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Transkriptomanalyse verschiedener Immunzellpopulationen aus dem Blut von Patienten mit Multipler Sklerose im Verlauf der Therapie mit Fingolimod

Inauguraldissertation
zur
Erlangung des akademischen Grades
Doktor der Medizin
der Universitätsmedizin Rostock

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https://doi.org/10.18453/rosdok_id00003734

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Datum der Einreichung: 22.01.2021

Datum der Verteidigung: 26.04.2022

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1. Einleitung

1.1. Multiple Sklerose

Multiple Sklerose (MS), eine chronisch-entzündliche Erkrankung des zentralen Nervensystems (ZNS). Sie ist die häufigste Ursache für erworbene Behinderung im jungen Erwachsenenalter. Weltweit sind ungefähr 2,8 Millionen Menschen an MS erkrankt [1]. Das durchschnittliche Erkrankungsalter liegt bei etwa 30 Jahren [2].

Die Ätiologie der Erkrankung ist bis heute ungeklärt, obwohl die pathophysiologischen Zusammenhänge immer genauer verstanden werden und zahlreiche Risikofaktoren bekannt sind [3]. Zu den wichtigsten Risikofaktoren für die Entstehung der MS zählt eine genetische Prädisposition sowie begünstigende Umweltfaktoren [4]. Die genetische Prädisposition zeigt sich in einem veränderten Risiko bei unterschiedlicher ethnischer Herkunft. Für Patienten mit nordeuropäischer Abstammung besteht ein deutlich höheres Erkrankungsrisiko [5]. Des Weiteren besteht eine Assoziation mit bestimmten humanen Leukozytenantigenen (HLA), vor allem mit der Variante HLA-DRB1*15 [6]. Es zeigt sich auch eine familiäre Häufung der Erkrankung. Für Kinder mit einem an MS erkrankten Elternteil liegt das Risiko im Laufe des Lebens an MS zu erkranken bei 1,45% [7]. Umweltfaktoren, die die Erkrankung begünstigen, sind bestimmte Virusinfektionen, vor allem die Infektion mit Epstein-Barr Virus (EBV) [8]. Ein weiterer Umweltfaktor ist das Rauchen, wodurch das Risiko, an MS zu erkranken, um bis zu 55% steigt [9].

MS kann abhängig von den betroffenen neurologischen Systemen sehr variable motorische oder sensible Defizite verursachen. Diese gehen mit einer Demyelinisierung, einem resultierenden axonalen Schaden sowie einer Gliose einher, die durch entzündliche Aktivität im ZNS bedingt sind [10]. Häufige Manifestationen sind die Retrobulbärneuritis, Paresen der Extremitäten, Sensibilitätsstörungen, Kleinhirnfunktionsstörungen sowie eine beschleunigte Hirnvolumenminderung mit neuropsychologischen Veränderungen [11,12]. Verlauf und Progredienz der Erkrankung können individuell sehr verschieden sein. Es werden mehrere Verlaufsformen unterschieden. Als klinisch isoliertes Syndrom (CIS) wird das erstmalige akute Auftreten neurologischer Defizite, die sich in der Regel vollständig zurückbilden, bezeichnet [13]. Bei circa 85% der MS-Patienten tritt die schubförmig remittierende MS (relapsing-remitting MS, RRMS) auf. Ein Schub ist charakterisiert durch das plötzliche Auftreten von neurologischen Defiziten über mehr als 24 Stunden. Nach einem Schub, gegebenenfalls unter entsprechender Therapie, bildet sich bei RRMS die Symptomatik oftmals zurück (Remission) [14]. Bei bis zu 80% der RRMS-Patienten konvertiert die Erkrankung innerhalb von 20 Jahren in eine sekundär progrediente Form (SPMS). Hierbei akkumulieren neurologische Defizite und es kommt zu einer zunehmenden Verschlechterung der Symptome ohne Remission [15]. Bei etwa 10-15% der

Patienten tritt eine primär progrediente MS (PPMS) auf, wobei die Symptome nicht schubförmig auftreten, sondern bereits zu Beginn eine kontinuierliche Zunahme der Beschwerden stattfindet [16].

Die Diagnose der MS wird anhand der modifizierten McDonald-Kriterien gestellt, wobei sowohl klinische Befunde als auch apparative Untersuchungen einbezogen werden [17]. Notwendig für eine sichere Diagnosestellung sind sowohl die zeitliche als auch die räumliche Dissemination sowie der Ausschluss anderer Differentialdiagnosen. Die zeitliche Dissemination ist klinisch durch das Auftreten von zwei oder mehr Schüben gegeben oder kann durch Hinweise auf unterschiedlich alte Läsionen in der Magnetresonanztomographie (MRT) festgestellt werden. In Ausnahmefällen kann die Diagnose auch bei fehlendem Nachweis der zeitlichen Dissemination unter Einbeziehung von Laborparametern gestellt werden. Räumliche Dissemination besteht, wenn in mehr als einer ZNS-Region klassische MRT-Läsionen nachweisbar sind oder klinische Evidenz dafür vorliegt [18]. Die individuelle Beeinträchtigung der Patienten kann von der Ausprägung der ZNS-Läsionen abweichen. Zur Beurteilung der Alltagseinschränkung und der Krankheitsprogredienz wird typischerweise die Expanded Disability Status Scale (EDSS) eingesetzt [19]. Dabei wird anhand des Grades der Symptomatik in acht verschiedenen funktionellen Systemen ein Wert für die Behinderung auf einer Skala von 0 bis 10 zugeordnet.

1.2. Die Rolle des Immunsystems bei MS

MS ist eine immunvermittelte Erkrankung, bei der autoreaktive Zellen des Immunsystems das ZNS infiltrieren und dort entzündliche und neurodegenerative Prozesse in Gang setzen. Dabei spielen verschiedene Immunzellpopulationen unterschiedliche Rollen in der Krankheitsentwicklung [20]. Autoreaktive CD8+ zytotoxische T-Zellen richten sich vor allem gegen Bestandteile des Myelins wie das Myelin Basische Protein und das Myelin Oligodendrozyten Glykoprotein, wodurch eine Schädigung der Myelinscheiden hervorgerufen wird [21]. Diese Schädigung hat zur Folge, dass die Axone der Nervenzellen ungeschützt sind und in der Folge auch beschädigt werden, was eine Störung der Nervenleitung und einen Ausfall der Funktion des betroffenen Areals bedeuten kann. Die CD4+ T-Helferzellen (TH), vor allem die Subtypen TH1 und TH17, spielen durch die Sekretion von proinflammatorischen Signalmolekülen wie Interferon-γ und Interleukin 17 eine Rolle im pathogenetischen Prozess der MS [22]. Diese verschiedenen autoreaktiven T-Zelltypen sind vor allem im peripheren Blut vertreten, migrieren aber im Krankheitsgeschehen durch die Blut-Hirn-Schranke auch ins ZNS und werden dort durch Antigenkontakt reaktiviert [23]. Am Krankheitsprozess beteiligte Zellen aus der B-Zellreihe sind unter anderem die antikörperproduzierenden Plasmazellen und Plasmablasten sowie antigenpräsentierende Gedächtnis-B-Zellen [24]. Die genauen Antigene, die pathologische B-Zell-Antworten auslösen und eine Differenzierung zu autoreaktiven Plasmazellen stimulieren, sind jedoch bisher nicht bekannt [25]. B-Zellen sind auch für die Regulation der T-Zell-Aktivität

relevant. Hierbei spielen vor allem die regulatorischen B-Zellen und naive B-Zellen eine Rolle. Diese sezernieren unter anderen proinflammatorische Zytokine wie den Tumor-Nekrose-Faktor α und Interleukin 6, aber auch das antiinflammatorische Interleukin 10 [26]. Wenn letzteres zu gering auftritt, kann eine chronische Entzündung begünstigt werden, da die regenerativen Prozesse nicht ausreichend stimuliert werden [27].

Die entzündliche Aktivität im ZNS zeigt sich im Liquor. Ein laborchemischer Parameter bei MS ist der IgG-Index. Hierbei werden die IgG-Spiegel in Liquor und Serum verglichen. Ist dieser Index erhöht, kann eine intrathekale IgG-Synthese oder eine Störung der Blut-Hirn-Schranke nachgewiesen werden [28]. Bei über 90% der Patienten sind oligoklonale Banden (OKB) in der isoelektrischen Fokussierung des Liquors nachweisbar [29]. Diese entstehen, wenn B-Zellen das ZNS infiltrieren und nach weiterer Differenzierung zu Plasmablasten und Plasmazellen intrathekal Immunglobuline (Ig), vor allem IgG, synthetisieren. Zur weiteren Untersuchung von pathophysiologischen Prozessen und Therapiemöglichkeiten bei MS wird häufig das Tiermodell der experimentellen autoimmunen Enzephalomyelitis verwendet. Hierbei wird durch aktive Immunisierung oder passiven Transfer von autoreaktiven T-Zellen eine entzündliche Reaktion ausgelöst, die der bei MS ähnelt [30].

1.3. Therapien bei MS

In der Therapie von MS ist aufgrund der unbekannten Ätiologie derzeit keine kausale Therapie möglich. Daher wird mit immunmodulatorischen Behandlungsstrategien in den Krankheitsverlauf eingegriffen [31]. Für die RRMS werden die Therapiemöglichkeiten unterteilt in verlaufsmodifizierende Therapie und Schubtherapie [32]. Die verlaufsmodifizierende Therapie dient der Kontrolle der Krankheitsaktivität mit dem Ziel, die Schubhäufigkeit bestmöglich zu reduzieren und somit auch eine Zunahme der kumulativen Behinderung zu verhindern. Hierbei werden in Deutschland Therapiemöglichkeiten für Patienten mit einer hochaktiven und einer moderaten Verlaufsform der MS unterschieden [33]. Zur Therapie von Patienten mit moderater Verlaufsform werden in Deutschland sowohl Interferon-β-Derivate und Glatirameracetat per injectionem eingesetzt als auch die neueren oral applizierbaren Medikamente Dimethylfumarat und Teriflunomid [34, 35]. Bei Vorliegen einer hochaktiven Verlaufsform der MS oder unzureichenden Therapieerfolgen stehen in Deutschland Fingolimod und die Biologicals Natalizumab, Alemtuzumab und Ocrelizumab zur Verfügung [36, 37, 38]. Andere Therapiemöglichkeiten sind Ozanimod, Cladribin sowie Mitoxantron [39, 40, 41]. Bei einem akutem Krankheitsschub ist eine gezielte Therapie zur raschen Remission nötig. Sie besteht in der Regel aus hochdosierten Methylprednisolongaben über 3-5 Tage. Bei persistierenden Schüben kann eine Plasmapherese oder Immunadsorption in Erwägung gezogen werden. Für die SPMS und die PPMS stehen weniger Therapiemöglichkeiten zur Verfügung. Bei SPMS mit nachgewiesener Krankheitsaktivität durch aufgelagerte Schübe oder neue MRT-Läsionen kann

die Therapie mit Interferon-β oder Siponimod erfolgen [42]. Es besteht außerdem die Möglichkeit zur regelmäßigen Kortisonstoßtherapie. In der Therapie der PPMS konnte bisher nur Ocrelizumab erfolgversprechende Ergebnisse erzielen [43]. Für die langfristige Alltagsbewältigung sind Rehabilitationsmaßnahmen wie regelmäßige Physiotherapie, Selbsthilfegruppen sowie eine psychotherapeutische und sozialmedizinische Anbindung wichtig [44].

Fingolimod war das erste zugelassene Medikament zur Therapie von RRMS, welches oral eingenommen werden konnte [45]. Bei einer regelmäßigen Gabe von 0,5 mg pro Tag konnte in der Zulassungsstudie die jährliche Schubrate gegenüber Placebo mehr als halbiert werden [46]. Die Progredienz der Behinderung konnte ebenfalls positiv beeinflusst werden. Es gab nur in 17,7% der Fälle innerhalb von 2 Jahren eine Erhöhung des EDSS-Wertes, während dies bei 24,1% der Placebo-Patienten der Fall war [47]. Auch die MRT-Befunde zeigten nach vier Jahren Therapie mit Fingolimod im Durchschnitt mit 4,5 vergrößerten oder neuen Läsionen weniger als die Vergleichsgruppe (durchschnittlich 6,5 neue oder vergrößerte Läsionen) [48]. Die häufigsten Nebenwirkungen von Fingolimod sind Herzrhythmusstörungen wie Bradykardien oder ein AV-Block I°/II° nach der ersten Gabe, die bei bis zu 5% der Patienten auftreten, in der Regel keine Therapie verlangen und selbstlimitierend sind [49]. Bei der Langzeittherapie mit 0,5 mg Tagesdosis treten Nebenwirkungen selten auf (bei etwa 4% der Patienten). Beschriebene Nebenwirkungen unter Fingolimod-Therapie sind verschiedene Infektionen, vor allem der oberen Atemwege, Kopfschmerzen, eine Erhöhung der Leberenzyme im Blut sowie ein Makulaödem [48, 50].

