from the membrane to the nucleus. During the evolution, several regulatory mechanisms have evolved to fine-tune both intensity and duration of the cytokine signalling including the "protein inhibitors of activated STATs" PIAS. The genome of rainbow trout contains eight pias genes, which encode at least 14 different pias transcripts. These transcript variants are expressed differently in various tissues and cells. This research provides evidence for pias gene multiplication and sub-functionalisation during salmonid evolution.

Highlights

- The genome of rainbow trout contains eight pias genes, which encode at least 14 different pias transcripts and differentially expressed in a tissue- and cell-specific manner.
- The knock-out of Pias factors in a CHSE cells using CRISPR/Cas9 technology failed, indicating the vital importance of the trout Pias factors.
- The overexpressed Pias factors modulated the transcript levels of NF-κB-dependant immune gene.
- Pias1 and Pias2 have shown a differential nuclear localisation

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PIAS Factors from Rainbow Trout Control NF-κB- and STAT-Dependent Gene Expression

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Abstract: Four 'protein inhibitors of activated STAT' (PIAS) control STAT-dependent and NF-κB-dependent immune signalling in humans. The genome of rainbow trout (*Oncorhynchus mykiss*) contains eight *pias* genes, which encode at least 14 different *pias* transcripts that are differentially expressed in a tissue- and cell-specific manner. *Pias1a2* was the most strongly expressed variant among the analysed *pias* genes in most tissues, while *pias4a2* was commonly low or absent. Since the knock-out of Pias factors in salmonid CHSE cells using CRISPR/Cas9 technology failed, three structurally different Pias protein variants were selected for overexpression studies in CHSE-214 cells. All three factors quenched the basal activity of an NF-κB promoter in a dose-dependent fashion, while the activity of an Mx promoter remained unaffected. Nevertheless, all three overexpressed Pias variants from trout strongly reduced the transcript level of the antiviral Stat-dependent *mx* gene in ifnγ-expressing CHSE-214 cells. Unlike *mx*, the overexpressed Pias factors modulated the transcript levels of NF-κB-dependent immune genes (mainly *il6*, *il10*, *ifna3*, and *stat4*) in ifnγ-expressing CHSE-214 cells in different ways. This dissimilar modulation of expression may result from the physical cooperation of the Pias proteins from trout with differential sets of interacting factors bound to distinct nuclear structures, as reflected by the differential nuclear localisation of trout Pias factors. In conclusion, this study provides evidence for the multiplication of *pias* genes and their sub-functionalisation during salmonid evolution.

Keywords: CRISPR/Cas9; innate immunity; immune regulation; JAK-STAT signalling; NF-κB

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1. Introduction

The signalling through Janus kinases (JAK) and signal transducers and activators of transcription (STAT) [1,2] transfer a wide range of information from the membrane to the nucleus of eukaryotic cells [3,4]. Upon the stimulus-dependent activation of specific cytokine receptors, the four mammalian JAK proteins (JAK1, JAK2, JAK3, and TYK2) become activated and phosphorylate several other associated proteins, including themselves, other receptor chains, and STAT factors [5] (Table S1). The seven mammalian STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) dimerise after phosphorylation and translocate into the nucleus, where they bind to cognate DNA elements. These binding sites are often in close proximity to the response elements of nuclear factor-κB (NF-κB).

The NF-κB/Rel family of transcription factors comprises five members (p65/RelA, RelB, c-Rel, and p50/NF-κB1, p52/NF-κB2) in most vertebrates [6]. Together, STATs and NF-κB/Rel factors co-regulate a variety of inflammatory genes [7], including cytokines and, in particular, interferons (IFN) [8]. During evolution, several regulatory mechanisms have evolved to fine-tune both the intensity and the duration of cytokine signalling [9–12], including the 'protein inhibitors of activated STATs' (PIAS; Figure 1) [13,14]. The human PIAS family is comprised of four members: PIAS1, PIAS2/PIASx, PIAS3, and PIAS4/PIASy. By contrast, only one PIAS protein is present in the lancelet (*Branchiostoma* sp.) [15].

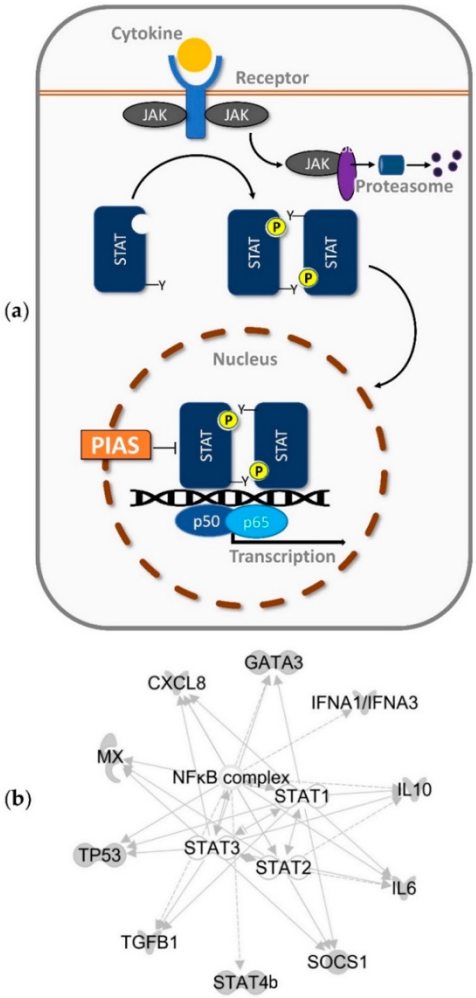

Figure 1. (**a**) Regulation of STAT/NF-κB-mediated pathways via PIAS; (**b**) target genes of the STAT/NF-κB-dependent signalling.

In fish, three orthologues of human PIAS1, PIAS2, and PIAS4 have been identified [16]. *Pias1* and *Pias4* are present as a pair of paralogue genes (a and b) in most fish species, but (pseudo-/allo-)tetraploid families, such as Salmonidae or Cyprinidae, are expected to encode additional *Pias* ohnologues.

The vertebrate PIAS proteins constitute a subfamily of E3 SUMO (small ubiquitin-related modifier) ligases. SUMO ligases tag their substrates post-transcriptionally with small ubiquitin-related modifiers to control the activity of transcription factors, affect the localisation of certain proteins, and inhibit or activate enzymes [17]. The characteristic functional motifs and domains are largely conserved across the vertebrate PIAS members. The N-terminal SAP (scaffold attachment factor A/B/acinus/PIAS) domain recognises and binds to A/T-rich DNA regions [18]. The PINIT motif [19] and two adjacent nuclear localisation signals (NLSs) allow for the retention of PIAS in the nucleus. A RING-finger-like zinc-binding domain (Siz/PIAS RING finger, SP-RING) [20] is followed by a SUMO-interacting motif (SIM) [21], and both are required for SUMO-protein ligase activity and interaction with other proteins. At the C-terminus, PIAS proteins contain a serine- and threonine-rich (S/T) region of unknown function [22].

PIAS factors employ the SUMOylation mechanism to negatively regulate the transcription of target genes by (i) blocking the DNA-binding activity of transcription factors [23–25]; (ii) recruiting histone deacetylases and other regulators, which modulate chromatin compaction [26]; and (iii) isolating transcription factors in specific subnuclear structures that are enriched with corepressor complexes [27,28]. Most studies have focused on the interaction of mammalian PIAS and STAT factors, but PIAS proteins can also block the activity of the NF-κB/Rel factors [15]. PIAS1 prevents the binding of RelA/p65 to the RELA response element of a distinct panel of mainly pro-inflammatory NF-κB-dependent genes [23,29], whereas PIAS3 suppresses the interaction of RelA/p65 and its coactivator CREB-binding protein (CBP) [30,31]. In addition to their inhibitory activities, PIAS proteins can also positively regulate the activity of certain transcription factors [32] (Table S2). Mammalian PIAS4, for instance, may activate NF-κB by SUMOylating the inhibitor of NF-κB kinase subunit gamma (IKBKG) [33].

The only study on the three PIAS4 orthologues of zebrafish (*Danio rerio*) revealed that Pias4a regulates the Ticam1 (TIR domain-containing adaptor molecule 1)/Trif-dependent Ifn and NF-κB pathways [34]. Comparatively more studies have revealed the involvement of Stat proteins in controlling teleostean Ifn pathways [35–37]. The present study comparatively studied the structure, expression, and function of the three PIAS orthologues in salmonid fish and detected indications for their sub-functionalisation.

2. Results

2.1. PIAS Genes Are Present as Multiple Gene Copies in Rainbow Trout and Chinook Salmon

We searched the NCBI gene database for orthologues encoding 'protein inhibitors of activated STAT' in two *Oncorhynchus* species: rainbow trout (*O. mykiss*) and Chinook salmon (*O. tshawytscha*). The PIAS-encoding genes *pias1*, *pias2*, and *pias4* were available, whereas a *pias3* orthologue was absent not only in the *Oncorhynchus* spp., but in all teleostean species. Notably, all three *PIAS* orthologues in the *Oncorhynchus* spp. were present as multiple gene copies on eight chromosomes in rainbow trout (Table 1), similar to Chinook salmon. A synteny analysis allowed us to deduce the ancestry of the pias genes (Figure 2). *Pias1* genes are characteristically located adjacent to *skor1* across the analysed vertebrate species, except for Chinook salmon (Figure 2a). The common adjacency to *morf4l1* and *uaca* indicates that the two *pias1* genes on chromosomes 26 and 30 of rainbow trout are ohnologues, *pias1a1* and *pias1a2*. *Pias1* on chromosome 2 is flanked by a different set of genes, suggesting that this is a paralogue of *pias1a1* and *pias1a2* and should be termed *pias1b*. The *pias2* genes on chromosomes 6 and 11 of rainbow trout are both in close proximity to *npr3*, *arid3a*, and *bmp3*, indicating a common origin (Figure 2b). The two ohnologous genes should be referred to as *pias2a1* and *pias2a2*. We note, in this context, that none of the genes flanking the mammalian *PIAS2* are located near their teleostean orthologues. All *pias4* genes are

flanked by *map2k2* and *onecut* across the analysed teleost species (Figure 2c). The two *pias4* genes on chromosomes 4 and 5 of rainbow trout are both adjacent to *zbtb7a* and *eef2* and should be termed *pias4a1* and *pias4a2*.

Table 1. *Pias* sequences identified in rainbow trout.

Nucleotide NCBI Accession Number	Chromosome	Gene	Transcript Isoform	CDS Length [nt]	UTR Length [bp] 5′	UTR Length [bp] 3′	Instability Motifs	Protein Length [aa]	Protein NCBI Accession Number
Pias1									
XM_036963708	26 (6) *	LOC110527003	pias1a1.1 (X1)	2130	41	1113	1 (3′ UTR)	709	XP_036819603
XM_036963709	26 (6)	LOC110527003	pias1a1.2 (X2)	2112	41	1113	1 (3′ UTR)	703	XP_036819604
XM_036961353	2	pias1b	pias1b	1968	78	3326	20 (3′ UTR)	655	XP_036817248
XM_036969029	30 (4)	LOC110521158	pias1a2	1929	140	1718	6 (3′ UTR)	642	XP_036824924
Pias2									
XM_036936540	11	pias2	pias2a2.1 (X1)	1965	289	3152	14 (3′ UTR)	654	XP_036792435
XM_036936542	11	pias2	pias2a2.2 (X2)	1950	286	3134	14 (3′ UTR)	649	XP_036792437
XM_036936543	11	pias2	pias2a2.3 (X3)	1920	411	3134	14 (3′ UTR)	639	XP_036792438
XM_036979193	6	LOC110525143	pias2a1.1 (X1)	1527	572	2502	2 (5′ UTR), 3 (3′ UTR)	508	XP_036835088
XM_036979194	6	LOC110525143	pias2a1.2 (X2)	1512	572	2502	2 (5′ UTR), 3 (3′ UTR)	503	XP_036835089
Pias4									
XM_021598984	4	LOC110521438	pias4a1.1 (X1)	1485	125	2933	24 (3′ UTR)	494	XP_021454659
XM_021598985	4	LOC110521438	pias4a1.2 (X2)	1455	60	2931	24 (3′ UTR)	484	XP_021454660
XM_021603817	5	LOC11052429	pias4a2	1497	132	3617	26 (3′ UTR)	498	XP_021459492
XM_021613940	8	pias4b	pias4b.1 (X1)	1491	410	696	1 (5′ UTR), 5 (3′ UTR)	496	XP_021469615
XM_036986356	8	pias4b	pias4b.2 (X2)	1422	498	696	1 (5′ UTR), 5 (3′ UTR)	473	XP_036842251

* Brackets indicate the former chromosomal location.

Pias4 on chromosome 8 is flanked by a different set of genes, including *lingo3*, and should thus be termed *pias4b*. The annotation of the *pias* genes from Chinook salmon was less obvious, as some genes have not yet been localised.

The lengths of the sequences coding for the three *pias* paralogues ranged from 1422 bp (*pias4b*) to 2130 bp (*pias1a1*, LOC110527003) in rainbow trout (Table 1).

Although the *pias*-encoding sequences in Chinook salmon are in part shorter, the orthologous sequences from rainbow trout and Chinook salmon share a high level of identity of up to 96% (Figure 3). The identity between salmonid *pias* genes and their human orthologues ranges between 35% and 76% (Figure 3a–c). *PIAS1* shares the highest degree of identity across vertebrate species (Figure 3a), while *PIAS4* is less well conserved across vertebrates (Figure 3c).