1.4. Wirkmechanismus von Fingolimod

Im Weiteren dreht sich diese Arbeit um die Therapie der MS mit Fingolimod, weshalb zunächst dessen Wirkmechanismus beleuchtet werden soll. Die Wirkung von Fingolimod basiert auf einer strukturellen Analogie des Moleküls zu Sphingosin, einem Sphingolipid, das nach Phosphorylierung zu Sphingosin-1-Phosphat (S1P) verschiedene Immunprozesse reguliert [51]. S1P fungiert als Signalmolekül der Migration von Lymphozyten (Abbildung (Abb.) 1). Es leitet die Zellen entlang eines steigenden Gradienten aus dem Gewebe durch die Lymphknoten in die efferenten Lymphgefäße und weiter ins Blut, wo eine besonders hohe S1P-Konzentration besteht. Außerdem ist die Migration von Zellen auch in anderen lymphatischen Organen wie Milz, Thymus und Knochenmark durch S1P organisiert [52]. Ein weiteres Molekül, welches für die Migration von Immunzellen durch die Lymphknoten relevant ist, ist der Homing-Rezeptor CCR7, der vor allem von naiven T-Zellen, zentralen Gedächtnis-T-Zellen und einigen B-Zellen exprimiert wird. Durch die Bindung der Liganden CCL19 und CCL21 an CCR7 wird die Einwanderung der Zellen in die Lymphknoten bewirkt [53].

Fingolimod wird nach der Aufnahme in die Zellen, wie auch Sphingosin, von Sphingosinkinasen zu seiner aktiven Form, Fingolimod-Phosphat, phosphoryliert. Es sind 5 verschiedene Rezeptoren für S1P (S1PR) bekannt, die besonders stark von T-Zellen aber auch von B-Zellen exprimiert werden. Fingolimod-Phosphat bindet an 4 dieser 5 Rezeptoren, S1PR1 und S1PR3-5, stimuliert jedoch nicht die gekoppelten Signalwege [54]. Durch die Bindung der Rezeptoren unter der regelmäßigen Gabe von Fingolimod werden diese internalisiert und abgebaut. Die entstehende reduzierte Stimulation durch S1P bewirkt, dass die Immunzellen ihre Migration nicht fortsetzen und somit in den Lymphknoten verbleiben. Dadurch zirkulieren mutmaßlich weniger autoreaktive Zellen im Blut, sodass diese nicht das ZNS infiltrieren können. Hierdurch wird eine Minderung der entzündlichen Aktivität bewirkt [55].

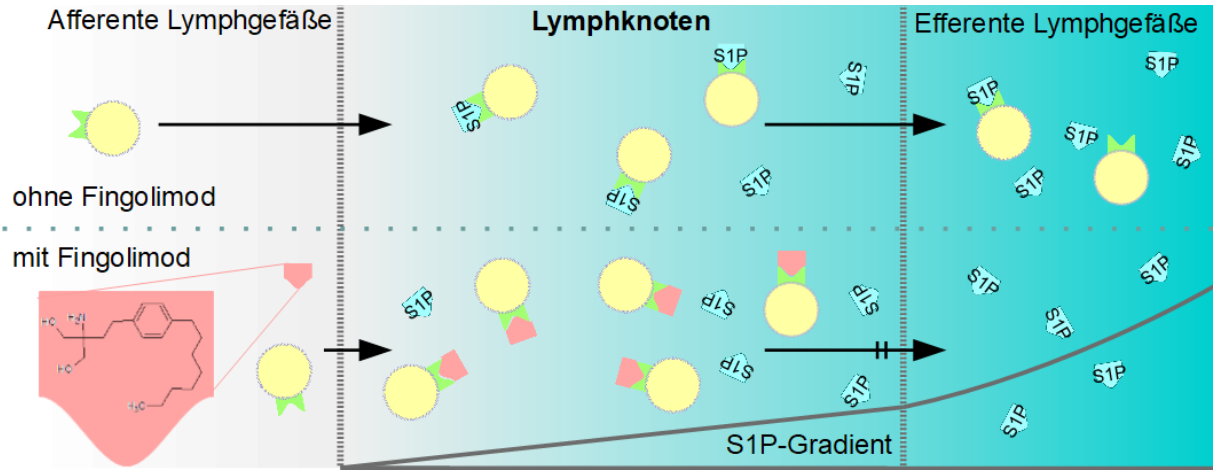


Abbildung 1: Migration der Immunzellen und Effekte der Therapie mit Fingolimod.

Die obere Hälfte der Abbildung zeigt die Regulation der Migration der Immunzellen durch Sphingosin-1-Phosphat (S1P) entlang eines S1P-Gradienten (türkis dargestellt, höhere Farbintensität entspricht einer höheren Konzentration an S1P). Die Immunzellen (gelb, Kreis) exprimieren verschiedene S1P-Rezeptoren (grün) und wandern über das Blut (nach Bindung von Chemokinen wie CCL19 und CCL21 in den hochendothelialen Venolen, nicht gezeigt) oder über afferente Lymphgefäße in die Lymphknoten ein [56]. In der unteren Hälfte der Abbildung ist die Migration der Immunzellen unter der Therapie mit Fingolimod (rote Form, inklusive Strukturformel) dargestellt. Hierbei wird durch Blockade und die (hier nicht dargestellte) Rezeptor-Internalisierung die Regulation der Migration der Immunzellen durch S1P unterbunden.

1.5. Transkriptomanalysen

In jeder Zelle eines Menschen liegt im Prinzip immer das gleiche Genmaterial vor und dennoch gibt es zahlreiche verschiedene Zellarten und -funktionen. Diese Vielfalt wird erreicht, indem das Genom, die Gesamtheit aller Gene, in variablem Umfang transkribiert wird [57]. Um also die Funktion von Zellen zu betrachten, reicht es nicht aus, deren Desoxyribonukleinsäure (DNA) zu analysieren, sondern es muss das Ausmaß betrachtet werden, in dem die unterschiedlichen Gene in Ribonukleinsäure (RNA) übersetzt werden. Das Transkriptom bezeichnet die Gesamtheit

aller Transkripte in einer Zelle zu einem bestimmten Zeitpunkt [58]. Des Weiteren können durch die vollständige Transkriptomanalyse auch Aussagen über die Expressionsniveaus nicht-protein-kodierender Transkripte getroffen werden. Hierzu gehört auch die Klasse der microRNA (miRNA). Dies sind kurze Transkripte, die posttranskriptional die Genexpression beeinflussen und bei zahlreichen zellulären und immunologischen Prozessen eine Rolle spielen [59]. Zur Genexpressionsanalyse stehen verschiedene Methoden zur Verfügung. Mithilfe der quantitativen Echtzeit-Polymerase-Kettenreaktion (rt-PCR) kann das Expressionsniveau von einzelnen Gentranskripten sehr sensitiv bestimmt werden. Es werden bestimmte Nukleotidsequenzen in mehreren Zyklen amplifiziert. Dabei werden Fluoreszenzsignale erzeugt, die nach jedem Zyklus gemessen werden [60]. Dies ist ein sehr aufwändiges Verfahren und es können nur ausgewählte bekannte Sequenzen untersucht werden, weshalb hiermit nicht das gesamte Transkriptom hinreichend analysiert werden kann. Die Verwendung von Microarrays, die mit Oligonukleotidsonden Millionen von Sequenzen abdecken können, ermöglicht das gesamte humane Transkriptom quantitativ zu untersuchen [61]. Eine neuere Möglichkeit zur RNA-Analyse ist die sogenannte *next-generation*-Sequenzierung. Hierbei werden alle Gentranskripte der Zellen sequenziert und auf diese Weise quantitativ bestimmt [62].

Eines der Ziele der Untersuchung des Transkriptoms ist die Identifizierung von Biomarkern. Ein Biomarker ist ein objektiv messbarer Indikator, der anzeigt, ob biologische Prozesse normal oder pathologisch ablaufen, und die Antwort des Organismus auf äußere Einflüsse messbar macht [63]. Biomarker sind wichtig für die Diagnosestellung, die Einschätzung der Krankheitsaktivität sowie die Prognose des Ansprechens auf verschiedene Therapien. Durch die Komplexität der pathophysiologischen Mechanismen bei MS sind bereits zahlreiche molekulare Biomarker postuliert worden, die jeweils auf einzelne Aspekte der Erkrankung abzielen. Nur wenige von ihnen sind jedoch im klinischen Alltag etabliert. Hier finden lediglich der IgG-Index sowie der Nachweis von OKB im Liquor Verwendung, die eine entzündliche Aktivität im ZNS anzeigen [64]. Biomarker, die bei MS mit hoher Sensitivität und Spezifität Hinweise auf die aktuelle entzündliche Aktivität oder sogar auf das Schubrisiko geben können, sind derzeit nicht ausreichend validiert. Auch für die individuelle Wirksamkeit und Verträglichkeit von einzelnen Medikamenten gibt es aktuell nur wenige molekulare Biomarker wie etwa der Nachweis von anti-JCV-Antikörpern, welche ermöglichen, das Risiko für eine progressive multifokale Leukenzephalopathie unter immunmodulatorischer Therapie (insbesondere Natalizumab) einzuschätzen. Für eine Übersicht von etablierten, molekularen Biomarkern bei MS sei auf Paul et al. verwiesen [65].

2. Fragestellungen

Bei der vorliegenden Studie zum Verlauf der Therapie mit Fingolimod bei Patienten mit Multipler Sklerose standen zum einen Aspekte der klinischen Wirksamkeit und Verträglichkeit im Vordergrund:

- Kann durch die Therapie mit Fingolimod eine Reduktion der Schubrate bei den in diese Untersuchung eingeschlossenen Patienten mit RRMS erreicht werden?
- Kann bei diesen Patienten durch Fingolimod eine Progression der Behinderung verhindert werden?
- Treten Nebenwirkungen auf, die eine Fortführung der Therapie mit Fingolimod unattraktiv machen?

Die Untersuchungen von Blutproben der Patienten zielten außerdem darauf ab, die Wirkung von Fingolimod auf zellulärer Ebene zu erfassen. Hierbei ergab sich die Frage, wie sich die Therapie mit Fingolimod auf die Anteile der verschiedenen Zellpopulationen (T-Zellen, B-Zellen, Monozyten bzw. NK-Zellen) im Blut auswirkt, beziehungsweise welche Zellen stärker in ihrer Migration innerhalb lymphogener Gewebe beeinflusst werden.

Außerdem wurden offene Fragen adressiert, die sich auf den Wirkmechanismus von Fingolimod auf molekularer Ebene beziehen:

- Sind bereits nach der ersten Gabe von Fingolimod Gene in ihrer Expression verändert und wie wirkt sich die dauerhafte Therapie mit Fingolimod auf das Transkriptionsprofil der weiterhin im Blut zirkulierenden Zellen aus?
- Gibt es Gene, die bei mehreren der untersuchten Zellpopulationen (T-Zellen, B-Zellen, Monozyten und NK-Zellen) in ihrer Expression verändert sind?
- Welche biologischen Funktionen haben die Genprodukte, die von Transkripten kodiert werden, die unter Therapie auf einem anderen Expressionslevel vorliegen?
- Gibt es Vorläufertranskripte von miRNA, die in Folge der Behandlung mit Fingolimod im Blut stärker oder schwächer exprimiert werden?
- Wie wirkt sich die veränderte Genexpression auf die Signaltransduktion von S1P aus?