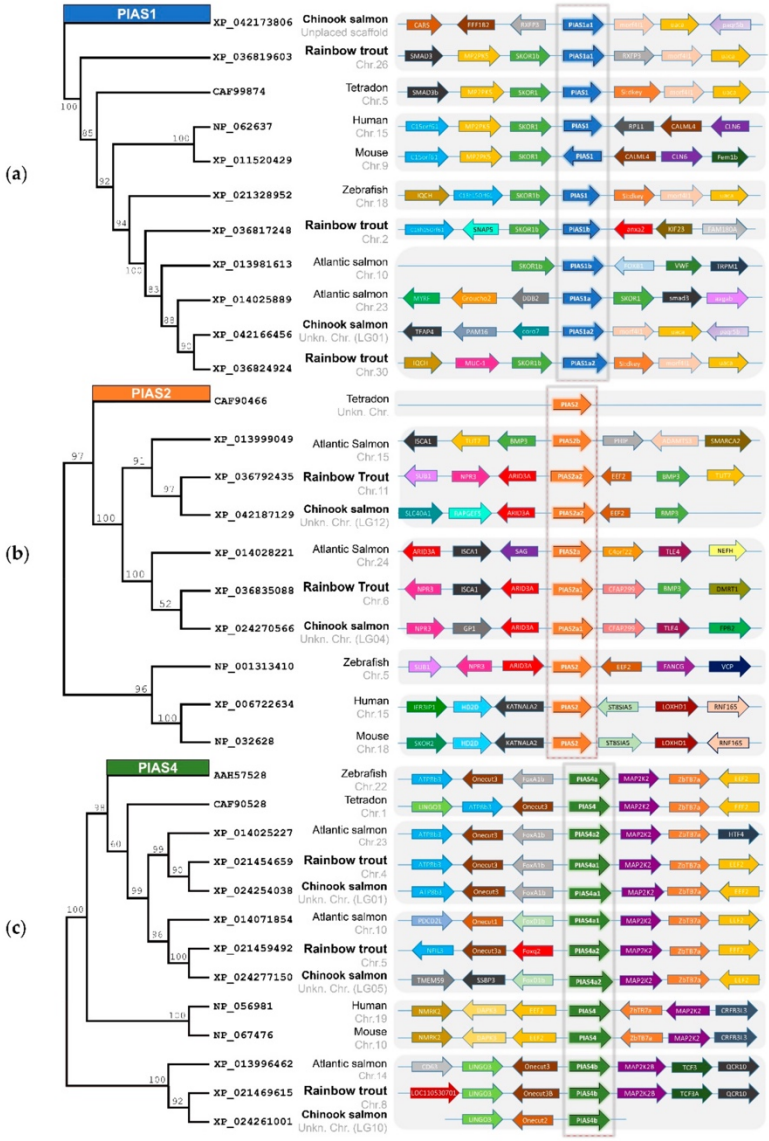

Figure 2. Phylogenetic relationship and synteny between the (**a**) *PIAS1*, (**b**) *PIAS2*, and (**c**) *PIAS4* genes from different vertebrate species. The bootstrap values of the phylogenetic analysis are given at the nodes of the tree. The NCBI protein accession codes, species names, and chromosomal location are listed between the phylogenetic and synteny analyses; the target species are labelled in bold. Arrows represent the reading direction of genes found in synteny; the same colours indicate orthologous genes. The figure is not scaled.

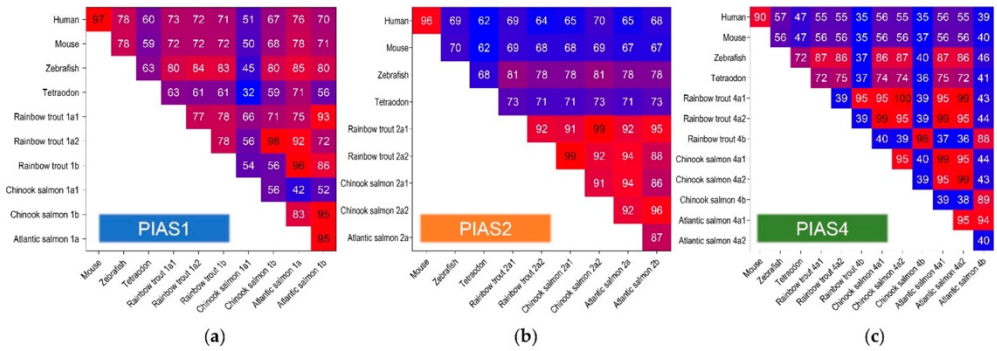

Figure 3. Sequence identity of (**a**) *PIAS1*, (**b**) *PIAS2*, and (**c**) *PIAS4* genes from different vertebrate species, listed to the left and below the individual graphs (different *PIAS* gene variants are indicated behind the species name). For NCBI protein accession codes, please refer to Figure 2.

The overall architecture of PIAS proteins is well conserved across vertebrates (Figure 4).

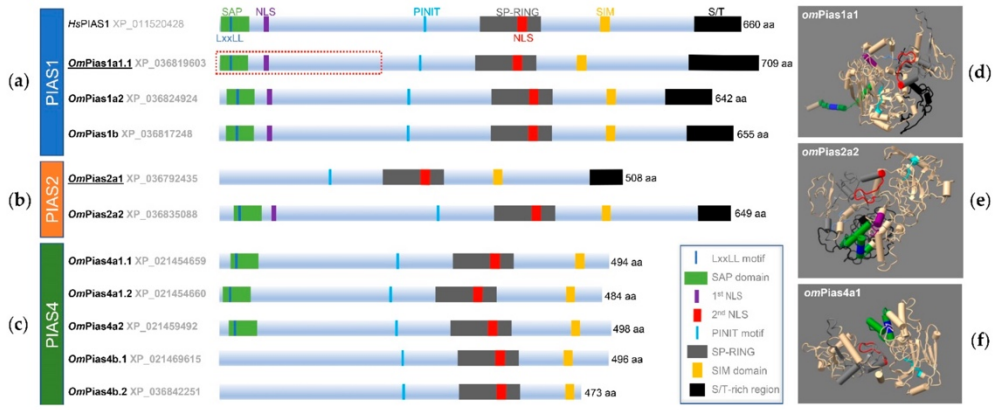

Figure 4. Representation of the domains and motifs characteristic of the variants of (**a**) Pias1, (**b**) Pias2, and (**c**) Pias4 in rainbow trout (Om). A schematic structure of the human (Hs) PIAS1 protein is included. The tertiary structures of (**d**) Pias1a1, (**e**) Pias2a2, and (**f**) Pias4a1 from rainbow trout were drawn using UCSF ChimeraX. The domains and motifs (**a**–**f**) are labelled according to the legend to the right of the Pias4 structures. The two underlined Pias variants were overexpressed in a cell model; the segment framed in red was overexpressed as a third 'truncated Pias1' variant.

All Pias variants identified in rainbow trout contain the PINIT motif, followed by the SP-RING and SIM domain. A KxKELYRRR motif (amino acid (aa) residues 56–64, Pias1a) and the nucleoplasmin nuclear targeting signal $(KK(x)_9KK)$ are signatures of nuclear proteins (aa 373–384, Pias1a; aa 358–369, Pias1b) and characterize the Pias orthologues from *Oncorhynchus* sp. (Figure S1). Some of the analysed variants diverge significantly from the canonical Pias structure. The N-terminal SAP domain is present in all Pias1 aa sequences (Figure 4a, Figure S1), but is absent in Pias2a1 (Figure 4b, Figure S2) and the two Pias4b ohnologues (Figure 4c, Figure S3) from rainbow trout. While a centrally located NLS is included in the SP-RING domain of all Pias proteins of rainbow trout, the N-terminal located NLS is absent in Pias2a1 and all Pias4 paralogs of rainbow trout. In addition, all

Pias4 protein variants of rainbow trout lack the S/T-rich region (Figure 4c, Figure S3), which is present in Pias1 and Pias2.

Notably, the *Oncorhynchus* Pias1b sequences are elongated at the N-terminus by a stretch of six amino acid residues, but this is absent in the mammalian counterpart (Figure S1). Conversely, more than 30 amino acid residues at the N-terminus extend the human Pias2 sequence (Figure S2). No signal peptide has been predicted for any Pias sequence from *Oncorhynchus* sp., but several disorder regions are present and are mostly located at positions conserved across vertebrate species (Figure S4).

The three-dimensional reconstructions of the three Pias factors from rainbow trout (Figure 4d–f) illustrate the well-conserved tertiary structure of the particular domains. The characteristics of the domains that are critical for nuclear localisation, SUMOylation, and zinc finger activity are located in a similar configuration to those observed in their human counterparts [38].

2.2. The Expression of Pias Genes Is Tissue and Cell-Type Specific

We used qRT-PCR to quantify the transcript levels of ten *pias* gene and transcript variants in all the selected tissues (brain, gills, head kidney, liver, muscle, spleen, and trunk kidney) of adult rainbow trout (Figure 5a–g). The *pias* gene variants did not show a uniform expression pattern; instead, they were regulated in a tissue-specific manner. *Pias1a2* was the dominantly expressed *pias* transcript variant (0.9 to 2.9 $\times$ 10^3 transcripts/µg RNA) in gills, liver, spleen, head kidney, and trunk kidney, while its paralogue *pias1b* was the dominant *pias* transcript in the brain (2.7 $\times$ 10^3 transcripts/µg RNA) (Figure 5a). In muscle, the overall *pias* transcript levels were low, with *pias4b.2* as the major *pias* transcript (1.4 $\times$ 10^3 transcripts/µg RNA) (Figure 5e).

As a complement to our qPCR measurements in the whole tissues, we also analysed the levels of the various *pias* transcript variants in four cell models: (i) the secondary cell line CHSE-214, a model for functional studies of our *pias* constructs; (ii) freshly isolated head-kidney cells from rainbow trout; (iii) a non-myeloid (mAb21N) fraction enriched with T- and B-lymphocytes, natural killer-like cells, and thrombocytes; and (iv) a myeloid (mAb21P) fraction enriched with dendritic cells, granulocytes, and monocytes/macrophages from the head kidney of rainbow trout (Figure 5h–k). Strikingly, the model cells revealed considerable transcript levels of *pias4a2* (>1 $\times$ 10^3 transcripts/µg RNA), which was more or less absent in the whole tissues previously analysed. By contrast, *pias4a1.2* was constitutively expressed in all the selected tissues but was almost absent in the cell models. The *pias4a1.1* transcript variant was absent or present at an undetectable level (<20 transcripts/µg RNA in the liver) across all analysed tissues and cell models. The expression of *pias4b* was also not detected in the CHSE-214 cells (Figure 5h). Despite this observation, the expression of the *pias1* and *pias2* transcript variants in CHSE-214 was roughly comparable to that of the head kidney, with *pias1a2* as the most strongly expressed variant (3.3 $\times$ 10^3 transcripts/µg RNA). The primary head-kidney cells had over 50% higher expression of various *pias* genes (*pias1a1*, *-1b*, *-2a1*, *-2a2*) compared with CHSE-214 cells. The expression of *pias* genes was even stronger in the non-myeloid cell fraction (up to 1.2 $\times$ 10^5 *pias* transcripts/µg RNA).

In the head-kidney cells and the non-myeloid cells, *pias2a2* was the most strongly expressed *pias* gene (>6.0 $\times$ 10^3 transcripts/µg RNA), while the *pias4a2* gene was most strongly expressed in the myeloid cell fraction (5.6 $\times$ 10^4 transcripts/µg RNA).

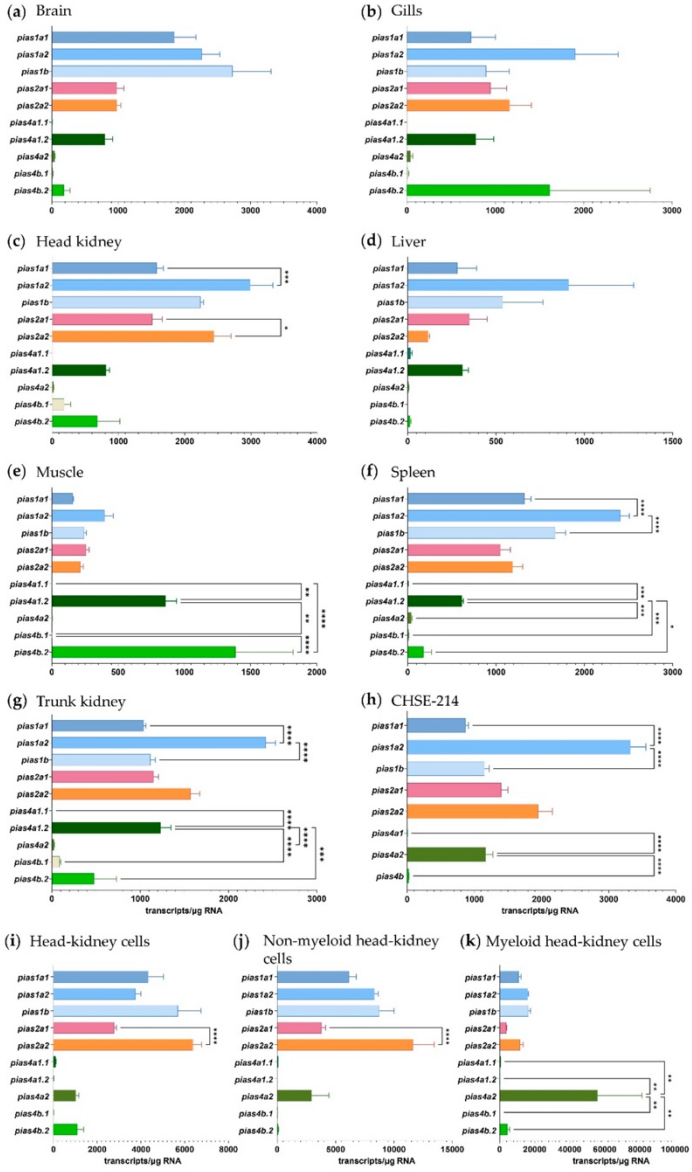

Figure 5. Levels of *pias* transcripts in rainbow trout tissues and salmonid cells (as listed above the diagrams). Bars represent the averaged copy numbers (n = 4) normalised against two reference genes; error bars represent the standard error of the mean. Asterisks represent significantly different transcript levels across ohnologues and transcript variants (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

We also quantified the expression of three *stat1* genes (Figure 6a–k), which are targeted by activated Pias factors. Overall, the qPCR expression data provided no obvious evidence for co-expression of *stat* and *pias* genes, as the transcript levels of *stat1a1* and *stat1a2* uniformly dominated over the *stat1b1* levels across all the tissues and cell models investigated.