3. Methoden

3.1. Studiendesign

Bei der vorliegenden Studie handelt es sich um eine longitudinale Beobachtungsstudie. Die Studienpopulation umfasste elf Patienten, die im Rahmen dieser Studie erstmals Fingolimod erhalten haben. Zuvor wurde bei ihnen mithilfe der überarbeiteten McDonald-Kriterien von 2010 [66] eine hochaktive RRMS diagnostiziert. Unter den Patienten waren 7 Frauen und 4 Männer im Alter von 26 bis 46 Jahren (Tabelle 1, Seite 13). Die vorhergehende Therapie bestand bei sieben der Patienten aus Interferon-β. Vier von den Patienten erhielten zuvor Glatirameracetat. Trotz dieser Medikationen traten jedoch innerhalb eines Jahres vor Beginn der Therapie mit Fingolimod 1 bis 3 (Ø 1,6) Schübe auf. Der EDSS-Wert lag zu Beginn der Therapie zwischen 1,5 und 5,5 (Ø 3,4). Die Umstellung auf Fingolimod erfolgte bei dem Großteil der Patienten (n=9) innerhalb eines Monats nach Absetzen der Vormedikation. Fingolimod wurde in der Standarddosierung von 0,5 mg täglich entsprechend der europäischen Zulassungskriterien appliziert. Die individuelle Entscheidung zur Therapie mit Fingolimod erfolgte bei allen Patienten bereits vor der vorliegenden Studie gemäß der damals aktuellen Leitlinie der Deutschen Gesellschaft für Neurologie zur Therapie von RRMS [33]. Nach Aufklärung über die Transkriptomstudie haben die Patienten freiwillig schriftlich in die Teilnahme an der Studie eingewilligt. Das Konzept der Studie wurde gemäß der Deklaration von Helsinki entwickelt und durch die Ethikkommission der Universität Rostock genehmigt (Ethikvotum Nr. A 2011 125). Die erhobenen Forschungsdaten wurden anonymisiert gespeichert und ausgewertet. Die Transkriptomuntersuchungen wurden durch die Firma Novartis teilweise finanziell unterstützt (Projektnr.: CFTY720DDE14T). Die Behandlung der Patienten einschließlich Diagnose und Therapie erfolgte jedoch nicht nach einem vorab festgelegten Prüfplan im Sinne einer klinischen Studie.

3.2. Probenentnahme und -verarbeitung

Zur Transkriptomuntersuchung von verschiedenen Zellpopulationen im Therapieverlauf wurden von den 11 Patienten zu 3 verschiedenen Zeitpunkten Blutproben zu je 20 ml in Röhrchen mit Ethylendiamintetraessigsäure (EDTA) gewonnen (Abb. 2). Dies erfolgte vor der ersten Gabe von Fingolimod sowie 24 Stunden (also vor der zweiten Gabe) und 3 Monate nach dem Therapiebeginn.

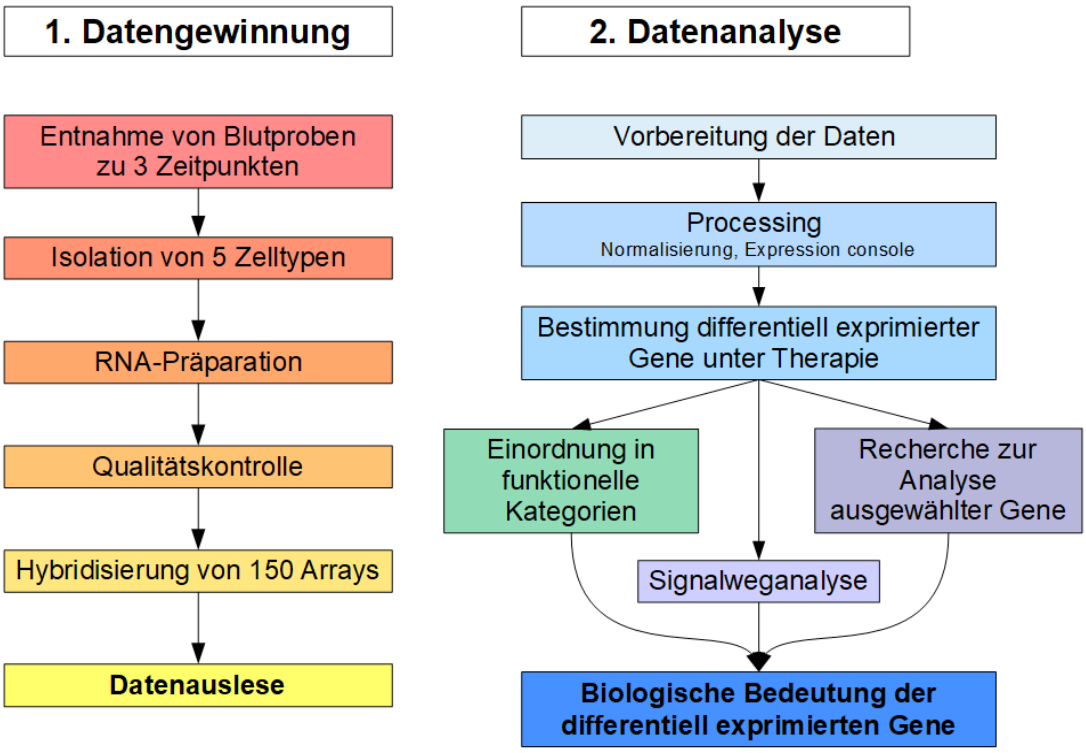


Abbildung 2: Übersicht über die einzelnen Arbeitsschritte. *Die Flussdiagramme zeigen den Ablauf der einzelnen Arbeitsschritte unterteilt in die Abschnitte Datengewinnung und den darauffolgenden Prozess der Datenanalyse.*

Für die zelltypspezifische Analyse erfolgte die Aufteilung der entnommenen 20 ml Blut in 5 mal 4 ml. Im Anschluss wurden den nun 5 Aliquoten zunächst verschiedene whole blood MicroBeads (Miltenyi Biotec®) zugegeben. Diese markieren spezifische Oberflächenmoleküle magnetisch, in diesem Fall CD4, CD8, CD14, CD19 und CD56. Die CD4+-Zellen sind vorwiegend T-Helferzellen [67]. CD8 ist auf zytotoxischen T-Zellen zu finden [68]. Durch die Selektion von CD19+-Zellen können B-Zellen isoliert werden. Monozyten tragen in der Regel das Oberflächenmolekül CD14 und natürliche Killerzellen (NK-Zellen) CD56 [69]. Im Anschluss wurden die so markierten Zellen mithilfe des automatischen *magnetic-activated cell separator* (autoMACS, Miltenyi Biotec®) positiv selektiert. Die unmarkierten Zellanteile wurden verworfen. Um die Effekte der Fingolimod-Therapie auf die Häufigkeit der Zellpopulationen im Blut zu bestimmen, wurden die selektierten Zellen unter dem Mikroskop gezählt. Nach der Zugabe von Lyse-Puffer wurden die Proben bis zur weiteren Verarbeitung bei -20°C gelagert. Die Gesamt-RNA der Zellen wurde mit dem mirVana isolation kit (Ambion) extrahiert. Die RNA-Konzentrationen wurden anschließend mit dem NanoDrop 1000 Spektrophotometer (Thermo Fisher Scientific) bestimmt. Zur Qualitätskontrolle der RNA wurden unter Verwendung des Agilent 2100 Bioanalyzer und RNA 6000 Pico Lab Chips (Agilent Technologies) RNA integrity numbers (RIN) bestimmt [70]. Diese zeigen auf einer Skala von 1 bis 10 an, ob eine ausreichende Integrität der extrahierten RNA gewährleistet ist.

3.3. Bestimmung differentiell exprimierter Gene

Zur Analyse der zellulären RNA wurden 150 Human Transcriptome Arrays (HTA) 2.0 (Affymetrix) eingesetzt [61]. Damit erfolgten für die 5 verschiedenen Zellpopulationen zu 3 Zeitpunkten Untersuchungen der je 10 Proben mit der besten RNA-Qualität. In der Untersuchung der CD8+-Zellen fehlt die Probe von Patient Nr. 2, bei den CD56+-Zellen ist es die Probe von Patient Nr. 11 und bei den CD4+-, CD14+- und CD19+-Zellen die Probe von Patient Nr. 10. Diese Arrays decken mit über 6 Millionen verschiedenen Oligonukleotidsonden alle derzeit annotierten protein-kodierenden und nicht-kodierenden humanen Transkripte und deren Isoformen ab [59, 69]. Die Sonden sind jeweils 25 Basen lang und haben eine komplementäre Sequenz zu einem Bereich eines entsprechenden Transkripts. Für jedes Transkript sind viele verschiedene Sonden vorhanden. Somit können über 67.500 Transkripte in ihrem Expressionsniveau beurteilt werden. Von diesen Transkripten sind etwa 70% protein-kodierende Transkripte und etwa 30% nicht-kodierende Transkripte [71]. Die Sonden werden bei der Auswertung zu Gruppen (engl. Probesets) zusammengefasst, die spezifisch für einzelne Exonfragmente, Exon-Exon-Grenzen oder Transkript-Cluster sind. Für gut annotierte Gene mit offiziellem HGNC-Gensymbol gibt es in der Regel genau ein Transkript-Cluster Probeset. Von den 150 Proben wurden jeweils 70 ng (bei CD4+-, CD19+- und CD56+-Zellen) bzw. 200 ng (bei CD8+- und CD14+-Zellen) der Gesamt-RNA genutzt, um daraus unter Verwendung des Affymetrix Whole Transcript Protokolls komplementäre RNA (cRNA) zu gewinnen. Mithilfe dieser cRNA wurde anschließend Einzelstrang-DNA generiert, die fragmentiert und biotinyliert wurde. Die Einzelstrang-DNA wurde nun bei 45 °C für 16 Stunden auf den HTA 2.0 Microarrays hybridisiert (Abb. 3). Um die Arrays von überschüssiger DNA zu befreien, wurden sie gespült und mit einem Streptavidin-Phycoerythrin-Konjugat unter Verwendung einer Affymetrix Fluidics Station 450 fluoreszenzmarkiert. Die Signale der Microarrays wurden schließlich mithilfe des GeneChip Scanner 3000 7G (Affymetrix) ausgelesen. Alle Prozesse wurden gemäß des Hersteller-Protokolls durchgeführt.

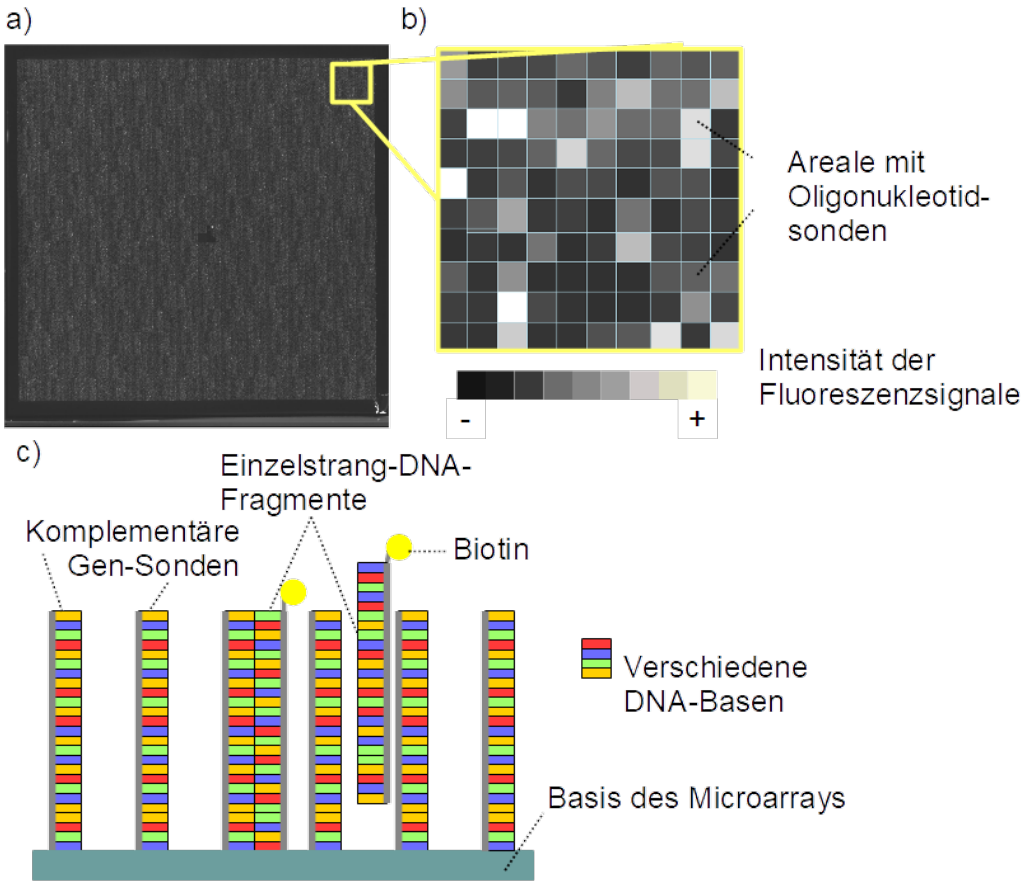


Abbildung 3: Genexpressionsmessung mittels Affymetrix-Microarrays: *a) Scan eines Affymetrix-Microarraychips als Darstellung der unverarbeiteten Rohdaten. b) Die quantitative Bestimmung der an die Gen-Sonden gebundenen Fragmente erfolgt über die Bestimmung der Intensität der Fluoreszenzmarkierung. c) Es sind zu den jeweils zu untersuchenden RNA-Abschnitten komplementäre, je 25 Basen lange Oligonukleotid-Sonden auf der Basis des Microarrays fixiert. Es werden aus der ursprünglichen RNA erstellte, biotinylierte Einzelstrang-DNA-Fragmente hinzugegeben und hybridisiert. Die den Sonden komplementären Fragmente binden nun an die fixierten Oligonukleotid-Sonden. Die restliche DNA wird anschließend ausgewaschen. In einem nächsten Schritt erfolgt die Fluoreszenzmarkierung des gebundenen Biotins. Nun kann die Auslesung des Arrays mit einem Scanner erfolgen.*

Die initiale Verarbeitung der Microarray-Scans wurde mit der Affymetrix GeneChip Console (AGCC)-Software, Version 4.0, vorgenommen. Die damit erstellten CEL-Daten wurden dann unter Nutzung der Affymetrix Expression Console (EC) einer RMA-Normalisierung unterzogen, die auch eine log2-Transformation der Daten einschließt [72], und in eine tabellarische Form gebracht. Die rohen und prozessierten Daten sind vollständig öffentlich zugänglich in der Gene Expression Omnibus-Datenbank [73] (SuperSeries GSE73174) hinterlegt [74]. Um die Veränderungen des Transkriptoms im Therapieverlauf zu analysieren, wurden die Daten mittels der Transcriptome Analysis Console 1.0 von Affymetrix weiterverarbeitet. Die RNA-Expressionsniveaus nach 24 Stunden und 3 Monaten wurden dazu jeweils mit der Baseline (vor 1. Gabe Fingolimod) verglichen. Die Kriterien für eine signifikante Genexpressionsänderung

waren hierbei ein t-Test *p*-Wert von <0,001 sowie ein Fold change von >1,5 oder <-1,5. Der Fold change (FC) beschreibt die relative Veränderung des Expressionsniveaus. Ein FC von 1,5 entspricht dabei einer Erhöhung der Expression des Gens auf das 1,5 fache. Ein negativer FC bedeutet eine Verringerung der Expression, bei einem FC von -1,5 auf 2/3 des vorherigen Wertes. Aufgrund der hohen Anzahl an differentiell exprimierten Genen bei CD4+-Zellen wurde hier der FC-Schwellenwert auf >2 bzw. <-2 erhöht.

3.4. Analyse der Genfunktionen und -interaktionen

Um die funktionellen Effekte der Expressionsveränderungen einzuschätzen, war es nötig, zu betrachten, in welchem Ausmaß die differentiell exprimierten Gene an biologischen Prozessen beteiligt sind. Für CD4+-Zellen wurde analysiert, ob bestimmte funktionelle Gruppen der Hauptkategorien „Molekulare Funktionen“ und „Biologische Prozesse“ der Gene Ontology (GO)-Datenbank [75] unter den differentiell exprimierten Genen stärker repräsentiert sind als andere. Für die Untersuchung der CD8+-Zellen wurden Funktionskategorien der SABiosciences-Datenbank verwendet. Mithilfe der Software GOstats wurde dann analysiert, ob eine dieser Gruppen innerhalb der untersuchten Genlisten statistisch überrepräsentiert ist [76]. Zur näheren Untersuchung der Transkripte, deren Varianten und den zugehörigen Genloci wurde der Genome Browser der University of California Santa Cruz verwendet [77]. Dieser vereint Daten vieler verschiedener Forschungsgruppen und Datenbanken wie GenBank und RefSeq. Zur Annotation ausgewählter Probesets für nicht-kodierende differentiell exprimierte Gene wurde im Genome Browser die durch die Oligonukleotidsonden repräsentierte genomische Sequenz auf bekannte Messenger-RNA (mRNA), Transfer-RNA (tRNA), miRNA und sonstige DNA-Ableseprodukte untersucht.

Zur weiteren Untersuchung der molekularen Effekte von Fingolimod wurden Interaktionen innerhalb der S1P-assoziierten Signalwege analysiert. Besonders durch Bindung des Rezeptors S1PR1 wird durch Fingolimod-Phosphat eine verlängerte Internalisierung bewirkt [78]. Zur Analyse der Expressionsveränderungen von Signalmolekülen wurde ein Schema der aktivierten Signalwege nach Bindung von S1P an S1P-Rezeptoren konstruiert. Wie in Kapitel (Kap.) 5.2 näher beschrieben, wurden aus 11 verschiedenen Übersichtsarbeiten Gene identifiziert, bei denen Einigkeit darüber bestand, dass sie im Metabolismus und den Wirkungskaskaden von S1P eine Rolle spielen. Das Schema beinhaltet somit die Synthese von S1P beziehungsweise die Phosphorylierung von Fingolimod, die Interaktionen mit den S1P-Rezeptoren und nachgeschaltete Prozesse. Außerdem wurde der Chemokin-Rezeptor CCR7 aufgrund seiner wichtigen Rolle in der Regulation der Lymphozytenmigration mit in die Signalweganalyse integriert. Das molekulare Netzwerk wurde schließlich durch Verwendung des Programms Cytoscape 3.1.0 jeweils unter Einbeziehung der Expressionsdaten der CD4+- und CD8+-Zellen graphisch dargestellt [79].

4. Ergebnisse

4.1. Studienpopulation

Für die meisten Patienten hatte die Umstellung der Therapie auf Fingolimod einen sehr positiven Effekt auf den Krankheitsverlauf. Bei 9 der 11 Patienten traten in den 12 Monaten nach dem Therapiebeginn keine neuen Schübe auf (Tabelle 1). Eine Patientin (Patient (Pat.) Nr. 6) hatte im zweiten Halbjahr nach Therapiebeginn 2 Schübe, führte die Therapie aber weiterhin fort. Bei einem weiteren Patienten (Pat. Nr. 7) trat bereits nach 3 Monaten Fingolimod-Therapie ein Schub auf. Es folgten zwei weitere Schübe, woraufhin die Therapie nach 8 Monaten abgebrochen wurde. Es wurde im Anschluss eine Therapie mit Alemtuzumab begonnen. Bei diesen beiden Patienten, die unter der Therapie mit Fingolimod Schübe erlitten, kam es außerdem zu einer deutlichen Verschlechterung des EDSS-Wertes ein Jahr nach Therapiebeginn mit Fingolimod im Vergleich zum Ausgangswert. Außerdem war noch bei einem weiteren Patienten (Pat. Nr. 2), bei dem jedoch kein Schub auftrat, eine leichte EDSS-Wert-Erhöhung um 0,5 Punkte zu beobachten. Bei 6 der 11 Patienten konnte ein Jahr nach Therapiebeginn eine Verringerung des EDSS-Wertes festgestellt werden. Bei 2 Patienten blieb der EDSS-Wert unverändert. Die Patienten Nr. 1 und Nr. 10 zeigten im ersten Therapiejahr leicht erhöhte Leberwerte (Kap. 5.2). Bei den anderen Patienten wurden in dieser Zeit keine Nebenwirkungen berichtet.

Tabelle 1: Klinische und demographische Daten der Patienten.

Patient Nr.	Alter	Krankheits-dauer in Jahren	Geschlecht	Letzte Therapie vor Fingolimod	Abstand zur Vortherapie in Monaten	EDSS: Baseline	EDSS: +1 Jahr	Anzahl Schübe Baseline -1J	Anzahl Schübe Baseline +1J
1	43	20	weiblich	IFN beta 1a	38	4,0	3,0	1	0
2	26	3	weiblich	IFN beta 1b	1	1,5	2,0	2	0
3	29	5	weiblich	GA	<1	2,5	1,5	3	0
4	43	1	weiblich	IFN beta 1a	1	4,0	3,5	2	0
5	45	12	weiblich	IFN beta 1a	2	3,0	3,0	3	0
6	33	2	weiblich	GA	<1	2,5	5,5	2	2
7	33	4	männlich	IFN beta 1a	<1	3,0	6,0	1	3
8	46	15	männlich	IFN beta 1b	<1	3,5	2,0	1	0
9	37	9	männlich	GA	1	5,5	4,0	1	0
10	31	7	männlich	GA	<1	3,5	2,0	1	0
11	46	9	weiblich	IFN beta 1b	<1	4,0	4,0	1	0

Die Tabelle enthält Informationen über die 11 untersuchten Patienten. Dabei sind zum einen demographische Daten wie Alter und Geschlecht dargestellt sowie krankheitsbezogene Daten. Diese betreffen die Krankheitsdauer, die letzte Therapie vor der Umstellung (Interferon (IFN), Glatirameracetat (GA)) auf Fingolimod und den zeitlichen Abstand zwischen dem Ende der Vortherapie und der ersten Dosis Fingolimod. Der Expanded Disability Status Scale (EDSS)-Wert vor und nach einem Jahr der Therapie mit Fingolimod sowie die Anzahl der Schübe in den 12 Monaten vor (Baseline-1J) und den 12 Monaten nach dem Therapiebeginn (Baseline+1J) mit Fingolimod sind ebenfalls angegeben.

4.2. Veränderungen in der Anzahl zirkulierender Immunzellen

Bereits 24 Stunden nach der ersten Gabe von Fingolimod zeigte sich im Mittel eine Reduktion in der Anzahl separierter CD4+-Zellen um 21,1% sowie der CD19+-Zellen um 14,2% (Abb. 4). Nach drei Monaten Therapie war die Anzahl der CD8+-Zellen um 59,5% reduziert, die der CD4+- und CD19+-Zellen sogar um 92,4% bzw. 87,0%. Die mittlere Anzahl von CD56+-Zellen war verglichen mit dem Ausgangswert um 15,7% verringert. Die geringste Reduktion zeigte sich in der Anzahl der CD14+-Zellen mit -5,3%.