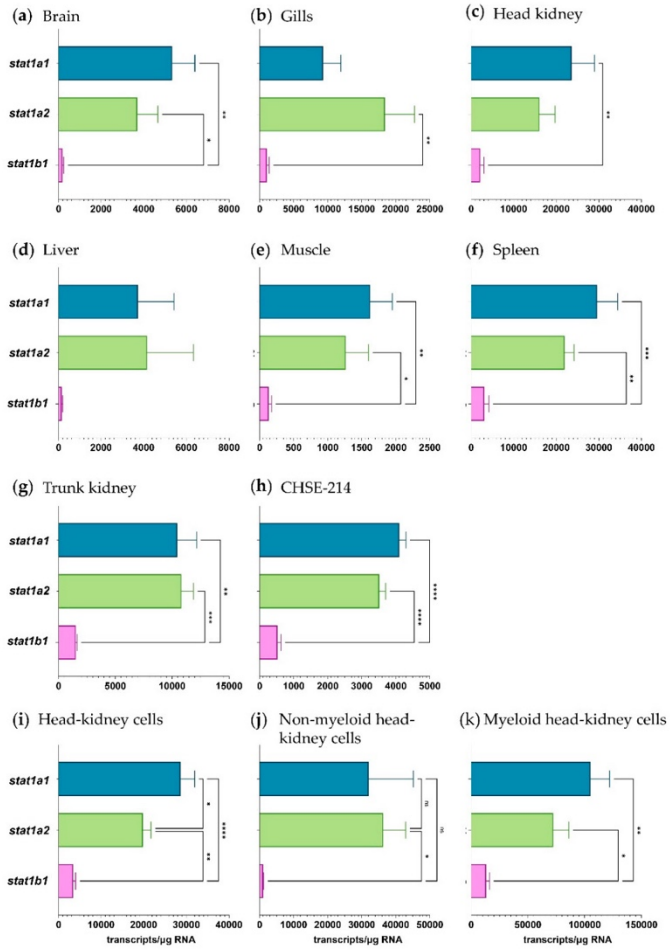

Figure 6. Expression profile of the *stat* genes in rainbow trout tissues and salmonid cells (as listed above the diagrams). Bars represent the averaged copy numbers ($n = 4$) normalised against two reference genes; error bars represent the standard error of the mean. Asterisks represent significantly different transcript levels (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

In general, the expression levels of the three *stat1* genes were highest in the leukocytes enriched with myeloid cells (1.1×10^5 *stat1a1* transcripts/µg RNA), as observed for *pias* expression. Remarkably, the transcript level of the three *stat* genes exceeded the *pias* levels, in general, by approximately 10-fold. Moreover, the expression levels of the three

stat1 genes were roughly 10 times lower in CHSE-214 cells than in the primary head-kidney leukocytes.

2.3. Pias1 and Pias2 Are Located in the Nucleus of Model Cells and Interact with NF-κB to Alter Transcriptional Responses

The prominent expression of *pias1* and *pias2* in CHSE-214 cells (Figure 5h) suggested that a CRISPR/Cas9 strategy might knock out both genes in the CHSE-214-derived cell line CHSE-EC [39]. The specific short guide (sg) RNA targets were located 20 bp upstream of the neighbouring 'protospacer adjacent motif' in exon 2 on *pias1*, in the first exon on *pias2*, and in the *mEGFP* (monomeric enhanced green fluorescence protein) gene. Cell sorting selected 48 GFP-negative single cells (Figure 7a), but only about 30 single cells generated a clonal cell line for each experiment. We sequenced the first 300 nt of the mEGFP gene and the sgRNA target exon of both *pias* genes. All sequenced clones had a deletion of one nucleotide in the ORF of the *mEGFP* gene, resulting in a frameshift and gene disruption (Figure 7b). Despite this observation, none of the mEGFP-negative clonal cell lines were mutated for the *pias1* or *pias2* genes. This suggests that the CRISPR/Cas9 system basically worked in CHSE-EC cells, and we can only speculate that a knock-out of the genes of interest might have been lethal for the cell [8]. In-silico analysis at DepMap Portal (https://depmap.org/; accessed on 1 October 2021) supports this assumption, since human *PIAS2* and *PIAS4* genes were designated as strongly selective.

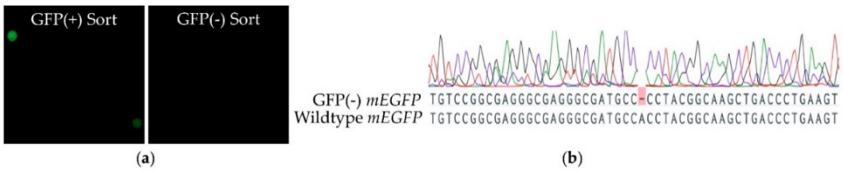

Figure 7. Knock-out targeting *pias1* and *pias2* in CHSE-EC cells. (**a**) Sorting of GFP-positive (+) and GFP-negative (-) cells; (**b**) genotype of GFP(-) clones.

Since the knock-out of both *pias1* and *pias2* genes failed, we selected three *pias* sequences for overexpression in the CHSE-214 cell model based on their explicit structural differences (cf. Figure 4a,b). Pias1 (709 aa) largely corresponds to the human orthologue, and Pias2 (508 aa) lacks the SAP domain and one of the two NLS, while the truncated Pias1 variant (233 aa) contains only the SAP domain and the respective NLS. Confocal microscopy revealed that full-length Pias1 and Pias2 from rainbow trout (both flagged with green fluorescent protein, GFP) apparently shared the same subcellular localisation in the nucleus of unchallenged or stimulated cells (Figure 8a,b) as the human orthologue [23,40]. Remarkably, Pias1 was more homogeneously distributed across the nucleus, while Pias2 was located at distinct nuclear spots.

The functions of the three Pias variants were explored by transient overexpression in CHSE-214 cells in separate approaches, together with a luciferase-reporter construct under the control of either (i) a rainbow trout *mx* promoter with an interferon-stimulating response element [41,42] or (ii) a human NF-κB-responsive *ELAM* (endothelial cell-leukocyte adhesion molecule) promoter [43]. Co-transfection with a construct expressing the ifnγ (encoded by the *ifng* gene, LOC100136413; NCBI acc. #AY795563) from Atlantic salmon, *Salmo salar*, simulated antiviral immune responses involving the production and secretion of ifnγ and the subsequent activation of the jak-stat pathway. The endogenous ifnγ synthesis induced a significant four-fold increase in the NF-κB promoter activity and approximately a two-fold increase in the *mx* promoter activity (both set as 1.0 in Figure 8c,d) over cells not transfected with the ifnγ-expression construct.

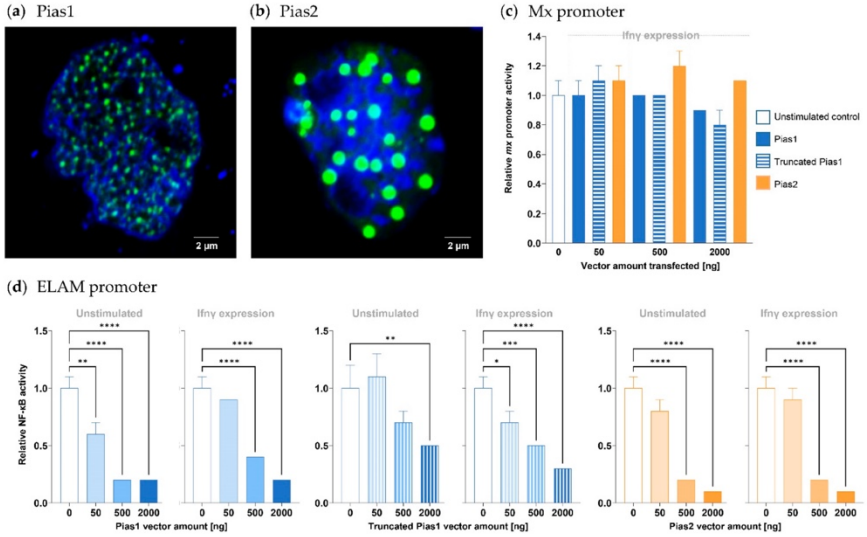

Figure 8. Overexpression of Pias factors in salmonid cell models. Confocal analysis of (**a**) GFP-tagged Pias1 (green) and (**b**) GFP-tagged Pias2 (green) in CHSE-214 cells; nuclei were stained with Hoechst 33342 dye (blue). The white scale bar represents 2 µm. The luciferase activity of CHSE-214 cells co-expressing (**c**) *mx*-reporter construct or (**d**) *ELAM*-reporter construct was determined in unstimulated control cells or ifnγ-expressing cells (as indicated above the graphs) co-expressing increasing concentrations of the *pias*-expressing vector (indicated on the abscissa). The luciferase activity in all cell cultures not expressing *pias* was set to 1.0. Statistical significance compared with the control group was assessed using one-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). The standard error of the mean (SEM) is indicated.

In cells that co-expressed one of the three Pias factors and the *mx*-reporter construct, we observed that neither different concentrations of transfected Pias-expressing vectors nor the additional endogenous expression of ifnγ affected the reporter-gene activity (Figure 8c). By contrast, we detected a significantly reduced reporter-gene activity in cells co-expressing one of the three Pias factors and the *ELAM*-reporter construct (Figure 8d). The use of 50 ng and more of Pias1-expression vector, 500 ng and 2000 ng of Pias2-expression vector, and 2000 ng of truncated Pias1-expression vector reduced the reporter-gene effectivity down to at least 0.6 (with $p < 0.01$). In other words, 50 ng of vectors expressing either Pias2 or truncated Pias1 were ineffective. The cells producing endogenous ifnγ underwent a similar Pias-induced effect, but this effect was not significantly different from that observed in unstimulated cells that did not produce ifnγ.

Having established that the three analysed Pias factors affected NF-κB activity, we used qPCR to test whether the overexpression of Pias1, truncated Pias1 or Pias2 modulates the transcription of a panel of early immune genes, which are regulated by the crosstalk of NF-κB and stat factors (cf. Figure 1b).

Ifnγ expression resulted in a ~two-fold increase in *mx* transcript levels and a slight, but significant, reduction in the *ifna1* transcript levels (Figure 9a). The use of 500 and 2000 ng of Pias1 and Pias2 both caused a 2.1- to 4.2-fold decrease in *il6* transcripts. The highest concentration of Pias2 also caused a significant decrease in *il10*, *ifna3*, and *stat4* copies by 1.6- to 2.0-fold. Of note, *tgfb* was the only gene that was significantly (two-fold) upregulated in concentration after the addition of 2000 ng of Pias1 (Figure 9a). The addition of 50 ng or 500 ng of Pias1 or truncated Pias1 from rainbow trout only slightly modulated the concentration of *mx* transcripts, but the addition of 2000 ng of one of the three Pias factors caused a strong decrease of more than ten-fold. The use of 500 ng of

Pias2 also significantly (7.4-fold) lowered the number of *mx* transcripts. Altogether, 2000 ng of Pias2 downregulated the transcript levels of eight studied genes (*cxcl8*, *tgfb*, *il10*, *il6*, *ifna1*, *ifna3*, *stat4*, and *mx*) to a greater or lesser extent; therefore, we determined the mRNA concentration of four additional genes (*gata3*, *mmp9*, *socs1*, and *tp53*; cf. Figure 1b, [7]). The use of 2000 ng of Pias2 also reduced the transcript levels of *mmp9*, *gata3*, and *socs1* by 1.9- to 3.3-fold, while the levels of *tp53* remained unchanged (Figure 9b).

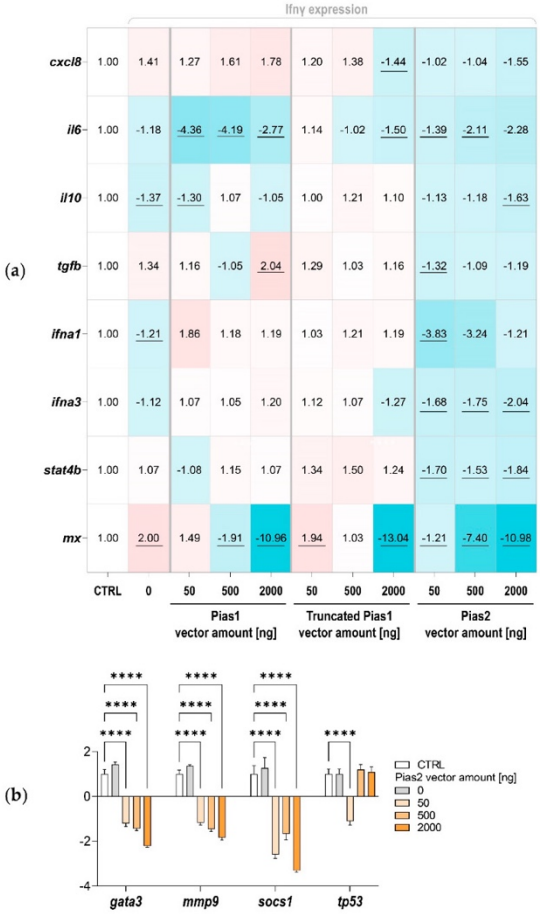

Figure 9. Expression profiling of *pias*-overexpressing CHSE-214 cells. (**a**) The heatmap and (**b**) bar chart illustrate the averaged fold-change values of the mRNA concentrations measured in cells transfected without (CTRL, set as 1.0) or with the *ifnγ*-expression vector (0 ng pias vector amount) together with *pias*-expression vectors (as indicated). The quantified transcripts are listed as gene symbols. All expression values shown in (**a**,**b**) were normalised against the geometric mean of two reference genes. Significantly different FC values compared to CTRL are underlined in (**a**). Statistical significance compared with the control group was assessed in (**b**) using one-way ANOVA (****, $p < 0.0001$); standard error of the mean (SEM) is indicated.