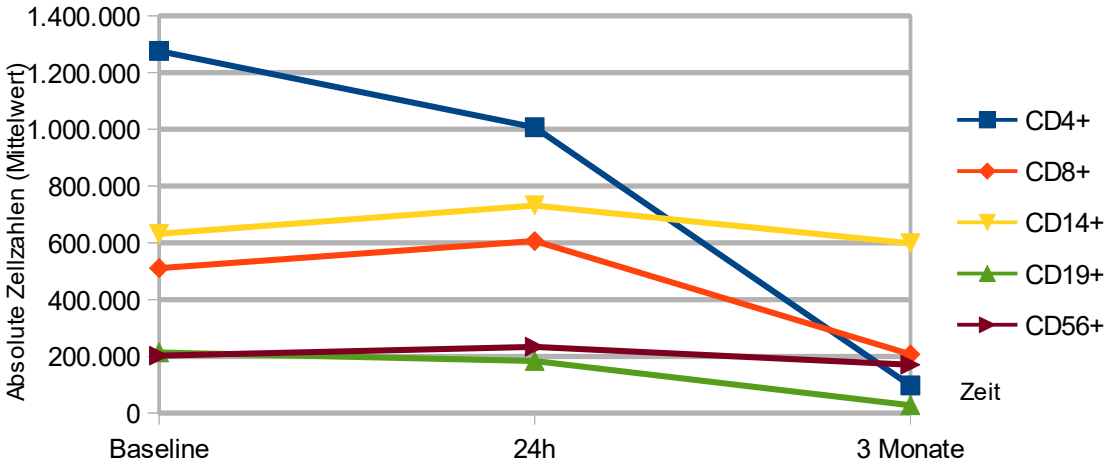


Abbildung 4: Effekt der Fingolimod-Therapie auf die Anzahl der Zellen verschiedener Zellpopulationen im Blut von MS-Patienten: *Absolute Veränderung der Anzahl von Lymphozyten und Monozyten pro 2 ml Blut. Es wurde für jeden Zeitpunkt jeweils der Mittelwert der verschiedenen Patientenproben gebildet.*

4.3. Veränderung der RNA-Expression in T-Zellen

Nach Qualitätskontrolle der RNA und Bestimmung der RIN-Werte wurden pro Zellpopulation für jeden Zeitpunkt 10 Proben mit ausreichender RNA-Qualität und -Konzentration für die Microarray-Analyse verwendet [74]. Für den Zeitpunkt 24 Stunden nach der 1. Gabe von Fingolimod konnten keine signifikanten Veränderungen der Genexpression der T-Zellen festgestellt werden. Bei den CD4+-Zellen war nur ein Gen (bzw. Probeset) differentiell exprimiert, bei den CD8+-Zellen keines. Das Transkriptom dieser Zellen hat sich im weiteren Studienverlauf im Vergleich zu den anderen Zellpopulationen aber am meisten geändert. So zeigte die Transkriptomanalyse der CD8+ T-Zellen nach 3 Monaten Therapie eine deutliche Veränderung: Es waren 861 Gentranskripte differentiell exprimiert, 80% davon zeigten signifikant höhere Expressionslevel als zu Therapiebeginn. Bei den CD4+ T-Zellen waren bei einem FC von >1,5 oder <-1,5 nach 3 Monaten Therapie 6489 Gene in ihrer Expression verändert (Tabelle 2, S. 15). Für die weitere funktionelle Analyse wurde deshalb ein FC-Grenzwert von >2 bzw. <-2 gewählt. Dieses Kriterium erfüllten 890 Gene. Hiervon waren 618 Gene stärker und 272 Gene schwächer exprimiert als vor Beginn der Therapie.

Tabelle 2: Transkriptomveränderungen der verschiedenen Zellpopulationen im Verlauf der Therapie mit Fingolimod.

Zelltyp	24 h nach Therapiebeginn		3 M nach Therapiebeginn	
	stärker exprimierte Gene	schwächer exprimierte Gene	stärker exprimierte Gene	schwächer exprimierte Gene
CD4+	1	0	2574	3915
CD8+	0	0	690	171
CD14+	0	0	1	0
CD19+	0	0	41	1
CD56+	0	0	0	0

Die Tabelle zeigt die Anzahl der Gene, die nach 24 Stunden (h) beziehungsweise 3 Monaten (M) Therapie differentiell exprimiert waren. Als Kriterien für eine differentielle Expression wurden ein Fold change von >1,5 oder <-1,5 und ein t-Test p-Wert von <0,001 gewählt. Adaptiert von Angerer et al. 2018 (Kapitel 5.3.). Die Anzahlen sind die jeweilige Anzahl gefilterter Probesets, diese werden zur besseren Lesbarkeit Gene oder Gentranskripte genannt.

Sowohl bei den CD4+- als auch bei den CD8+-Zellen konnte eine Überrepräsentation verschiedener funktioneller Gengruppen unter den differentiell exprimierten Genen festgestellt werden. Bei den CD4+-Zellen wurden unter Einbeziehung der Hauptkategorien „Biologische Prozesse“ und „Molekulare Funktionen“ aus den untergeordneten GO-Gruppen mit Odds Ratio >7 jeweils die 10 Gruppen mit dem niedrigsten Signifikanzwert gefiltert. Darunter befanden sich vor allem Gruppen, die mit der Regulation der Aktivität von Immunzellen assoziiert sind. Unter der Hauptkategorie „Biologische Prozesse“ fanden sich z. B. die Gengruppen „Positive Regulation der Lymphozyten-Aktivierung“, „Regulation der Zytokinsekretion“ und „Zytokinsekretion“. Zu den überrepräsentierten „Molekularen Funktionen“ gehörten vorwiegend GO-Gruppen, die mit der Modulation des Immunsystems assoziiert sind (Kap. 5.1). Bei den CD8+-Zellen wurden 16 Gruppen der SABiosciences-Datenbank ermittelt, die besonders viele Gene enthielten, die unter der Therapie mit Fingolimod differentiell exprimiert waren. So wurde beispielsweise eine Überrepräsentation von Genen der Gruppe „G-Protein-gekoppelte Rezeptoren“ beobachtet. Außerdem wurden unter den differentiell exprimierten Genen Vorläufertranskripte für miRNA gefunden. Hierzu gehören beispielsweise hsa-mir-216b und hsa-mir-548c bei den CD4+-Zellen sowie hsa-mir-548j und hsa-mir-644a bei den CD8+-Zellen.

Die Analyse des S1P-assoziierten Geninteraktionsnetzwerkes zeigte, dass Fingolimod als funktioneller S1P-Rezeptor-Antagonist durch Bindung entsprechender G-Protein-gekoppelter Rezeptoren in die durch diese vermittelten Signalwege eingreift. Es wurden Effekte der verminderten Wirkung von S1P festgestellt. Die Veränderungen der Expression der Gene innerhalb der S1P-assoziierten Signalwege der CD4+ und CD8+-Zellen unter Fingolimod-Therapie zeigte, dass der CCR7-Rezeptor sowohl bei CD4+-Zellen (FC=-2,05) als auch bei CD8+-Zellen (FC=-6,52) auf mRNA-Ebene signifikant schwächer exprimiert wurde als zu Therapiebeginn. Nach 3 Monaten Therapie waren bei den CD8+-Zellen insgesamt 6 Gene der Signalwegmoleküle signifikant stärker exprimiert. Hierzu gehörten die Signalmoleküle MAPK1,

PIK3CG, PLCB1, PLCG2 und ROCK2. Außerdem war das S1PR5-Gen höher exprimiert. Bei den CD4+-Zellen gab es, neben dem vermindert exprimierten Gen CCR7, einige S1P-Signalweggene, die stärker exprimiert waren als zu Therapiebeginn. Darunter waren zum Beispiel PIK3CG (FC=2,13) und die Rho-Kinasen ROCK1 (FC=1,58) und ROCK2 (FC=1,87) (Kap. 5.1).

4.4. Veränderung der RNA-Expression in B-Zellen

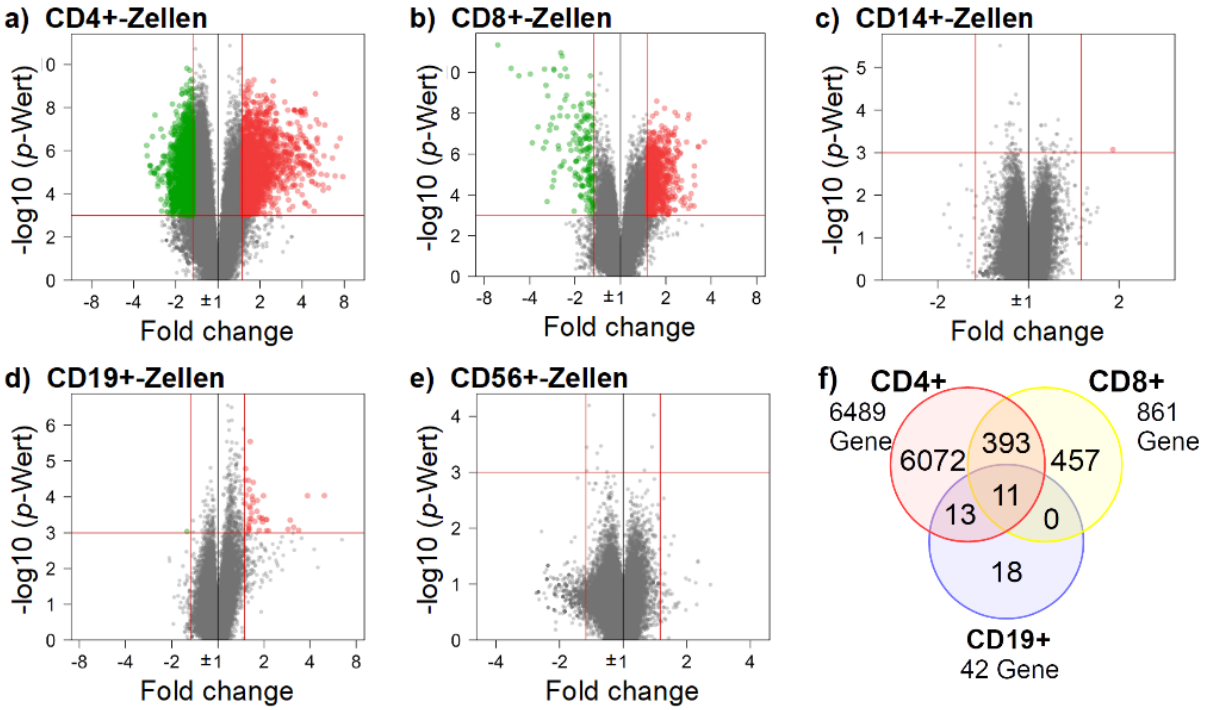
Bei den CD19+ B-Zellen war eine signifikante Veränderung der Genexpression nach 3 Monaten Therapie zu sehen (Tabelle 2, S. 15). Obwohl 24 Stunden nach der ersten Gabe von Fingolimod kein Gen den Kriterien für eine differentielle Expression entsprach, waren es nach 3 Monaten Therapie 42 Gene. Von diesen Genen war der Großteil (n=41) stärker exprimiert als vor Therapiebeginn. Ein Gentranskirpt (GenBank-Nr: DQ584601) war schwächer exprimiert als vor Therapiebeginn. Zu den Transkripten mit veränderter Expression unter Fingolimod gehörten (nach Affymetrix-Annotation) 25 protein-kodierende sowie 17 nicht-kodierende Transkripte. Außerdem wurden Vorläufertranskripte für miRNA (hsa-mir-326 und hsa-mir-3120) gefunden, die in ihrer Expression nach 3-monatiger Therapie verstärkt waren.

4.5. Änderung der RNA-Expression von Monozyten und NK-Zellen

Schon die Anzahl der Monozyten und NK-Zellen im Blut zeigte im Verlauf der Therapie mit Fingolimod nur eine geringe Veränderung (Kap. 4.2). Die molekularen Effekte waren bei diesen beiden Zellpopulationen ebenfalls sehr gering. Bei den CD56+ NK-Zellen entsprach weder nach 24 Stunden noch nach 3 Monaten Therapie ein Gen den Kriterien für eine differentielle Expression. Bei den CD14+ Monozyten konnte nach 3 Monaten Therapie ein nicht protein-kodierendes Gentranskirpt (Ensembl-Name: AC097634.1) als höher exprimiert eingestuft werden. Nach 24 Stunden Therapie zeigte sich hier auch keine signifikante Veränderung im Transkriptionsprofil (Tabelle 2, S. 15).

4.6. Vergleichende Analyse der Genexpressionsveränderungen

Es gab große Unterschiede im Ausmaß der Veränderung des Expressionsprofils der verschiedenen untersuchten Zellpopulationen. Während die B- und T-Lymphozyten in ihrer Genexpression beeinflusst wurden, zeigten die NK-Zellen und Monozyten keine signifikanten Veränderungen auf Transkriptomebene. Diese Unterschiede sind in Abb. 5 gegenübergestellt. Um festzustellen, ob bestimmte Gene unabhängig vom Zelltyp durch Fingolimod in ihrer Expression beeinflusst werden, wurde analysiert, wie viele Gene nach drei Monaten Therapie sowohl bei CD4+-, CD8+- als auch CD19+-Zellen differentiell exprimiert waren (FC >1,5 oder <-1,5, *p*-Wert <0,001).