3. Discussion

PIAS proteins interact with more than 60 different proteins linked with transcriptional processes [23–25] and fine-regulate not only the STAT-dependent pathway but also NF-κB-signalling. Four different Pias proteins are encoded in humans, whereas PIAS3 has been lost in fish. In the present study, we found eight Pias-encoding genes in *Oncorhynchus* sp., and these likely result from two additional genome duplications in teleosts [44] and salmonids [45]. These eight genes encode at least 14 different pias transcripts. We speculate that the fish-specific and salmonid-specific duplications expanded the pias gene family in fishes by additional members, thereby compensating for the loss of PIAS3. The question that remains is why the number of Pias proteins in trout is still twice as high as that in humans.

In previous studies, we inspected the diversity of ohnologue and paralogue genes in salmonid fish that encode immune inhibitors [14]. We repeatedly found that structural modifications of the protein may fundamentally impact its function [46,47]. However, all three Pias1 proteins from rainbow trout strongly resemble the prototypical PIAS architecture of their human orthologue. This structural conservation suggests that the three Pias1 proteins from rainbow trout are functionally homologous to their counterparts in other vertebrates. One of the two Pias2 proteins from trout also resembles human PIAS1, while its paralogue and one of the Pias4 paralogues lack the SAP domain and one NLS. Similarly, all Pias4 proteins from trout lack the ST-rich region (as their mammalian counterparts), which is vital for the pleiotropic interactions associated with SUMO. Confocal microscopy provided evidence that Pias1 (with its SAP domain and two NLS) and Pias2 (without a SAP domain and with only one NLS) differentially localise in the nucleus. The 'dot-like signatures' of Pias2 from trout have also previously been observed for Pias4 from zebrafish [34]. Mammalian PIAS proteins interact with different (sets of) other (transcription) factors [48,49]. The dissimilar localisation of Pias1 and Pias2 from trout probably results from their cooperation with different sets of proteins that bind to distinct nuclear structures. The LxxLL motif, in particular, has been established to interact with nuclear receptors and co-receptors [49]; this motif is present in Pias1 but missing in Pias2 from rainbow trout.

Expression profiling of the individual *pias* variants from trout provided further evidence of their sub-functionalisation. While the *stat1* transcripts were expressed in relatively similar ratios to each other across the tissues analysed, the *pias* transcripts showed a rather tissue-specific expression pattern. Although *pias1a1* is the predominantly expressed gene in most tissues and cell fractions, the *pias4* transcripts, which do not encode an ST-rich region, dominated in muscle and in cell fractions enriched with dendritic cells, granulocytes, and monocytes/macrophages. The non-myeloid cell fraction contained mostly *pias2a2* transcripts, which are structurally quite similar to the *pias1a1* transcripts. The non-myeloid fraction is enriched with lymphocytes and thrombocytes and may also contain hematopoietic progenitor cells. The murine PIAS1 pathway has been reported to regulate self-renewal and differentiation of hematopoietic stem cells [50]. Therefore, the role of *pias1* and *pias2* in immunity and haematopoiesis remains an open topic for research in trout. The myeloid cell fraction expressed the highest levels of *pias* transcripts in general, thereby underpinning the importance of the Pias factors in immunophysiology.

Functional in vitro studies on Pias proteins in a non-mammalian model have only been carried out for Pias4 from zebrafish [34] to date. Therefore, we restricted our studies to one representative of Pias1 and Pias2 from rainbow trout and a truncated variant of Pias1 for comparative purposes. Since our expression studies on the salmonid fish cell model revealed that all transcript variants of both *pias1* and *pias2* were abundantly detectable, our aim was to knock out both factors using CRISPR/Cas9 technology. Since this approach was not successful, we were left with overexpression, a well-established method [51], to gain first insights into the function of trout Pias factors.

Both Pias1 and Pias2 from rainbow trout reduced the basal activity of NF-κB in unstimulated cells to a similar extent and in a dose-dependent fashion. This was not expected, as St2/Il1rlL1, another established inhibitor of NF-κB signalling, failed to modulate the

basal level of NF-κB in our previous in vitro experiments in rainbow trout [52]. However, a study on murine PIAS1 has pointed to its potential to reduce NF-κB activity in a dose-dependent manner [23]. Murine PIAS1 directly interferes with the binding of NF-κB p65 to its corresponding response elements [48], and this mechanism is apparently conserved in fish. Interestingly, both Pias1 and Pias2 were similarly efficient in lowering the level of activated NF-κB, even though Pias2 lacks the SAP domain with the intrinsic LxxLL motif. A point worth mentioning in this context is that the expression of a mammalian PIAS3 mutant with point mutations in the LxxLL motif also did not interfere with the NF-κB activity [31].

In contrast to NF-κB-dependent promoters, Pias1 and Pias2 from rainbow trout did not modulate the activity of a trout *mx* promoter. Mx is a potent effector of antiviral defence [53]. We observed that overexpressed *pias1*, *pias2* and truncated *pias1* from trout strongly reduced the transcript level of *mx* in ifnγ-expressing cells. Apart from this effect, the truncated Pias1 variant did not modulate the transcript levels of any of the selected immune genes. Pias1 and, to a lesser extent, Pias2 lowered the transcript level of *il6*. In this regard, we note that the JAK-STAT signalling pathway is also known as the IL6 signalling pathway [54], as IL6 activates the cascade and thus also regulates its own expression. In contrast to the full-length and truncated Pias1 variants, Pias2 also reduced the transcript levels of several other NF-κB-dependent genes (*ifna3*, *stat4*, and *il10*) and, beyond those, the STAT-dependent genes *socs1*, *gata3*, and *mmp9*. These apparently different efficiencies in expression regulation likely reflect another consequence of the structural differences between Pias1 and Pias2 from trout. Previous studies have demonstrated that the overexpression of PIAS proteins enhanced the SUMOylation of nuclear receptors [55]. Since Pias1 and Pias2 from trout contain the required SP-RING and SIM domains, both should be capable of transferring SUMO proteins and thus altering the activity of transcription factors. The SAP domain is crucial for the translocation of transcription factors to the nuclear periphery [18], and for this reason, only Pias1, but not Pias2, from trout should be capable of an alternative regulatory mechanism that does not involve SUMO tags.

In conclusion, this study provides evidence for the multiplication of *pias* genes and their sub-functionalisation during salmonid evolution. For the functional analysis, we largely relied on the widely used CHSE-214 cell line. We note, in this regard, that this model cell is characterised by certain immunocompetence [56,57], although it cannot represent the complex interactions of different cell populations that tailor immune responses in vivo. For this reason, the CHSE cell line is a helpful tool, but it alone is not sufficient to map out the multiple functions of the Pias factors. Subsequent studies may investigate the influence of Pias proteins from trout using different immune cell subsets and in vivo.

4. Materials and Methods

4.1. Sampling and Cell Sorting

Rainbow trout (*O. mykiss*) were obtained from a local commercial fish farm 'Forellenzucht Uthoff GmbH', Neubrandenburg (Germany). Four fish were euthanised using an overdose of benzocaine (100 mg/L, Sigma-Aldrich/Merck, Steinheim, Germany) in compliance with the relevant European guidelines on animal welfare (Directive 2010/63/EU on the protection of animals used for scientific purposes) and were approved by the institute's ethics board (approval ID: FLI 28/17). For the preparation of leukocyte suspensions, head kidneys were homogenized separately in 5 mL of 1% newborn calf serum (NCS)/phosphate-buffered saline (PBS) buffer (FB buffer). Cell suspensions were centrifuged at 4 °C at 290 g for 5 min and then resuspended in 3 mL FB. A Percoll gradient to discard erythrocytes was prepared as described previously [58]. One million leucocytes were labelled for 30 min at 4 °C with the monoclonal antibody 21 (mAb21, [59]), which recognises cells from a myeloid lineage. Thereafter, the cells were washed by centrifugation at 300× *g* and 4 °C for 5 min in 700 µL MACS Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). The pellet was resuspended in 200 µL of secondary antibody solution containing anti-mouse IgG-conjugated magnetic beads (Miltenyi Biotec), followed by a 30 min incubation at 4 °C

and a final washing step. The cells were resuspended in 500 µL MACS buffer (Miltenyi Biotec, Germany) and placed into ice-cold racks to perform the magnetic separation in the autoMACS Pro Separator (Miltenyi Biotec). The sorting was conducted using the Possel_S program. After the separation, the enriched (mAb21-positive) cell fraction consisted of >95% myeloid cells, and the depleted (mAb21-negative) fraction consisted mostly of B- and T-lymphocytes, as well as thrombocytes. Both fractions were centrifuged, and the resulting pellets were resuspended in 350 µL RLT lysis buffer (Qiagen, Hilden, Germany) for RNA extraction and gene expression analysis.

4.2. RNA Isolation, cDNA Synthesis, and Quantitative PCR (qPCR) Analysis

RNA was isolated from the brain, gills, head kidney, trunk kidney, liver, muscle, and spleen of rainbow trout first with TRIzol (Thermo Fisher Scientific, Bremen, Germany) and then with the RNeasy Mini Kit (Qiagen), including an in-column DNase treatment for 30 min. For RNA isolation from cells, we used the ISOLATE II RNA Micro Kit (Bioline/Meridian Bioscience, Luckenwalde, Germany). RNA quantity was determined with a NanoDrop One^c (Thermo Fisher Scientific), and the integrity was assessed by agarose-gel electrophoresis. Total RNA was reverse-transcribed using a SensiFAST cDNA Synthesis Kit (Bioline/Meridian Bioscience).

The expression of (a) paralogues and ohnologues of *pias* genes in different tissues and cells of *Oncorhynchus* and (b) various immune genes in CHSE-214 cells transfected with *pias*-expressing vectors was profiled by establishing a panel of oligonucleotides (Table 2) using the Pyrosequencing Assay Design software (v.1.0.6; Biotage, Uppsala, Sweden) to amplify specific fragments between 95 and 195 bp.

The cDNA input into the individual RT-qPCR assays was equivalent to 2.5 ng total RNA isolated from cells and 75 ng total RNA isolated from tissues. The analyses were conducted with a LightCycler 96 instrument (Roche, Mannheim, Germany) using a SensiFAST SYBR No-ROX Kit (Bioline/Meridian Bioscience). Melting curve analyses validated the amplification of distinct products. In addition, we validated the size and quality of the PCR products on 1.5% agarose gels. Standard curves were generated based on the crossing points of 10-fold dilutions containing 10^3 to 10^6 copies of a PCR-generated standard fragment. The copy number was calculated for each fragment based on linear regression of the standard curve and relative to the amount of input RNA. Each expression value of the target genes was divided by the geometric mean of the reference genes *eef1a1* (eukaryotic translation elongation factor) [60] and *rps5* (ribosomal protein S5) [61].

Table 2. Primers used in this study.

Gene Symbol	Primer Sequence 5′→3′ (Sense, Antisense)	Nucleotide NCBI Accession Number Used for Primer Design		Fragment Length [bp]
		O. mykiss	*O. tshawytscha*	
Quantitative PCR Analysis (*Oncorhynchus mykiss*, *O. tshawytscha*)				
Pias1a1.1	GTTGGAAGGCACCTTCTGTGTT, CTACGGTCCAAAGGCATCAGG	XM_036963708	XM_031812568	108
Pias1b	GGAGCTACTCTATGGCGGTGT, ATCAGGAACCCAGACCATTCCA	XM_036961353	XM_042317872	99
Pias1a2	TAGGCAGGAATTTCTCCATGGC, AGAGAAGTTAACAGCTGACCCG	XM_036969029	XM_042310862	140
Pias2a1.1	GTGTGCATCTCCAGGGACTTTT, CTAAGAATGGAGTGGAACAGAAG	XM_036979193	XM_024414775	195
Pias2a2.1	GAGCTACGGAGCATGGTGTCA, AACTTTATCGACGCCGCTATCC	XM_036936540	XM_042331195	185
Pias4a1.1	ATTGGAAGCAGAGAACCGTCGA, ATTTTCGGGTGTCTGACCTGCA	XM_021598984	XM_024398270	158
Pias4a1.2	GCCTGCTAGGCTGGGAAACTA, CGCAGTAAAAGTGGTCTGAAGC	XM_021598985	—	99
Pias4a2	AGGAGGAGGGGGGAGGAGG, CGGACTGACCCCACAAACTGA	XM_021603817	XM_024421382	144
Pias4b.1	ACATAGCAGAAGCAATTAGGTTGT, AATCTGCTGGTGAGGGCAGTG	XM_021613940	XM_024421383	146
Pias4b.2	ACAAAGGCCCCGGAGTGAACA, GGGAGGGGAGTCAAGCTACAT	XM_021613941	—	129
Stat1a1 (stat1-1)	GAGAGCATCGACTGGGAAAATGT, AAACAACTTCCTGCTACAACACAA	NM_001124707	XM_024426102	131
Stat1a2 (stat1-2)	CCCCGTTCACATGGCCATGAT, CATAGAGACCGACAGAGAAAACA	XM_021608237	XM_042324083	95
Stat1b1 (stat1ab)	GGCCATGATAATCTGTAACTGTC, ACGTTAAAGACCTGAGGAACCG	XM_021579196	XM_042306329	150
Cxcl8	ATATAACACTTGTTACCAGCGAGA, ATTACTGAGGAGATGAGTCTGAG	HG917307	XM_024415648	106
Il6	GTGTTAGTTAAGGGGAATCCAGT, CCTTGCGGAACCAACAGTTTGT	NM_001124657	XM_024404411	128