<u>Abbildung 5</u>: Differentiell exprimierte Gene der im Blut zirkulierenden CD4+-, CD8+-, CD14+-, CD19+- und CD56+-Zellen nach 3 Monaten Fingolimod-Therapie: *Volcano-Plot-Darstellung der Genexpressionsänderungen nach drei Monaten Therapie mit Fingolimod im Vergleich zu Therapiebeginn bei CD4+-Zellen (a), CD8+-Zellen (b), CD14+-Zellen (c), CD19+-Zellen (d) und CD56+-Zellen (e). Auf der x-Achse ist jeweils der Fold change (FC) dargestellt, auf der y-Achse negativ logarithmisch der t-Test p-Wert. Gene, die den Kriterien für eine differentielle Expression (FC>1,5 oder <-1,5; p-Wert <0,001) entsprachen, sind farbig markiert. Dabei spricht rot für einen FC >1,5 und grün für einen FC <-1,5. Das Venn-Diagramm in f) zeigt die Überlappungen der Genlisten und somit die Schnittmengen der Gene, die bei mehr als einem Lymphozyten-Zelltyp differentiell exprimiert waren.*

Es konnten 404 Gene gefunden werden, die nach 3 Monaten Fingolimod-Therapie sowohl bei CD4+- als auch bei CD8+-Zellen differentiell exprimiert waren. Darunter waren 11 Gene außerdem auch bei den CD19+-Zellen differentiell exprimiert. Es wurden keine Gene gefunden, die bei CD19+- und CD8+-Zellen in ihrer Expression verändert waren, jedoch nicht bei CD4+-Zellen. Allerdings waren 13 der 42 bei CD19+-Zellen differentiell exprimierten Gene ebenfalls bei CD4+-Zellen differentiell exprimiert, aber nicht bei CD8+-Zellen (Abb. 5f). Zu den 11 Genen, die bei allen drei Zellpopulationen differentiell exprimiert waren (Tabelle 3, S. 18), gehörten 3 protein-kodierende Transkripte (IQGAP2, MYBL1 und PTPN12) und 8 nicht-kodierende Transkripte. Bei den beiden Patienten, die unter der Therapie mit Fingolimod Schübe erlitten (Pat. Nr. 6 und 7), zeigten sich keine erkennbaren Abweichungen in den Transkriptomänderungen im Vergleich zu den anderen Patienten. So war beispielsweise das Transkript CCR7 bei allen Patienten sowohl in CD4+- als auch in CD8+-Zellen zum 3-Monats-Zeitpunkt niedriger exprimiert als zu Therapiebeginn. Die Studie war angesichts der geringen Fallzahl nicht dafür ausgelegt, Unterschiede in der Genexpression, die mit dem klinischen Verlauf der Patienten korrelieren, valide zu erkennen.

Tabelle 3: Liste von Genen, die bei im Blut von MS-Patienten zirkulierenden CD4+, CD8+ und CD19+-Zellen signifikante Veränderungen in der Expression zeigten.

Nr.	Probeset	Gensymbol	Genort
1	TC02004620.hg.1	---	chr2(-):145143042-145143557
2	TC02004622.hg.1	---	chr2(-):145251832-145254445
3	TC02004623.hg.1	---	chr2(-):145268952-145277958
4	TC05002969.hg.1	---	chr5(-):39383148-39393457
5	TC05000368.hg.1	IQGAP2	chr5(+):75699074-76003957
6	TC07000495.hg.1	PTPN12	chr7(+):77166415-77269388
7	TC08002460.hg.1	---	chr8(-):104395306-104396082
8	TC08001286.hg.1	MYBL1	chr8(-):67474410-67525529
9	TC08002364.hg.1	---	chr8(-):67476954-67525175
10	TC10002487.hg.1	---	chr10(-):17256245-17271983
11	TC12000011.hg.1	---	chr12(+):890299-890424

Die angegebenen Transkript-Cluster der HTA 2.0 Arrays wurden vom Hersteller Affymetrix definiert [61]. Die Tabelle zeigt diejenigen Gentranskripte, die bei CD4+-, CD8+- und CD19+-Zellen mit einem Fold change (FC) >1,5 und einem t-Test p-Wert <0,001 nach 3 Monaten Fingolimod-Therapie im Vergleich zu Therapiebeginn differentiell exprimiert waren, sortiert nach Genomposition. Für 8 der entsprechenden Genorte sind zwar Gentranskripte in den Datenbanken annotiert, aber noch keine offiziellen HGNC-Gensymbole festgelegt worden (---). Die Angabe des Genorts bezieht sich auf das humane Referenzgenom GRCh37/hg19. Die Gene sind entweder auf dem Plusstrang (+) oder auf dem Minusstrang (-) des DNA-Doppelstrangs lokalisiert.

5. Manuskripte

5.1. Manuskript 1

Friess J, Hecker M, Roch L, Koczan D, Fitzner B, Angerer IC, Schröder I, Flechtner K, Thiesen HJ, Winkelmann A, Zettl UK.

Fingolimod alters the transcriptome profile of circulating CD4+ cells in multiple sclerosis.

Sci Rep. 2017 Feb 3;7:42087. doi:10.1038/srep42087.

Journal Impact Factor 2019: 3,998

www.nature.com/scientificreports

OPEN

Fingolimod alters the transcriptome profile of circulating CD4+ cells in multiple sclerosis

Received: 26 August 2016
Accepted: 04 January 2017
Published: 03 February 2017

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Multiple sclerosis is a demyelinating disease affecting the central nervous system. T cells are known to contribute to this immune-mediated condition. Fingolimod modulates sphingosine-1-phosphate receptors, thereby preventing the egress of lymphocytes, especially CCR7-expressing CD8+ and CD4+ T cells, from lymphoid tissues. Using Affymetrix Human Transcriptome Arrays (HTA 2.0), we performed a transcriptome profiling analysis of CD4+ cells obtained from the peripheral blood of patients with highly active relapsing-remitting multiple sclerosis. The samples were drawn before the first administration of fingolimod as well as 24 hours and 3 months after the start of therapy. Three months after treatment initiation, 890 genes were found to be differentially expressed with fold-change >2.0 and t-test p-value < 0.001, among them several microRNA precursors. A subset of 272 genes were expressed at lower levels, including CCR7 as expected, while 618 genes showed an increase in expression, e.g., CCR2, CX3CR1, CD39, CD58 as well as LYN, PAK1 and TLR2. To conclude, we studied the gene expression of CD4+ cells to evaluate the effects of fingolimod treatment, and we identified 890 genes to be altered in expression after continuous drug administration. T helper cells circulating in the blood during fingolimod therapy present a distinct gene expression signature.

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) affecting more than 2.3 million people worldwide. It is a common cause of chronic neurological disability in young adults[1,2]. Disease onset is typically between 20 and 40 years of age, with a prevalence 3 times higher for women than for men. The majority of patients (~85%) have the relapsing-remitting form of MS (RRMS), which is characterised by neurological flares (relapses) followed by periods of stability (remission)[3,4].

Genetic and environmental factors are known to contribute to the development of MS[5,6]. The pathogenesis of MS includes inflammatory and neurodegenerative mechanisms presumably driven by the migration of autoreactive lymphocytes across the blood-brain barrier (BBB)[7–9]. A dysregulated adaptive immune response by T cells is thought to play a role in MS, leading to demyelination and axonal injury within the CNS. In particular, CD4+ T helper 1 cells (Th1) and CD4+ T helper 17 cells (Th17), which differentiate from naive CD4+ T cells in the presence of the cytokines IL-6, IL-23 and TGF-beta[10,11], were shown to promote neuronal damage and BBB disruption[12,13]. Recent studies suggest that microRNA (miRNA) are implicated in T helper cell differentiation[14]. MicroRNA are small non-coding RNA regulating gene expression by binding to mRNA targets, which results in translational inhibition and/or mRNA degradation[15]. There is also growing evidence that miRNA are involved in the pathogenesis of autoimmune diseases, and some miRNA are discussed as biomarkers for MS[16,17].

Several disease-modifying therapies with proven clinical benefits are available for the treatment of RRMS. They allow to reduce the rate and severity of relapses and the number of new brain lesions seen in magnetic resonance imaging (MRI)[18,19]. The first approved oral medication for highly active RRMS was fingolimod, a sphingosine-1-phosphate (S1P) receptor modulator[20–22]. Fingolimod has been demonstrated to reduce clinical and MRI disease activity in patients with RRMS, but adverse effects, including a temporary decrease in the heart rate after initial administration, have been reported as well. S1P is a bioactive metabolite formed from

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Patient	Gender	Age in years	Disease duration in years	Previous treatment	Treatment gap in months	EDSS at baseline	EDSS after 12 months	Relapses during 12 months prior to fingolimod	Relapses during 12-month follow-up
Pat01	female	43	20	IFN-beta-1a sc	38	4.0	3.0	1	0
Pat02	female	26	3	IFN-beta-1b sc	1	1.5	2.0	2	0
Pat03	female	29	5	glatiramer acetate	<1	2.5	1.5	3	0
Pat04	female	43	1	IFN-beta-1a im	1	4.0	3.5	2	0
Pat05	female	45	12	IFN-beta-1a im	2	3.0	3.0	3	0
Pat06	female	33	2	glatiramer acetate	<1	2.5	5.5	2	2
Pat07	female	46	9	IFN-beta-1b sc	<1	4.0	4.0	1	0
Pat08	male	33	4	IFN-beta-1a sc	<1	3.0	6.0	1	3
Pat09	male	46	15	IFN-beta-1b sc	<1	3.5	2.0	1	0
Pat10	male	37	9	glatiramer acetate	1	5.5	4.0	1	0
Median (range)		40 (26–46)	7 (1–20)		1 (0–38)	3.3 (1.5–5.5)	3.3 (1.5–6.0)	1.5 (1–3)	0 (0–3)

Table 1. Clinical and demographic data of the 10 patients with MS. The table provides gender, age at study onset, disease duration and previous treatment for each patient selected for the transcriptome profiling. The time span between the last injection of the previous immunomodulatory treatment and the first oral administration of fingolimod is given as well (treatment gap). Additionally, the EDSS and the number of relapses before the start of fingolimod therapy (baseline) as well as after a clinical follow-up period of one year are given. EDSS: Expanded Disability Status Scale, IFN: interferon, im: intramuscular, sc: subcutaneous.

sphingosine. It functions as a major regulator of immune cell trafficking[23]. As a structural analogue of natural sphingosine, fingolimod can be phosphorylated to produce fingolimod-phosphate, which binds to S1P receptors expressed on lymphocytes. Whereas S1P binding results in internalisation and recycling of the S1PR1 receptor (S1P1), phosphorylated fingolimod causes prolonged internalisation and degradation, thus acting as a functional antagonist[24,25]. Therefore, fingolimod affects the competing chemotactic signalling in secondary lymphoid organs of egress-promoting S1P receptors and homing receptors such as CCR7. In the absence of S1P1, CCR7+ lymphocytes are unable to override the retention signals in lymphoid tissues[23].

As a consequence, patients treated with fingolimod show a reduction in peripheral lymphocyte counts[26]. After 12 months of therapy, CD19+ B cells are reduced from ~6% to <2% and CD4+ T cells are reduced from ~32% to <6% within the total lymphocyte population[27]. Regarding CD4+ T cells, the absolute counts of naive T cells, central memory T cells (TCM), effector memory T cells (TEM) and regulatory T cells (Treg) were all found to be decreased in peripheral blood during therapy[28]. However, the effects vary considerably for the different cell subpopulations. While the egress of CCR7+ naive T cells and CCR7+ TCM from lymph nodes is strongly inhibited by fingolimod, CCR7- TEM are generally spared[29,30]. Moreover, CD4+ T cell subsets producing pro-inflammatory cytokines (IFN-gamma and IL-17) are significantly reduced in response to fingolimod treatment, while the frequency of circulating Treg is increased[31,32]. By this means, fingolimod is thought to prevent the infiltration of the CNS by autoaggressive T cells.

The aim of the present study was the evaluation of differential gene expression in response to fingolimod in CD4+ cells obtained from blood samples of RRMS patients in order to achieve a better understanding of MS and the drug's molecular mechanisms of action. For this purpose, a longitudinal gene expression profiling was performed in the course of fingolimod therapy. Using high-resolution Affymetrix HTA 2.0 microarrays, it was not only possible to quantify all human protein-coding transcripts at the exon level, but also to measure the levels of non-coding transcripts, including precursors of mature miRNA.