Table 2. *Cont.*

Gene Symbol	Primer Sequence 5′→3′ (Sense, Antisense)	Nucleotide NCBI Accession Number Used for Primer Design		Fragment Length [bp]
		O. mykiss	*O. tshawytscha*	
Il10	TGCCCAGTGCAGACGTGTACC, TACACCACTTGAAGAGCCCCG	NM_001245099	XM_042324963	137
Tgfb	ATCAGGGATGAACAAGCTGAGG, CGGAGAGTTGCTGTGTGCGAA	XM_021591332	XM_024397891	161
Ifna1	TTGAAGAGAGCAAATGTATGATGG, TCCTGTACAGCCTACAGTTCATT	XM_024434105 (representative for *LOC110538045*, *LOC110538046*, *LOC110538047*,*LOC110538053*, *LOC110538937*, *LOC118937709*)	XM_024434105 (representative for *LOC112259401*, *LOC112259404*, *LOC121847201*, *LOC121847202*, *LOC112258510*)	173
Ifna3	CCAACATCACTTTACAGACACATA, GGGACAAGAAAAACCTGGACGA	XM_024432928 (representative for *LOC110511235*, *LOC110517168*, *LOC110538043*, *LOC110538058*)	XM_024389910 (representative for *LOC121838839*, *LOC112225816*, *LOC112258507*, *LOC112258508*)	140
Stat4b	ACCTCATCAAAAGCTCCTTTGTG, TTCACCACCAAAGTCAGATTGCT	XM_024388828	XM_024388828	112
Mx	GTAGCGGTATTGTAACACGATGC, TCGTGAAGCCCAGGATGAAATG	XM_036958922	XM_024415949	158
Gata3	CCACCTCCTCCACATAGTAGTC, GACCTGCCGGGGAACCGTG	XM_036957437	XM_042311479	160
Mmp9	TGCCAAGATAGAGGCTACAGTC, TGTCTTGGACCCATAGAGATAGT	XM_036986917	XM_024376362	181
Socs1	ACGGATTCTGCGTCGGAAAATAT, ACACAGTTCCCTGGCATCCGT	XM_036973400	XM_042313671	91
Tp53	GAATTTGAACCTGGTGGCAGTTC, CACCTCAAACAGACTCGGATCA	NM_001124692	XM_024394883	115
Construction of PIAS-expression constructs				
Pias1a1	ATGCAAGCTTATGGCGGAGAGTGCGGAACT, CATACCAGACGTGATCTCGTTAGACGAATTCGCAT	XM_036963708		1968
Pias2a1	ATGCAAGCTTATGATCCTGACAAGAAAAATGGCGG, ACATCATCTCAGACATCATCTCATTGGACGAATTCGCAT	XM_036936540		1965

Table 2. *Cont.*

Gene Symbol	Primer Sequence 5′→3′ (Sense, Antisense)	Nucleotide NCBI Accession Number Used for Primer Design		Fragment Length [bp]
		O. mykiss	*O. tshawytscha*	
	SgRNA target sequence			
Pias1a2	AGTAACACTTGTAGCTCTGA GCGTAGCCTAGTAACACTTG TGTTTGTTGCGTCCTGCGTA		XM_024407180	20
Pias1b	TCTACAATAACACAAAAAGA ACACGACTCTGCAAGAGGGT TCTGTCAATCCATCTACAAT		XM_024410569	20
Pias2a1.1	ACACGTCGTAGAACGGGAGA GAGGGATGAGAGGGGCGGGC CCAGCAGCCCGCCCCTCTCA		XM_024414775	20
Pias2a2	CCATTTTTCTTGTCAGGATC GCGAAGCCCAGTAACACTTG TAGCTCCTCAAATTCCGCCA		XM_024407486	20
mEGFP	GGCGAGGGCGATGCCACCTA		[39]	20
	Sequencing primers for GFP(-) cells			
Pias1a2	TTAGTTGTTCTATTCGTGTGTCCTA, TTACACACACTGTGTGTACAAAACA		XM_024407180	500
Pias1b	GACCCCACTGCCTTTGTTTCAAACC, CATTCCTCCAAGGAGACAACCACCAG		XM_024410569	500
Pias2a2	AGTCTAAGCTTGACATCCATGAAAG, GTGTAGGCATTGGCTTAGCAATGC		XM_024407486	360
Pias2a1.1	CCCAAGGCGGTAGACAGTAGTCT, ACTGGGCTTTATGTTTCTGGTGACG		XM_024414775	500
mEGFP	ATGGTGAGCAAGGGCGAGCTG, GTCCTCCTTGAAGTCGATGCCCT		[39]	500

4.3. Construction of Pias Expression Constructs

We amplified the open reading frames (ORF) of trout *pias1a1.1* (XM_036963708) and *pias2a1* (XM_036936540) using the oligonucleotide primers listed in Table 2. To this end, we performed standard PCRs using the Platinum Taq High-Fidelity DNA Polymerase (Thermo Fisher Scientific). The resulting amplicon was subcloned into pGEM-T Easy (Promega, Walldorf, Germany), retrieved by digestion with the restriction enzymes *Hind*III and *Eco*RI, and inserted into the mammalian expression vector v280 that had been previously double-digested with the above restriction enzymes. The resulting plasmids (v280_pias1, v280_pias2) were used for functional analyses.

We identified the subcellular localisation of Pias1 and Pias2 from rainbow trout by inserting the respective sequences in an expression vector flagged with green fluorescent proteins (GFP). In detail, we inserted a fragment coding for GFP at the 3′-end of the CDS of the v280_pias1 and _pias2 plasmid. The GFP fragments had previously been amplified from commercial vectors (GFP: pAM505, NCBI-nucleotide accession code: AF140578) and inserted into the v280 clone [47,62] using the restriction sites for *Hind*III and *Eco*RI. The truncated *pias1* variant was produced by digesting the GFP-v280_pias1 plasmid with *Bam*HI to cut off 1357 bp of the downstream ORF. The two *Bam*HI restriction sites (GGATCA and GGATCC) were located at positions 609 to 611 in the ORF of *pias1* and immediately downstream of the GFP sequence. The ends of the linearised plasmid were subsequently joined using the T4 ligase (Promega).

4.4. Transfection, Luciferase Assay, and Confocal Microscopy

Endotoxin-free preparations (ZymoPure II Plasmid Maxi Prep Kit, ZymoResearch/Biozol, Eching, Germany) of the expression constructs for *pias1*, *pias2i*, and truncated *pias1* were transfected into CHSE-214 cells (Chinook salmon embryo-214; order ID: 91041114-1VL, Sigma-Aldrich/Merck) using the X-tremeGENE HP DNA Transfection Reagent (Roche). For co-transfection assays in six-well plates, we used 50 ng of the *ELAM* or the *mx* promoter constructs and increasing concentrations (50, 500, and 2000 ng) of the respective *pias*-expression construct. Three wells of each row were left as unstimulated controls, while the other three were additionally co-transfected with 50 ng of a vector coding for ifnγ [63]. The total DNA concentration of each transfection mixture was adjusted to 2500 ng/assay by adding the empty cloning vector. Finally, the luciferase activity of the cell lysates was measured with the Dual-Luciferase Reporter Assay System (Promega) with a Lumat LB9501 luminometer (Berthold, Bad Wildbad, Germany). Values were normalised against the protein concentration of the CHSE-214 cell extracts. Each transfection was assayed in triplicate; each transfection experiment was performed three times.

CHSE-214 cells transfected with the vector expressing GFP-tagged *pias1* or *pias2* from rainbow trout were fixed with 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) and subsequently inspected by confocal microscopy (LSM 780; Carl Zeiss Microscopy, Jena, Germany), equipped with a 63× oil-immersion DIC objective. For staining the nuclei, Hoechst 33342 dye (1 mg/mL; Sigma-Aldrich/Merck) was added to the medium 30 min before fixation.

4.5. Strategy for the Generation of a Pias-Knock-Out Cell Line

The genetically modified CHSE cell line CHSE-EC that stably expresses Cas9 and monomeric enhanced green fluorescence protein (mEGFP) [39] was chosen as the terminus a quo for this study. These cells were grown at 20 °C in Eagle Minimal Essential Medium with Earle's salts (MEM) (Sigma-Aldrich) supplemented with 500 mg/mL G418 (Sigma-Aldrich), 30 mg/mL hygromycin (Thermo Fisher Scientific), 100 U/mL penicillin, 100 µg/mL streptomycin, 1% non-essential amino acids (NEAA; Biochrom AG), 2 mM L-glutamine (Biochrom AG), and 10% foetal bovine serum (FBS; Thermo Fisher Scientific).

Each sgRNA was designed on the first 100 nt of the coding sequence. The sgRNAs were synthesised using a 120 nt blunt-ended oligo (Sigma Aldrich/Merck) as a template and a RiboMAX Express T7 kit (Promega). The resulting product was purified using TRIzol

(Thermo Fisher Scientific), then resuspended in RNAse-free and DNAse-free water and quantified with a NanoDrop One© (Thermo Fisher Scientific) before the transfection.

CHSE-EC cells were transfected with 100 ng mixed sgRNA (Table 2), together with 100 ng sgRNA targeting the mEGFP per 10 µL of cell suspension as previously described [39]. Transfected cells were plated onto a 25 cm^2 flask and passaged weekly for 4 weeks. The mEGFP-negative cells were suspended in 2 mL MEM and sorted using a MoFlo XDP high-speed cell sorter (Beckman Coulter, Krefeld, Germany) with an incorporated air-cooled Coherent Sapphire laser (488 nm, 100 mW). The cells were sorted through a 70 µm nozzle at 60 psi in purify mode into 24-well plates and cultured with 1 mL MEM, weekly renewed for 4 months. The genomic DNA of CHSE-EC cells was isolated using DNeasy Blood & Tissue Kits (Qiagen) following the standard protocol. Sequencing primers (Table 2) were used to validate the success of the KO strategy.

4.6. Data Analysis

A parametric *t*-test or nonparametric Mann-Whitney U-test and GraphPad Prism software v.9 for macOSX were used to evaluate the statistical significance of the qRT-PCR data and reporter-gene measurements.

Alignment and phylogenetic reconstructions were performed to compare multiple Pias nucleotide and amino acid (aa) sequences using the 'build' function of ETE3 v3.1.1, as implemented on the GenomeNet site (https://www.genome.jp/tools/ete/, accessed on 1 March 2021) [64]. The tree was constructed using fasttree (with slow NNI and MLACC=3) to make the maximum-likelihood NNIs more exhaustive [65]. The gene synteny was determined using Genomicus v1.01 (https://www.genomicus.bio.ens.psl.eu/genomicus-100.01/cgi-bin/search.pl; accessed on 1 March 2021).

The three-dimensional structure was obtained using UCSF ChimeraX, offered as free software (http://www.rbvi.ucsf.edu/chimerax, accessed on 1 March 2021) [66]. Signal peptides were predicted using SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/ accessed on 1 March 2021). Disordered protein regions were predicted using PrDOS [67] (http://prdos.hgc.jp/cgi-bin/top.cgi accessed on 1 March 2021). An upstream analysis was performed using the Ingenuity program (Ingenuity Pathway Analyses/Qiagen accessed on 1 June 2021) to evaluate the target genes of STAT/NF-κB-dependent signalling.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Ethics Board (approval ID: FLI 28/17).

Informed Consent Statement: Not applicable.

Data Availability Statement: The *pias*-cDNA sequences and associated metadata have been submitted to the 'European Nucleotide Archive' under accession number PRJEB47768.

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Abbrevations

Aa, amino acid(s); CDS, coding sequence; CHSE, Chinook salmon embryo; CRISPR, clustered regularly interspaced short palindromic repeats; ELAM, endothelial cell-leukocyte adhesion molecule; GFP, green fluorescent protein; hpi, hours post infection; IFN, interferon; JAK, Janus kinase; LG, linkage group; mEGFP, monomeric enhanced green fluorescence protein; NCS, newborn calf serum; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; nt, nucleotide(s); PBS, phosphate-buffered saline; SAP, scaffold attachment factor A/B/acinus/PIAS; sgRNA, short guide RNA; SIM, SUMO-interacting motif; SP-RING, Siz/PIAS RING finger; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor; WGD, whole-genome duplication.

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Study III: The Early Immune Response of Lymphoid and Myeloid Head-Kidney Cells of Rainbow Trout (Oncorhynchus mykiss) Stimulated with Aeromonas salmonicida

Previous studies highlighted the importance of head kidney as a highly relevant immune organ, in which myeloid cells play a major role in the innate and adaptive immune response. Upon stimulation with inactivated *A. salmonicida,* cells derived from the head kidney of naïve rainbow trout, have been magnetically sorted with a monoclonal antibody mAB21 to obtain one (Mab21-positive) fraction enriched with myeloid cells, and one (Mab21-negative) fraction enriched with lymphocytes and thrombocytes.
The profiled panel of 43 immune-relevant genes specific for each cell fractions revealed an overall downregulation of the complement pathway and cytokine production at the considered timepoints.

Highlights

- Our results document an overall downregulation of the complement pathway and cytokine production at the considered time points.
- The expression of diverse proinflammatory (IL10, SERPING1, NKIRAS2a, RIP2K) factors analysed in myeloid and lymphoid cells was downregulated during earlier stimulation.
- A2M expression appeared completely inhibited in the treated myeloid cell fraction across all phases of the stimulation.

fishes

MDPI

Communication

The Early Immune Response of Lymphoid and Myeloid Head-Kidney Cells of Rainbow Trout (*Oncorhynchus mykiss*) Stimulated with *Aeromonas salmonicida*

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Abstract: The teleost head kidney is a highly relevant immune organ, and myeloid cells play a major role in this organ's innate and adaptive immune responses. Because of their complexity, the early phases of the innate immune reaction of fish against bacteria are still poorly understood. In this study, naïve rainbow trout were stimulated with inactivated *A. salmonicida* and sampled at 12 h, 24 h and 7 d poststimulation. Cells from the head kidney were magnetically sorted with a monoclonal antibody mAB21 to obtain one (MAb21-positive) fraction enriched with myeloid cells and one (MAb21-negative) fraction enriched with lymphocytes and thrombocytes. The gene expression pattern of the resulting cell subpopulations was analysed using a panel of 43 immune-related genes. The results show an overall downregulation of the complement pathway and cytokine production at the considered time points. Some of the selected genes may be considered as parameters for diagnosing bacterial furunculosis of rainbow trout.

Keywords: *Aeromonas*; NF-κB; myeloid; inflammatory; innate immune response; rainbow trout

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1. Introduction

Aquaculture is the fastest-growing food production sector, accounting for about half of all food fish consumed globally [1]. Disease outbreaks have been a massive stumbling block to the growth of the aquaculture industry and have severely impacted the industry's economic expansion in many countries [2]. To prevent pathogenic outbreaks and ensure economic success, fish health management aims for achieving optimum conditions in aquaculture [1]. Although prophylactic methods are currently applied, bacterial, viral and parasitic diseases still occur [3]. Therefore, greater knowledge of the molecular host–pathogen interactions over the course of an infection is critical for developing molecular tools and effective vaccines to prevent and reduce losses because of disease outbreaks.

The fish innate immune system comprises mucosal barriers, several unspecific pathogen-resistance components (pattern recognition receptors, enzymes, complement components, etc.) and cells (monocytes/macrophages, dendritic cells, granulocytes) [4,5]. Upon intraperitoneal (i.p.) stimulation, the innate defence is immediately activated, and myeloid cells, including monocytes/macrophages and granulocytes, are attracted to the peritoneum. Subsequently, lymphoid cells, including B-, T- and NK cells, are activated, which are part of the adaptive immune system. The induction of adaptive immunity involves time-consuming processes, such as cell proliferation, cell differentiation and cell mobilisation [6].

Despite these defence mechanisms, bacteria such as *Aeromonas salmonicida* ssp. *salmonicida*, which is the causative agent of furunculosis, can still invade fish. This Gram-negative

bacterium causes haemorrhagic and necrotic lesions in the gills, gut and muscle that are highly lethal [7,8]. A closer examination of the virulence factors of *A. salmonicida* shows a type III secretion system (T3SS). This is a syringe needle–shaped protein complex responsible for the transfer of toxins from the bacterial cytoplasm to the cytoplasm of the host cell. The T3SS effector proteins AexT, AopH, Ati2, AopP, AopO, AopN and ExsE modulate the host's immune response by interfering with those inflammatory responses initiated by MAP kinases or NF-κB proteins [9–12] (Figure 1). The ability of macrophages to trigger inflammation and interact with other immune cells is significantly reduced when pathogens block the NF-κB signalling pathways [13].

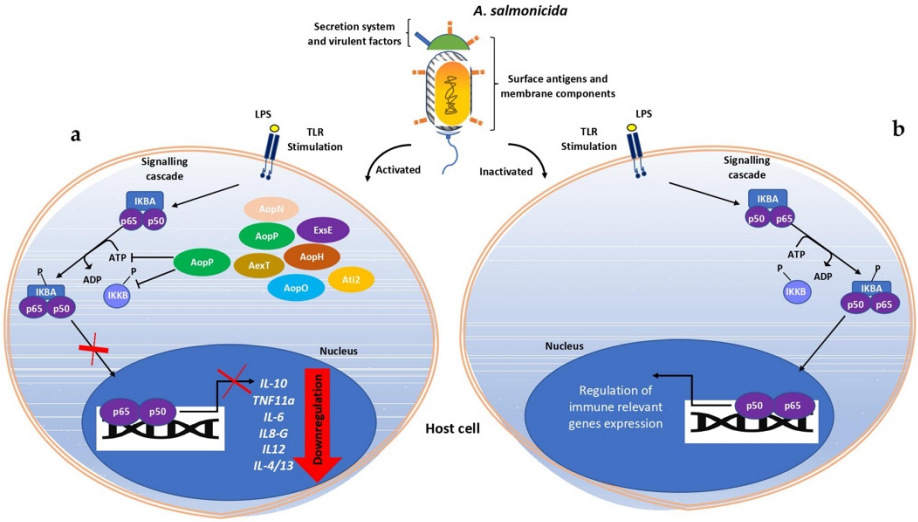

Figure 1. Graphic representation of (**a**) an infection with activated and (**b**) stimulation with inactivated *Aeromonas salmonicida* bacteria. In both scenarios, *A. salmonicida* activates the TLR-NF-κB pathway. (**a**) Previous investigations have documented the intracellular effects of AexT, Ati2 and AopP on inflammatory gene expression, whereas the impact of AopH, AopO and AopS has been predicted based on cytotoxic homologs from other bacterial species. (**b**) Stimulations with inactive bacteria do not contain an active T3SS system; the host cell response is directed against molecular antigens and induces immune-relevant gene expression. Abbreviations: LPS, lipopolysaccharide; TLR, toll-like receptor; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

Certain aspects of the early immune response to *A. salmonicida* of teleostean cells of myeloid origin remain unclear, for example, their expression profiles and their kinetics during the first 24 h poststimulation (p.s.) with the bacteria. To provide a better understanding of the early immune response to *A. salmonicida* in rainbow trout (*Oncorhynchus mykiss*), we investigated two leukocyte fractions from the head kidney at different time points after stimulation with *A. salmonicida*. To this end, we used a panel of 43 genes representing the most common markers for dendritic cells, macrophages, lymphocytes, thrombocytes, complement components and anti- and proinflammatory factors.

2. Materials and Methods

2.1. Ethics Statement

The experiment was approved by the State Office for Agriculture, Food Safety and Fisheries (approval number LALLF 7221.3-2-042/17), according to the German and Euro-

pean guidelines on animal welfare (Tierschutzgesetz, Tierschutz-Versuchstierverordnung, Directive 2010/63/EU).

2.2. A. Salmonicida for Stimulation Experiments

An aliquot of *A. salmonicida* ssp. *salmonicida* (A.s.s.)—the highly virulent strain JF 5505—from stock cryo-preserved batches was cultivated on tryptic soy broth media (TSB, Becton Dickinson, Heidelberg, Germany) at 15 °C for 24 h. Bacterial suspension was inactivated in 1.5% paraformaldehyde (PFA) for 1.5 h at 4 °C, and afterwards, a sample was plated out on TSB agar plates to prove successful inactivation. The inactivated A.s.s. bacteria were washed twice with TSB media by centrifugation at 4000× *g* for 10 min at 4 °C. The pellet was resuspended in TSB 25% glycerol at a concentration of 1.5×10^8 bacteria/mL. For intraperitoneal immunisation, the bacteria were washed once with phosphate-buffered saline (PBS) and set to a concentration of 1×10^7 bacteria/mL. Injections were prepared under aseptic conditions in sterile 1× PBS.

2.3. Fish

The Born strain of rainbow trout (*O. mykiss)* weighing 20 g to 100 g was purchased without gender selection from the commercial trout breeding farm Forellenzucht Uthoff GmbH, Neubrandenburg (Germany). The fish were kept in 300 L glass aquaria in a partially recirculating water system at a constant 12 °C and 12 h light:12 h dark period for both the summer and winter experiments. They were fed twice per day with commercial dry food pellets (Aminoforte, Kronen-Fisch, Wesel, Germany). A total of 24 trout were used for the experiments. All manipulations of the fish were done after anaesthesia with benzocaine. The fish received a single i.p. injection containing 100 µL of 1×10^7 bacteria/mL.

The highly virulent strain JF5505 of the inactivated *A. salmonicida* ssp. *salmonicida* was diluted in 200 µL PBS, and 5×10^5 CFU were i.p. injected using a 20 G needle. During this stimulation trial, the fish weighed between 30 g and 50 g. The control fish were injected with 200 µL of PBS. At 12 h, 24 h, and 7 d poststimulation, four fish per treatment group were euthanised using an overdose of benzocaine (100 mg/L, Sigma, Steinheim, Germany) (Figure 2) and sampled.

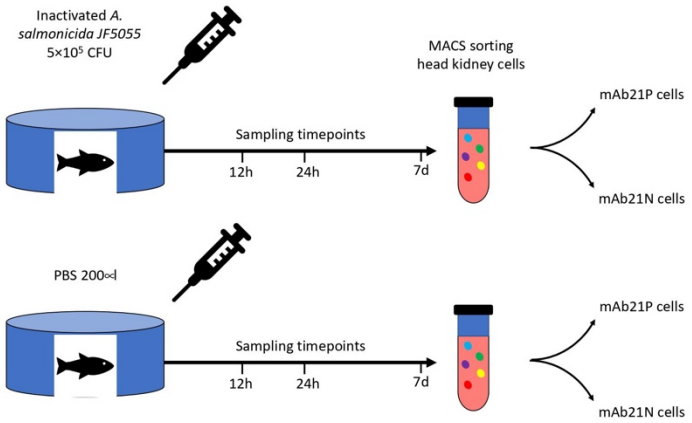

Figure 2. Overview of the exposure conditions, subsequent sampling at specific time points and magnetic separation with a monoclonal antibody (MAb21) against a myeloid lineage marker to separate a MAb21-positive (MAb21P) fraction of myeloid cells from a depleted MAb21-negative (MAb21N) cell fraction enriched in lymphocytes and thrombocytes.

2.4. Sorting of Head Kidney Cells

Pooled suspensions of viable leukocytes from the head kidney of four fish per treatment were prepared, as described previously [13]. Afterwards, 1×10^6 cells were incubated with the monoclonal antibody 21 (MAb21, previously validated as specifically recognising a lineage marker on all cells from myeloid lineage) for 30 min at 4 °C. Then, the cells were washed with 700 µL of MACS Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) and sedimented by centrifugation for 5 min at 300× *g* at 4 °C. The cell pellet was resuspended in 200 µL of antimouse IgG magnetic beads (Miltenyi Biotec, Germany) following the manufacturer's recommendations.

After a final washing step with 700 µL of MACS buffer (as described above), the cells were resuspended in 500 µL of MACS buffer (Miltenyi Biotec, Germany). For magnetic separation in the autoMACS Proseparator (Miltenyi Biotec, Germany), the Possel_S programme was used. The resulting enriched cell fraction (MAb21P, myeloid cells) and depleted cell fraction (MAb21N, lymphocyte and thrombocytes enriched) were centrifuged, and the pellets were resuspended in 350 µL of lysis buffer RLT (Qiagen, Hilden, Germany) for further RNA extraction and gene expression analysis.

2.5. Primer Design and Biomark qPCR Measurements

The qPCR oligonucleotide primers (Table 1) were designed using pyrosequencing assay design software (v.1.0.6; Biotage, Uppsala, Sweden). All the analysed genes were selected based on previous publications on teleosts. Each primer pair was tested prior to RT-qPCR measurements in a standard PCR reaction using the HotStar-Taq (Qiagen, Germany) following a standard protocol. The resulting PCR products were visualised on agarose gels to assess product size and quality. Subsequently, primer pairs were tested in a quantitative PCR analysis using the LightCycler-96 system (Roche, Mannheim, Germany).

Table 1. Primers used in the study, amplicon length and function of each analysed gene. The left column reports the specific cell population.

Cell Population	Gene Symbol	Primer 5′–3′	Length (bp)	Accession No.	Function	Ref.
Dendritic cells	*TAP1*	CACTCCTGGCAGGGGCTACTT	176	XM_021559784	Antigen presentation	[14]
		CCTTATTTCATACGCTTTGGAGC				
	TAP2	CATCTGTGAGACGTTTATCCCTT	99	XM_024386707	Antigen presentation	[15]
		TCATGTACGCCATTGGAGGCAT				
	CD83	GTCTGCATTCTAGCTGCCTACT	128	XM_021593617	Immune cell interactions	[16]
		ACGTAAGCCTGGGGTCCAGTA				
	CD209	ATCTCTCAGGTACCGGAAGAGT	127	HG428763	Intercellular adhesion, antigen uptake	[17]
		GACTGTCTGGAGAGAGGAGCA				
Macrophages	*LYG*	GCAGGTTGACAAGCGCTACCA	118	BT073825	Hydrolyzation of the bacterial cell wall	[18]
		AAAGGGGGAATTTCAGCCTACAA				
	DAA (MHCII)	CAGTGATTCAGATGGAGTGAATAT	131	FR688130	Cell surface proteins with a key role in adaptive immunity	[19]
		AGATTTCCTTCCCTGGATATTATG				
	CD80/86	GCGTCGGCTGCTTCGAAGGT	152	NM_001160477	Co-stimulation of T-cells	[20]
		AGACTCCCAAACCACCTGTATG				
	CD68	GACACTGGAAAGACAGGAGTATT	115	XM_021578316	Scavenger receptor and antigen processor	[21]
		TTCAAGGAGGGCTTCATCACCT				
	SPIC	CACCTGGTCCTGCATCAGAAG	127	NM_001124513	Immune gene expression	[22]
		CTGGGACTATCACGCCACTCA				