Methods

Study Population. Ten patients of Western European descent, aged between 26 and 46 years, were asked to participate in this study at the Department of Neurology, University of Rostock. Among them, seven were females and three were males. The patients were diagnosed with highly active RRMS according to the revised McDonald criteria from 2010[33]. Routine medical care was provided to all patients. They were treated and monitored according to the European Medicines Agency labels, following the consensus treatment guidelines and recommendations of the German Society of Neurology. Prior to the study, three of the patients received glatiramer acetate (GA), while seven received interferon-beta (IFN-beta). All patients showed relapses despite immunomodulatory treatment. Therefore, they switched to fingolimod treatment at standard dose of 0.5 mg once daily. Most patients (n = 8) were prescribed fingolimod within one month or less after discontinuing their previous medication. At the start of fingolimod therapy, the patients had a median disease duration of 7 years (range, 1 to 20). On the Expanded Disability Status Scale (EDSS), a measure of functional disability in MS[34], they reached scores between 1.5 and 5.5 (Table 1).

As part of our research on MS, this study was approved by the University of Rostock's ethics committee and carried out according to the Declaration of Helsinki. All patients gave written informed consent to participate in this study before the first blood sampling.

CD4+ Cell Isolation and RNA Extraction. Blood samples were obtained for the longitudinal analysis of the patients' CD4+ cell transcriptome. A total of 20 ml peripheral venous blood was collected from each of the 10 RRMS patients into EDTA (ethylenediaminetetraacetic acid) blood tubes at three different time points: before the

first application of fingolimod (baseline), after 24 hours (before the second application) as well as after 3 months of treatment.

The blood samples were magnetically labelled with Whole Blood CD4 MicroBeads (Miltenyi Biotec, Germany) in order to isolate cells expressing the surface molecule CD4, which is primarily found on T helper cells but also on a subset of monocytes. The CD4+ cells were magnetically separated (positive selection) according to the manufacturer's instructions using the autoMACS separator (Miltenyi Biotec). The unlabelled fraction was rejected. After addition of lysis buffer, the samples were stored until further use at −20 °C.

Total RNA was extracted using the mirVana isolation kit (Ambion, TX, USA). RNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, DE, USA). RNA integrity numbers (RIN) as estimates of the quality of RNA[35] were determined with an Agilent 2100 Bioanalyzer using RNA 6000 Pico LabChips (Agilent Technologies, CA, USA).

Microarray Hybridisation and Data Preprocessing. The cellular RNA was analysed with Human Transcriptome Arrays (HTA) 2.0, the latest generation of Affymetrix microarrays, which contain more than six million distinct oligonucleotide probes (25 bases in length) covering >67,500 coding and non-coding transcripts[36]. Approximately 70 percent of the probes on these high-resolution GeneChip microarrays match to the exons of protein-coding transcripts and the remaining 30 percent match to exon-exon splice junctions and non-coding transcripts according to the current gene annotation. The probes can be summarised into gene level, exon level and splice junction probe sets. HTA 2.0 microarrays are designed with 10 probes for each unique exon fragment (PSR) and 4 probes per exon-exon splice junction (JUC). This high coverage provides deep insights into basically all human transcripts[36].

From each of the 30 samples, cRNA was prepared from 70 ng total RNA according to the Affymetrix Whole Transcript (WT) protocol. The cRNA was used to generate single-stranded DNA, which was fragmented and biotinylated. The labelled single-stranded DNA was hybridised for 16 hours at 45 °C on the Affymetrix HTA 2.0 microarrays following the manufacturer's protocol. Subsequently, the microarrays were washed and stained with a streptavidin-phycoerythrin conjugate in an Affymetrix Fluidics Station 450. Signal amplification with antibodies was applied. The microarrays were scanned with a GeneChip Scanner 3000 7G (Affymetrix, CA, USA). Raw data were extracted from the scanned images using the Affymetrix GeneChip Command Console (AGCC) software version 4.0.

Preprocessing and quality control of the microarray data were done via the Expression Console 1.3.1 software (Affymetrix). For passing the quality control, signal intensities of hybridisation controls had to be within bounds. The robust multi-array average (RMA) algorithm[37] was used with default parameters to perform background correction, quantile normalisation and log2 transformation of the raw fluorescence intensity values. Probe set summarisation was realised by the software according to two standardised workflows. For the "gene level analysis", each probe set contains all probes matching a single gene (transcript cluster; TC). For the "exon level analysis", each probe set matches a single exon fragment or splice junction. Each resulting expression profile consisted of 70,523 gene level probe sets and 914,585 exon level probe sets. The gene level profiles included 44,699 probe sets for coding transcripts, 22,829 probe sets for non-coding transcripts and 2,995 other probe sets (e.g., hybridisation controls). The raw and preprocessed HTA 2.0 microarray data are publicly available at NCBI's Gene Expression Omnibus (GEO) database (accession numbers GSE73079 and GSE73080).

Filtering of Differential Expressed Genes. The gene level profiles of the samples from the 10 patients at the 3 time points were analysed for differentially expressed genes during fingolimod treatment using the Affymetrix Transcriptome Analysis Console 1.0 (TAC) software. The filtering was done by comparing the gene expression levels immediately before the initiation of fingolimod therapy with those after 24 hours and after 3 months. Fold-changes (FC) and t-test p-values were calculated to determine significantly differentially expressed genes. A p-value threshold of 0.001 was used, which is more stringent than the default value of 0.05 in TAC. For FC, a cut-off at ±2.0 was chosen. The FC is the ratio of the gene expression at baseline and during therapy. It is calculated by TAC on the expression values in linear scale. Accordingly, a FC of −2.0 corresponds to RNA levels reduced by 50% and a positive FC of +2.0 corresponds to RNA levels increased by 100% in response to therapy.

Analysis of Gene Functions. We examined to what extent genes that were found to be differentially expressed during therapy are involved in specific biological processes. For this purpose, we performed a gene set enrichment analysis using functional categories of the database Gene Ontology (GO). We used GOstats[38], a R/Bioconducter software package, to calculate p-values and odds ratios (OR) for overrepresentation of GO gene sets in the list of differentially expressed genes. As a reference gene list, we used all measured genes that were expressed by CD4+ cells. Genes with an expression value <4 for all samples were not considered to be expressed, eliminating 26,268 of the 70,523 gene level probe sets from this analysis. To confine the result to specific gene groups, only GO terms of the categories "biological process" and "molecular function" with less than 200 members in the reference list (size) were tested. Moreover, GO terms comprising less than 4 differentially expressed genes (count) and GO terms with OR <7 were discarded.

S1P Pathway Analysis. Another objective was the study of molecular interactions within the S1P-associated pathways. Therefore, we constructed a model of the S1P pathway, which we have described in more detail elsewhere[39], to investigate the extent to which pathway components are modulated at the transcript level in response to therapy. Briefly, genes involved in S1P signalling were gathered from 11 review articles to compile a consensus pathway. This pathway includes the synthesis of natural S1P and the conversion of fingolimod as well as the interactions with S1P receptors. Moreover, the chemokine receptor CCR7, which plays a critical role for the homing of lymphocytes, was integrated. The S1P pathway was then visualised with the open source software Cytoscape

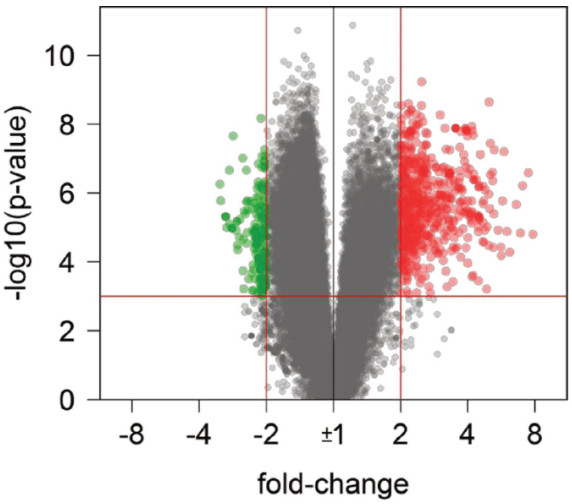


Figure 1. Volcano plot of gene expression changes. The transcriptome of circulating CD4+ cells from RRMS patients receiving fingolimod therapy for 3 months was compared to baseline. The x-axis specifies the fold-changes (FC) and the y-axis specifies the negative logarithm to the base 10 of the t-test p-values. Red vertical and horizontal lines reflect the filtering criteria (FC = ±2.0 and p-value = 0.001). FC > +2.0 indicates transcript levels increased by >100%, whereas FC < −2.0 indicates transcript levels reduced by >50%. Red and green dots represent probe sets for transcripts expressed at significantly higher (n = 618) or lower (n = 272) levels during therapy, respectively.

3.1.0[40], where nodes (e.g., genes) and edges (e.g., interactions) are the building blocks of the molecular network. Changes in CD4+ cell expression after 3 months of fingolimod treatment compared to baseline were highlighted in colour in the pathway.

Analysis of Alternative Splicing. Research has shown that the tens of thousands of human genes produce more than a hundred of thousands of different transcript isoforms. For instance, exons of a gene may be included within or excluded from the final mRNA (exon skipping). A major advantage of the high-resolution design of Affymetrix HTA 2.0 microarrays is that measuring and analysing transcript isoforms has been made possible. When analysing the data of the >6 million oligonucleotide probes with the exon level workflow, 914,585 probe sets (PSR and JUC) can be distinguished. This facilitates the examination of different transcriptional variants. If all the exons of a certain alternative splice variant have high signal intensities, we can infer that this transcript variant is expressed. As an example, we visualised the baseline data of patient Pat07 for the gene CX3CR1 at the exon level.

Results

Patients and Sample Information. Most RRMS patients showed a benefit from fingolimod treatment. Within a clinical follow-up period of one year, only two patients (Pat06 and Pat08) still had relapses. These two patients also showed a rapid disease progression in the first 12 months, whereas the remaining patients did not show a marked worsening of disability according to the EDSS (Table 1). Most patients continuously received fingolimod treatment during the one-year follow-up. Only Pat08 discontinued the therapy after 8 months and switched to alemtuzumab after 13 months.

The number of lymphocytes circulating in the blood of the patients decreased significantly after the start of fingolimod treatment. After 24 hours and 3 months, the numbers of separated CD4+ cells were reduced, on average, by >20% and >90%, respectively. The quality of cellular RNA was assessed by Bioanalyzer and estimated as RIN. The average RIN of all 30 samples obtained before and during fingolimod therapy was 9.3. Therefore, sufficient amounts of high quality RNA were available for the microarray analysis. Positive selection of CD4+ cells from whole blood was confirmed by high mRNA levels of CD4 in all samples (Supplementary Fig. S1).

Transcriptome Dynamics. Based on the data of the 70,523 gene level probe sets (transcript clusters), we searched for genes with significant expression changes in response to fingolimod therapy. None of the genes matched the filtering criteria (p-value < 0.001 and FC > + 2.0 or < −2.0) for the time point comparison of 24 hours after first administration of fingolimod versus baseline. In contrast, substantial transcriptome changes were seen after 3 months (Fig. 1). In total, 890 genes were filtered to be expressed at significantly higher levels (n = 618) or lower levels (n = 272) (Table 2, Supplementary Table S1). All of them had a false discovery rate <0.05 when correcting for multiple testing, and 651 genes were labelled as protein-coding and 239 genes were labelled as non-coding (Fig. 2).

Probe set	Symbol	Entrez	Location	Probes	P-value	Fold-change
TC07001546.hg.1	FGL2	10875	chr7 (q11.23)	30	1.47E-05	6.896
TC11003451.hg.1	MS4A7	58475	chr11 (q12.2)	145	2.20E-06	6.610
TC0X000171.hg.1	CYBB	1536	chrX (p11.4)	168	5.26E-05	6.183
TC12003207.hg.1	CLEC12A	160364	chr12 (p13.2)	137	1.34E-05	6.016
TC12000591.hg.1	IRAK3	11213	chr12 (q14.3)	183	1.72E-06	5.731
TC12001796.hg.1	DUSP6	1848	chr12 (q21.33)	139	6.67E-05	5.665
TC01001346.hg.1	MNDA	4332	chr1 (q23.1)	103	3.77E-05	5.650
TC12001215.hg.1	CLEC7A	64581	chr12 (p13.2)	208	1.21E-05	5.474
TC02000398.hg.1	PLEK	5341	chr2 (p13.3)	140	5.60E-08	5.268
TC12000309.hg.1	LRRK2	120892	chr12 (q12)	649	4.08E-06	5.240
TC11001839.hg.1	MS4A6A	64231	chr11 (q12.2)	233	4.46E-07	5.176
TC08000383.hg.1	LYN	4067	chr8 (q12.1)	240	2.54E-07	5.160
TC17000103.hg.1	CD68	968	chr17 (p13.1)	110	2.98E-07	5.079

Table 2. Top genes expressed at higher mRNA levels in response to fingolimod. Gene expression in peripheral blood CD4+ cells was analysed in RRMS patients receiving fingolimod therapy. After 3 months of treatment, 890 genes were identified to be differentially expressed relative to pre-treatment levels (Supplementary Table S1). A subset of 13 probe sets representing protein-coding genes was filtered even with fold-change > + 5.0. This table gives the Affymetrix HTA 2.0 transcript cluster (TC) probe set identifier, the official gene symbol, the Entrez database identifier, the genomic location, the number of 25mer oligonucleotide probes for each probe set as well as t-test p-values and fold-changes.