Table 1. *Cont.*

Cell Population	Gene Symbol	Primer 5′–3′	Length (bp)	Accession No.	Function	Ref.
B- and T-cells	*CD79B*	TGAACCTCTCAGTGGCTTTAAAC	111	XM_021565350	Signalling through B-cell receptors	[23]
		TCTGTGTGGTGTCGGACCGAA				
	PAX5	AAGTATCCGTCCCGGGGTGAT	144	NM_001124682	Development of lymphoid progenitors	[24]
		GACTATTGGCTGAGAGAGTGTG				
	TARP	GAGAAGTGGAGTGGGACCAGA	105	XM_021619909	Antigen presentation	[25]
		AGGTGAGTGACGGGGGACAC				
Pan-T-cells and Thrombocytes	*GAPDH*	TCAACGGATTTGGCCGTATTGG	134	NM_001124246	Influence cytokine production	[26]
		GTTCAAGTATGACTCCACCCAC				
	CS	CACCTTCAACGAGGTTTACCCC	131	XM_021610150	Metabolic regulator	[27]
		GAGATGTGCTTTTGGATCTTGTC				
	CD18	AGTGAGTTGAGGGTTACATAGGA	148	XM_021579244	Leukocyte migration and adhesion	[28]
		GTATGTTCCAAAACAGTGATCAAC				
	CD41	CAGTTTAGCAACAAAACCATCAGT	161	XM_021624569	Leukocyte migration and adhesion	[29]
		TGACACAAAAGGTGATGAGGTTTA				
	CD36	GACTGTTACAAAGGAATCGGTCAT	112	XM_021577070	Ligand binding	[30]
		ACAGTTCTGGCTCTTTGACGTG				
Natural Killer cells	*GZMB*	TCCTTTCCTCTGCTGGAGCCT	94	XM_021598076	Destruction of infected and/or transformed cells	[31]
		TATATGGTCTCTCTGCAACACAG				
	PRF1	GCGGGTATTACAGCTATCGAGTA	161	XM_021558434	Destruction of infected and/or transformed cells	[32]
		ATTACATTACCAAGGTGAGCCTG				
	KLRD1 (*CD94*)	TGGGGCAACGATCGGCTCAAA	114	XM_021559366	"Missing-self" discrimination	[33]
		GAATCCTGCCACAGCAGTGGA				
Complement system	*CFD*	GACAAGTCATGAGCCCCAAG	151	NM_001246346	Serine protease activity of the alternative complement pathway	[34]
		GTGCCGAAAGTGGGTATTGT				
	CFH	GCTGGACCAAGACACTTGGC	166	NM_001124410	Complement regulation	[34]
		CCTCTACCGGGGGTTGGTG				
	CFI	ACCCAGTGTTTGCAAGAGAACC	167	XM_021593383	Inactivation of C3b/C4b factors	[34]
		CAGTTGGCGATCAGAGAGACG				
	C3-1	AGCCTCTGACCAGGGAGATATT	164	L24433	Opsonisation, elimination of pathogens	[34]
		GACGATGTCAGGGAGTTTGAAC				
	C1r/s	AACCAGAGGGGACTCTGTCCA	182	NM_001124380	Initiation of classical complement pathway	[35]
		TGGACAGAGTCCCCTCTGGTT				
Immune regulation	*NFKBIA*	AACCCTGGAGGAAAACAGTGAC	153	NM_001124368	Inhibition of NF-κB pathways	[36]
		GAACAATCAGAGACAGACGGCG				
	SERPINB1	TACCAGTTCGTTGAGACGTTCC	116	NM_001124515	Reduction of tissue damage; cell differentiation; immune activation	[37]
		ATCAAGAACCTATTGGCGGAGG				
	SERPING1	AAGGAATGACGAACGGCAAACG	169	NM_001124379	Activation of the C1 complex	[37]
		TCAGCTGTCTCACAGTAGTACAT				
	CD59	GATTGAGTGGGCAAAGTATTGTAT	167	XM_021606996	Inhibition of the membrane-attack complex	[38]
		CATACCCTGTTACATAACATTGCT				
	IL10	TGCCCAGTGCAGACGTGTACC	137	NM_001245099	Anti-inflammatory function	[39]
		TACACCACTTGAAGAGCCCCG				
	SOD2	TCCCTGACCTGACCTACGAC	201	XR_00247449	Oxidative stress	[40]
		GAGGTTTAATGGAGGAGGCC				
	A2M	GGGAGGAAGGATGAGATGAGTA	184	XM_021582312	Inhibition of cytokine-induced inflammation	[41]
		CTAACAGTGGAGCTTCAGGACC				

Table 1. *Cont.*

Cell Population	Gene Symbol	Primer 5′–3′	Length (bp)	Accession No.	Function	Ref.
Inflammation	*RIPK2*	TGTTGGCGAAAGGGAGAGGAAT GTACATGAGCAATGGCTCTCTG	105	KJ184523	Modulation of innate and adaptive immune responses	[42]
	NKIRAS2a	TGCATGTCTGCCTGTCTCTTTTT TGAGCCCGCAATATGATTGGCA	201	XM_021557705	Regulation of NF-κB signalling	[43]
	RANKL	GAGAGCATCGACTGGGAAAATGT TGTTCTGGGTACTCTGACACCA	125	XM_021620403	Regulation of interactions between T-cells and dendritic cells	[44]
	IL2RG (CD132)	ACCCCCAATGTAAACTGCCTGA TTTCAGCAGCAGGTTCATCAAAG	112	NM_001124356	Cytokine signalling involved in the stimulation of phagocytosis	[45]
	IL6	GTGTTAGTTAAGGGGAATCCAGT CCTTGCGGAACCAACAGTTTGT	128	NM_001124657	Proinflammatory cytokine and anti-inflammatory myokine	[46]
	CXCL8	ATATAACACTTGTTACCAGCGAGA ATTACTGAGGAGATGAGTCTGAG	106	HG917307	Chemoattraction	[47]
	IL12	ACATTCAGTGAGAGTGCGTGTC ACAAGGGGATCCTTCCTCACAA	118	HE798148	Differentiation of naïve T-cells	[48]
	IL4/13	CTGTCAGAGGAACTTCTGGAAAC GTGAAAAATGACGCGTTTGGTGA	131	NM_001246341	Regulation of inflammatory processes	[49]
	IL2RB (CD122)	AGAGGACAGTGGCGGTAATGAT CTCACAACCTCCAAGGACTGTT	94	XM_021622445	Cytokine signalling involved in T-cell-mediated immune responses	[50]
	IL1B	GAGAGTGCTGTGGAAGAACATAT ATGAATGAGGCTATGGAGCTGC	157	NM_001124347	Inflammation	[51]

The cell pellets were resuspended in 350 µL RLT buffer (Qiagen, Germany) and stored at −70 °C. RNA was isolated from these samples in separate tubes using TRIzol (Invitrogen, Karlsruhe, Germany) and subsequently purified with the ISOLATE II RNA Micro Kit ((Bioline/Meridian Bioscience, Luckenwalde, Germany). The concentrations of the individual RNA aliquots were adjusted at 2 ng/5 µL or 5 ng/5 µL. After cDNA synthesis using the reverse transcription master mix (Fluidigm, South San Francisco, CA, USA), the cDNA aliquots were individually preamplified in 13 (5 ng RNA input) or 15 cycles (2 ng RNA input) using the PreAmp master mix (Fluidigm, CA, USA) and subsequently treated with exonuclease I (New England BioLabs, Ipswich, MA, USA). Multiplex RT-qPCR was conducted using the Biomark HD system and EvaGreen fluorescence dyes (Bio-Rad, Hercules, CA, USA), as previously described [15]. In brief, the 48.48 Fluidigm gene expression biochips were first primed in the MX integrated fluidic circuit (IFC) controller (Fluidigm, South San Francisco, CA, USA) before being loaded with the preamplified cDNA samples and eventually analysed using the Biomark HD instrument (Fluidigm, CA, USA). The raw RT-qPCR results were retrieved with instrument-specific analysis software (v. 3.0.2; Fluidigm, CA, USA). The geometric means of the copy numbers of the reference genes *EEF1A1* [16] and *RPS5* [17] were used to normalise the expression data.

2.6. Data Analysis

Heatmaps and data visualisation were performed using MacOS GraphPad 9 or Windows, GraphPad Software, San Diego, CA, USA (https://www.graphpad.com, accessed on 1 October 2021).

3. Results

3.1. Basal Expression Profiles of Selected Immune Genes in Two Fractions Enriched in Myeloid Cells or Lymphocytes and Thrombocytes

For the three sampling time points of 12 h, 24 h and 7 d after stimulation with inactivated *A. salmonicida*, head kidney cells were separated into a MAb21P fraction enriched in myeloid cells and a depleted MAb21N cell fraction comprising mainly lymphocytes and

thrombocytes (Figure 3). The purity after magnetic sorting was higher than 95%. Both fractions were used to profile a panel of 43 immune genes using multiplex qPCR.

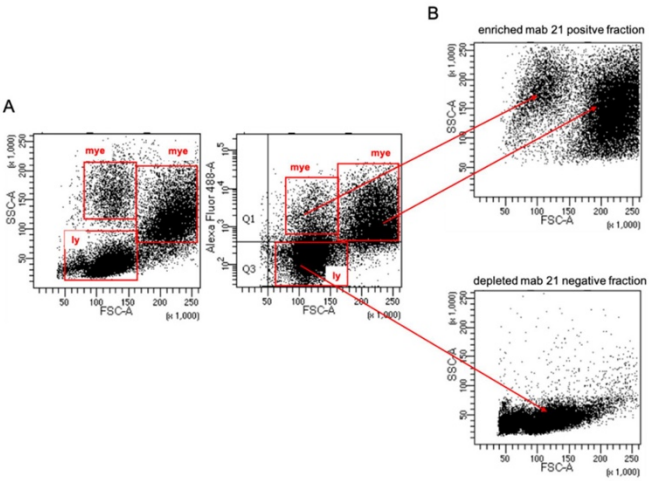

Figure 3. (**A**) Magnetic-activated sorting of head kidney cells from rainbow trout using the myeloid lineage marker specific monoclonal antibody MAb21 resulted in (**B**) an enriched myeloid cell fraction (MAb21-positive myeloid cells) and a depleted lymphoid cell fraction (MAb21-negative lymphocytes and thrombocytes).

The basal expression of the selected lineage marker genes validated the successful separation of the myeloid MAb21-positive cell fraction (expressing *CD209*, *LYG* and *SPI-1*) from the MAb21-negative fraction containing thrombocytes (*CD36*) and B-lymphocytes (*CD79b*, *PAX5*) and T-lymphocytes (*TCR*) (Figure 4).

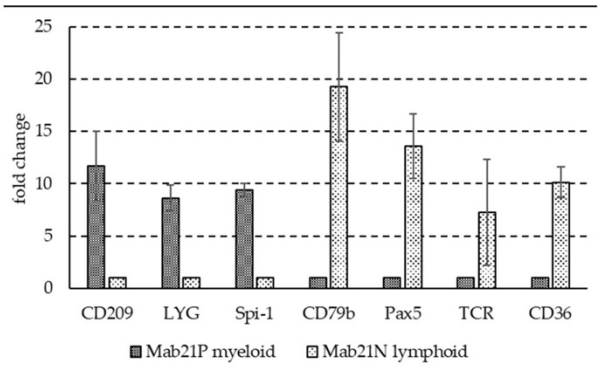

Figure 4. Basal expression of the selected lineage marker genes in MAb21-positive myeloid cells and MAb21-negative lymphoid cells.

A comparison of the kinetics of these genes after i.p. injection of PBS indicated that the average basal expression did not change significantly (data not shown).

3.2. Immune Gene Expression Profiling in Two Fractions Enriched in Myeloid Cells or Lymphocytes and Thrombocytes after Stimulation with A. salmonicida

The average expression ratios of the *A. salmonicida*–treated group compared with the PBS-treated control groups are presented in heatmaps as log2 fold change values (Figures 5 and 6).

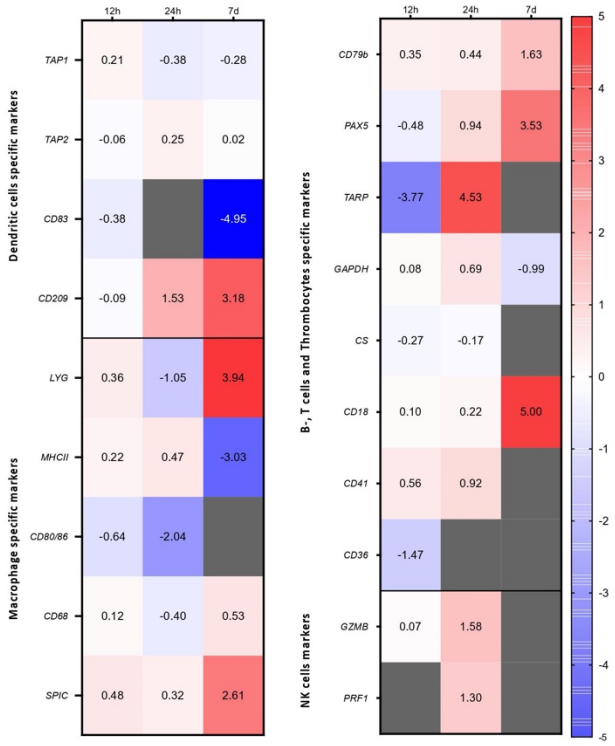

Figure 5. Expression modulation of immune genes specific to myeloid cells (left panel) and to lymphocytes and thrombocytes (right panel) after peritoneal injection of *A. salmonicida* into rainbow trout. The heat map is representative of four samples and illustrates the average expression ratios as log2 FC values of the *A. salmonicida*–stimulated group relative to the control group ($n = 4$) for the immune genes listed at the left margin of each panel. *EEF1A1b* and *RPS5* were used as reference genes to normalise the data. Grey fields indicate that the calculation of FC values failed because of undetectable expression in the treated or control groups. Increased and decreased transcript levels in the samples of the stimulated group compared with the controls are indicated by red and blue fields, respectively, according to the legend on the right.

Generally, the expression patterns in both the MAb21P and MAb21N fractions did not change strongly after intraperitoneal stimulation.

Compared with the PBS control groups, *CD209* was upregulated in the MAb21P fraction at 24 h p.s. (log2 FC = 1.5) and 7 d p.s. (log2 FC = 3.2), while *CD80/86* was downregulated at 24 h p.s. (log2 FC = −2). After 7 d p.s., the expression levels of *LYG*, *DC-SIGN* and *SPIC* (log2 FC > 2.6) increased. *CD83* underwent a strong downregulation at

7 d p.s. (log2 FC = −5), accompanied by a downregulation of *DAA/MHCII* at 7 d p.s. (log2 FC = −3) (Figure 5).

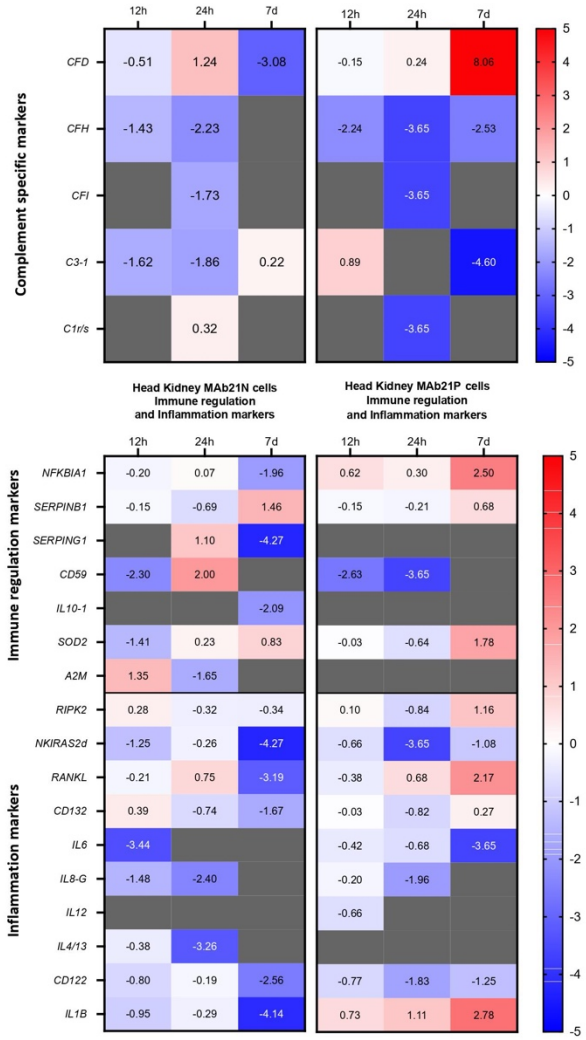

Figure 6. Expression modulation of genes specific to inflammation and immune regulation after p.i. injection of inactivated *A. salmonicida* into rainbow trout. The heat map is representative of four samples and illustrates the average expression ratios as log2 FC values of the stimulated group relative to the control groups (n = 4) for the given immune genes. *EEF1A1b* and *RPS5* were used as reference genes to normalise the data. Grey fields indicate that the calculation of FC values failed because of undetectable expression in the treated or control groups. Increased and decreased transcript levels in the samples of stimulated rainbow trout compared with controls are indicated by red and blue fields, respectively, according to the legend on the right.

In the PBS control groups, we did not detect *CFI*, *IL10* or *IL12* at 12 h p.s.; *CFH*, *C3-1*, *C1r/s*, *CD59*, *SERPING1*, *CFI*, *A2M* or *IL12* at 24 h p.s.; and *IL10* at 7 d p.s. *IL4/13* was not detectable in the controls across all time points.

In the stimulated groups, we did not detect *C1r/s*, *SERPING1* or *IL4/13*, at 12 h p.s.; *CFH*, *C1r/s*, *CD59*, *CFH*, *C3-1*, *C1r/s*, *CD59*, *SERPING1*, *CFI* or *IL4/13* at 24 h p.s.; and *CD80/86*, *C1r/s*, *CD59*, *CFI* or *CXCL8* at 7 d p.s. *A2M* and *IL12* were not detectable in stimulated fish across all time points.

3.3. Expression Profiles of Characteristic Markers in the Cell Fraction Enriched with Lymphocytes and Thrombocytes

The transcript level of *TARP* was strongly reduced in the MAb21N fraction 24 h p.s. (log2 FC = −3.8), followed by a marked increase 7 d p.s. (log2 FC = 4.5). *PAX5* was mildly upregulated in the MAb21N fraction at 24 h p.s. (log2 FC = 0.9), but its expression further increased at 7 d p.s. (log2 FC = 3.5), together with *CD18* (log2 FC = 5). *CD79B* was modestly upregulated across all time points (log2 FC between 0.4 and 1.6). The expression levels of *PRF1* increased at 24 h p.s. (log2 FC = 1.3) (Figure 5).

In the PBS control groups, we did not detect *PRF1*, *IL10* or *IL12* at 12 h p.s.; *CD36*, *CD94*, *IL10*, *CD59*, *A2M* or *IL12* at 24 h p.s.; and *CD41*, *CD36*, *GZMB*, *PRF1*, *CD94*, *CD59*, *SERPING1*, *A2M*, *IL6*, *CXCL8*, *IL12* or *IL14/13* at 7 d p.s.

In the groups stimulated for 12 h, we did not detect *CD94* or *SERPING1*; both *IL10* and *IL6* were not detectable at 24 h p.s.; and *TARP*, *CD36*, *GZMB*, *CD59*, *SERPING1*, *IL6* and *IL12* were all not detected at 7 d p.s.

3.4. Expression Patterns of Complement- and Cytokine-encoding Genes in the Two Cell Fractions from the Head Kidney

At 12 h p.s., the levels of *CD59* (log2 FC = −2.3) and *SOD2* (log2 FC = −1.4) were reduced in the MAb21N fraction. The *SERPING1* level was upregulated at 24 h p.s. (log2 FC = 1.1) and strongly downregulated at 7 d p.s. (log2 FC = −4.3). *A2M* was initially upregulated at 12 h p.s. in the MAb21N (log2 FC = 1.4), followed by a downregulation at 24 h p.s. (log2 FC = −1.7).

The genes encoding cytokines were generally downregulated in both fractions, except for *IL1B*. This gene was upregulated with a log2 FC of 1.1 (24 h) and 2.8 (7 d) in the MAb21P fraction but simultaneously strongly downregulated (log2 FC of −4.1 at 7 d) in the MAb21N fraction.

In both fractions, *CXCL8* and *IL4/13* were downregulated after 24 h p.s. (log2 FC < −1.5). *IL10* and *IL12* were downregulated only at one time point during stimulation in the MAb21N (7 d p.s.) and MAb21P fractions (12 h p.s.), respectively.

RIP2K, *SOD2* and *RANKL* were upregulated (log2 FC ≥ 1.2) in the MAb21P fraction 7 d p.s. *NFKBIA* levels were downregulated (log2 FC = −2) in the MAb21N fraction but upregulated (log2 FC = 2.5) in the MAb21P fraction at 7 d p.s. *SERPINB1* levels were mildly downregulated in both cell fractions (log2 FC < −0.7), followed by a mild upregulation at 7 d p.s. in the MAb21N (log2 FC = 1.5) and MAb21P fractions (log2 FC = 0.7).

The expression of the complement genes in both fractions was generally downregulated, except for *CDF*, which showed a strong upregulation (log2 FC > 8) at 7 d p.s. in the MAb21P fraction (Figure 6).

4. Discussion

In morphology and function, the head kidney of fish corresponds to the bone marrow of mammals and is one of the most important haematopoietic organs in bony fish. Macrophages and other cells of myeloid origin differentiate in the head kidney. These cells have a core function in innate immunity against a diverse and broad array of pathogens [52] because they are involved in phagocytosis, radical production and cytokine secretion, much like their mammalian counterparts [4].

The aim of the current study was to profile the expression of the specific genes involved in the early immune response of rainbow trout after intraperitoneal stimulation with

inactivated *A. salmonicida* ssp. *salmonicida* in two fractions of the head kidney: myeloid cells (MAb21P) and lymphocytes/thrombocytes (MAb21N).

Our results show an overall downregulation of the genes characteristic for cells of myeloid origin, except for *LYG*, *SPIC* and *CD209*, which appeared progressively higher expressed at the later time points analysed. *CD209* is an important mediator of dendritic cell/T-cell clustering and T-cell activation [53]; *SPIC* regulates haematopoietic cell differentiation, proliferation and apoptosis [22]; and *LYG* is involved in the response against bacterial infections [54]. The upregulation of the genes encoding *CD209*, *SPIC* and *LYG* indicates an effective activation of the early immune response, which is supported by the increased expression of the inflammation marker *IL1B*. The downregulated levels of *CD83*, *CD80/86* and *DAA* in the cells of myeloid origin may result from restricted NF-κB activation [55] and might indicate the reduced potential of leukocytes to produce cytokines in the head kidney [56–59]. It is more likely that the observed results indicate that the cells of myeloid origin migrate to the peritoneum to promote the first phase of inflammation at the site of stimulation, thereby leaving only a remaining fraction of unstimulated cells in the head kidney [60].

In the fraction enriched in lymphocytes and thrombocytes, the initially down- and subsequently upregulated level of transcripts coding for T-cell receptor gamma (*TARP*) could be explained by a process known as 'state-dependent inactivation'. Following its full downregulation, the T-cell receptor complex adapts to conditions of continuous stimulation [61]. *PAX5* and *CD79B* were progressively upregulated after stimulation. A similar regulation has been previously detected in the Chinese sucker (*Myxocyprinus asiaticus*) after being stimulated with inactivated *Aeromonas hydrophila*, suggesting increased levels of signal transduction and B-cell activation [23]. In Nile tilapia challenged with inactivated *A. hydrophila* [62], integrin β (*CD18*) was regulated during the early stimulation phase, indicating the recruitment and activation of leukocytes.

The downregulation of *CD36* may reflect a specific signalling pathway that activates thrombocytes [63]. Unlike their human counterparts, fish thrombocytes are nucleated cells that can phagocyte and regulate the immune response [64]. Thrombocytes from rainbow trout express genes that encode the proteins involved in activation and aggregation, antigen presentation and immune modulation [65]. *CD36* can be downregulated by toll-like receptor (TLR) ligands through TLR-induced cytokines [66], as observed in previous studies [29,67,68].

The early phase of stimulation revealed modulated levels of *PRF1* and *GZMB* in the MAb21N fraction, suggesting the regulation of apoptotic mechanisms by cytotoxic T-lymphocytes and NK cells [31,69].

Complement evasion strategies by pathogenic organisms have previously been reported for different Gram-negative bacteria [35,70]. The expression of complement components in myeloid cells was strongly inhibited in the first 12 h p.s. The strong downregulation of *CFH* and *CFI* at each time point analysed might indicate that the inhibition of the complement system by *A. salmonicida* is not unique to the early stage of an infection. It has been reported that several bacteria, including *A. salmonicida*, express and modify surface antigens such as outer membrane proteins, capsules and lipopolysaccharide (LPS) to inhibit the complement system [71,72]. Additionally, *A. salmonicida* may promote the immunosuppressive state of fish [73]. Although we used a highly virulent strain of *A. salmonicida*, the cells were inactivated. We cannot rule out the possibility that the membrane antigens of *A. salmonicida* interfered with the proinflammatory response, as reflected by the downregulation of *IL6* and a moderately increased *IL1B* level at the early time points. These proinflammatory cytokines promote the transcription of complement-related genes [74], which were consequently also at low levels. In the myeloid-depleted cell fraction, we did not observe any relevant regulation patterns in the first two time points analysed.

The expression of diverse proinflammatory factors was downregulated in both fractions during the earlier stimulation. The expression of *A2M* coding for A2-macrotubulin was obviously completely inhibited in the myeloid cell fraction across all phases of stim-

ulation. The acquisition of metal ions by pathogens is vital for their survival. Because A2-macrotubulin binds to iron, zinc and copper, its downregulation may be a further hallmark of the suppressed early immune response by *A. salmonicida* [41]. All of the above genes maintained essentially the same expression pattern 7 d after stimulation.

5. Conclusions

In conclusion, gene expression profiling in two head kidney cell fractions determined the transcriptional response to inactivated *A. salmonicida* at different time points. Our results have shown that despite the increased expression levels of myeloid cell markers, the expression of proinflammatory and anti-inflammatory cytokines was lower than in the control groups, which may reflect the migration of activated myeloid cells into the peritoneum. The current study provides valuable information about the immune components that should be targeted when developing and evaluating innovative vaccine formulations to provide effective protection against bacterial pathogens such as *A. salmonicida*. This will improve the knowledge of the early phase of the immune response to understand the regulation of immune-related genes upon inactivated bacteria stimulations.

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Publications and conferences

Peer-reviewed works (*part of the doctoral thesis**)**

*Characterisation of the teleostean κB-Ras family: The two members NKIRAS1 and NKIRAS2 from rainbow trout influence the activity of NF-κB in opposite ways.
Fabio Sarais, Henrike Rebl, Marieke Verleih, Sven Ostermann, Aleksei Krasnov, Bernd Kollner, Tom Goldammer, Alexander Rebl.
Fish & Shellfish Immunology Volume 106, November 2020, Pages 1004-1013
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*PIAS Factors from Rainbow Trout Control NF-κB- and 2 STAT-dependent Gene Expression.
Fabio Sarais, Sophia Kummerow, Ruth Montero, Henrike Rebl, Bernd Köllner, Tom Goldammer, Bertrand Collet and Alexander Rebl.
International Journal of Molecular Science, Int. J. Mol. Sci. 2021, 22(23), 12815
DOI: https://doi.org/10.3390/ijms222312815

*The Early Immune Response of Lymphoid and Myeloid Head-Kidney Cells of Rainbow Trout (*Oncorhynchus mykiss*) Stimulated with *Aeromonas salmonicida*.
Fabio Sarais, Ruth Montero, Sven Ostermann, Alexander Rebl, Bernd Köllner and Tom Goldammer.
Fishes 2022, 7(1), 12;
DOI: https://doi.org/10.3390/fishes7010012

Conference articles

Genomic and biological characterization of inhibitors and activators of the NF-kB pathway in rainbow trout (*Oncorhynchus mykiss*)
Fabio Sarais, Henrike Rebl, Marieke Verleih, Bernd Kollner, Tom Goldammer, Alexander Rebl.
https://doi.org/10.1016/j.fsi.2019.04.255

Declaration of authorship

I hereby certify that I have written this thesis independently and without outside help, that I have not used any aids or sources other than those indicated by me, and that I have marked the content and literal passages taken from the works used as such

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Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

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Curriculum vitae

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