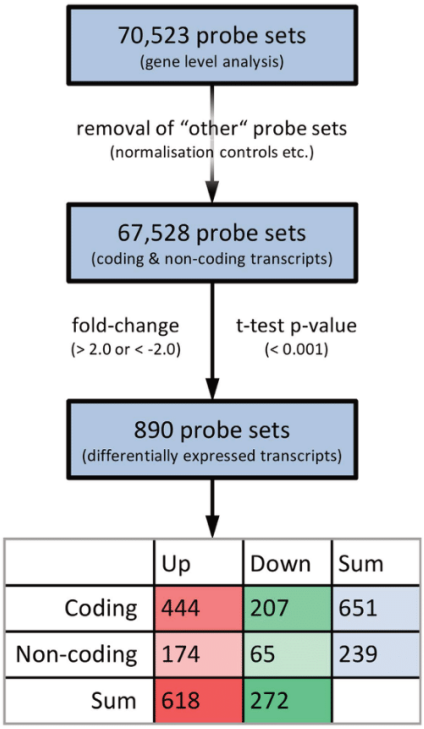
The gene CCR7 (FC = −2.05), for example, was found to be expressed at lower levels and the genes CD58 (FC = 2.01), CX3CR1 (FC = 3.39), CCR1 (FC = 2.84) and CCR2 (FC = 2.36) were found to be expressed at higher levels in response to therapy in all of the 10 individual patients (Fig. 3). Moreover, several major histocompatibility complex (MHC) genes were significantly increased in expression after 3 months compared to pre-treatment levels, including HLA-DRA (FC = 4.70) and HLA-DRB1 (FC = 3.71). There were also 12 differentially expressed precursor miRNA (Table 3), including hsa-mir-216b (FC = −2.09), hsa-mir-142 (FC = 2.20) and hsa-mir-548c (FC = 2.73). Probe sets for these precursor miRNA contain oligonucleotide probes matching only to the sequence of the short stem-loop formed by the primary miRNA transcript. Interestingly, both the precursor miRNA hsa-mir-4668 (FC = 2.05) as well as the respective primary miRNA, the host gene UGCG (FC = 2.16), were identified to be expressed at increased levels during therapy.

Associations to Gene Functional Categories. A GO term enrichment analysis was performed to functionally characterise the genes, which were identified to be differentially expressed in the course of therapy. For the two major GO categories "biological process" and "molecular function", the top 10 GO terms associated to the filtered genes with OR >7 were determined. Overlapping and distinct gene sets were found to be significantly overrepresented (Table 4, Supplementary Table S1).

Twenty differentially expressed genes were assigned to the GO term "cytokine secretion" (GO:0050663, p-value = 1.50E-15, OR = 14.64). This GO term contains genes, which are related to processes that modulate the release of cytokines from cells. Of these 20 genes, CCR7 was lowered in expression, and the remaining 19 genes, including CSF1R, LYN, S100A12 and SYK, were elevated in expression during fingolimod treatment. A subset of 17 of these genes also belongs to the GO terms "protein secretion" (GO:0009306) and "regulation of cytokine secretion" (GO:0050707). The GO term "positive regulation of lymphocyte activation" (GO:0051251, p-value = 8.16E-13, OR = 7.18) contained 25 genes, e.g., LYN, PAK1 and TNFSF13B, which all showed enhanced expression in response to therapy.

The GO terms of the "molecular function" category "MHC class II receptor activity" (GO:0032395, p-value = 2.64E-11, OR = 89.81) and "IgG binding" (GO:0019864, p-value = 2.34E-08, OR = 66.86) were also highly enriched. From the list of differentially expressed genes, 8 human leukocyte antigen (HLA) class II genes and 6 Fc receptor genes (e.g., FCGR2A-C) were associated to these two gene sets. All of these genes were elevated in expression after 3 months of treatment relative to baseline. Similar and overlapping GO terms were identified to be significantly overrepresented (e.g., "IgG binding" and "immunoglobulin binding") due to the hierarchical relationships in the GO database structure.

Effects within the S1P Pathway. Fingolimod is phosphorylated by sphingosine kinase 2 (SPHK2) to fingolimod-phosphate, which acts as an agonist on S1P receptors, especially S1P1. Binding of phosphorylated fingolimod to S1P1 results in internalisation of this receptor and loss of its signalling functions, leading to sequestration of lymphocytes in lymph nodes. The S1P-associated pathways are characterised by the interplay of numerous protein interactions and enzyme activities. The molecular network shown in Fig. 4 is a compilation of the physiological pathway for S1P synthesis as well as the therapeutic pathway with fingolimod[39]. Also shown is the competition between CCR7 and S1P receptor signalling, which have different chemotactic consequences. CCR7 engagement by its ligands, the chemokines CCL19 and CCL21, mediates the homing of naive T cells and TCM to lymph nodes, and the binding of S1P to S1P1 overrides this retention signal to promote lymphocyte egress. We analysed the changes in expression of the coding genes participating in the S1P pathway (Fig. 4).



	Up	Down	Sum
Coding	444	207	651
Non-coding	174	65	239
Sum	618	272	

Figure 2. Identification of differentially expressed genes in CD4+ cells. In total, 890 probe sets were filtered when comparing the transcript levels after 3 months of therapy with the pre-treatment levels. This figure shows the number of all gene level probe sets (top), the number of probe sets after removal of normalisation controls (middle) and the number of probe sets for differentially expressed transcripts satisfying t-test p-value < 0.001 and fold-change $> +2.0$ or < -2.0 (bottom). The table is giving the numbers of up-regulated (green) or down-regulated (red) and coding or non-coding transcripts.

Certain components were expressed at higher levels (FC $> +2.0$) three months after the start of fingolimod therapy (PI3K, PLC and ceramidase ASAH1). Rho kinases were notably elevated in expression as well (FC $= 1.58$ and FC $= 1.87$ for ROCK1 and ROCK2, respectively), whereas CCR7 was less abundant (FC $= -2.05$) relative to pre-treatment transcript levels. These changes reflect shifts in CD4+ cell subpopulations as well as regulatory effects, e.g., due to intracellular signalling and feedback mechanisms.

Alternative Transcript Variants of CX3CR1. To demonstrate the high coverage of the HTA 2.0 microarrays used in this study, we visualised the CD4+ cell gene expression data of an exemplary gene, CX3CR1, at exon level. This gene is encoded on chromosome 3. Five transcript variants of this gene are known to result from alternative transcription start sites. Figure 5 shows the signal intensities summarised from probes matching to distinct exon fragments and exon-exon splice junctions. The shorter transcript isoforms of CX3CR1 were found to be expressed at higher levels in comparison to the longest isoform.

Discussion

To our knowledge, this is the first study investigating the transcriptome changes in response to fingolimod therapy in peripheral blood CD4+ cells of RRMS patients. For this purpose, we used high-resolution, high-coverage Affymetrix HTA 2.0 microarrays with >6 million probes for protein-coding and non-coding genes. Fingolimod administrated once daily reduces peripheral lymphocyte counts within a few hours after the first dose, but this initial effect is transient and normal values appear after one day[41,42]. Homing of lymphocytes to lymph nodes over longer periods is established only after continued dosing of fingolimod. Over a two-week period, the lymphocyte counts continue to decrease, reaching a nadir at 20–30% of baseline values[21]. This correlates with the transcriptome dynamics as changes in expression were seen in our study only after 3 months. No gene matched our filtering criteria after 24 hours, but after 3 months of treatment, we identified 890 genes to be significantly differentially expressed (Fig. 1, Supplementary Table S1). The majority of the genes (n = 618) were expressed at higher transcript levels during therapy.

As discussed in the following, we defined a comprehensive signature of therapy-responsive genes in circulating CD4+ cells and detected gene expression changes consistent with previous studies in the literature. For instance, as expected, the chemokine receptor CCR7 was significantly reduced in expression after 3 months of

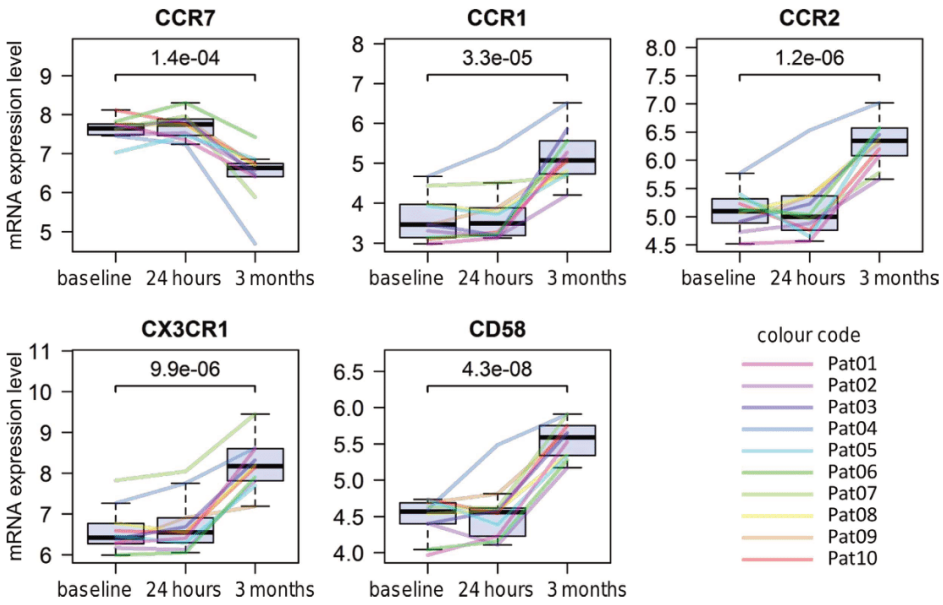


Figure 3. Longitudinal time course profiles of 5 selected genes. The figures show the individual gene expression levels at baseline as well as 24 hours and 3 months after fingolimod treatment initiation. Expression values are given in log2 scale as RMA normalised probe set summarised signal intensities. Different colours highlight the data for each of the 10 RRMS patients in the study. While CCR7 mRNA levels were reduced in CD4+ cells in response to fingolimod therapy, an elevated expression was measured for the other genes shown. The p-values were calculated by t-test.

Probe set	Symbol	Entrez	Location	Probes	P-value	Fold-change
TC17000728.hg.1	hsa-mir-21	406991	chr17 (q23.1)	30	4.18E-07	4.376
TC17001729.hg.1	hsa-mir-142	406934	chr17 (q22)	30	1.18E-05	2.204
TC02001872.hg.1	hsa-mir-216b	100126319	chr2 (p16.1)	30	8.72E-08	−2.093
TC12000573.hg.1	hsa-mir-548c	693129	chr12 (q14.2)	30	7.24E-05	2.731
TC07000452.hg.1	hsa-mir-590	693175	chr7 (q11.23)	30	4.21E-05	2.641
TC10000348.hg.1	hsa-mir-605	693190	chr10 (q21.1)	30	8.41E-07	−2.058
TC20000241.hg.1	hsa-mir-644a	693229	chr20 (q11.22)	30	7.74E-08	3.846
TC04001648.hg.1	hsa-mir-3140	100422896	chr4 (q31.3)	30	2.25E-05	2.083
TC10000815.hg.1	hsa-mir-4295	100422909	chr10 (q25.2)	30	1.08E-05	2.037
TC02000369.hg.1	hsa-mir-4434	100616419	chr2 (p14)	10	1.24E-04	2.259
TC05000673.hg.1	hsa-mir-4461	100616209	chr5 (q31.1)	30	2.13E-04	−2.055
TC09000561.hg.1	hsa-mir-4668	100616114	chr9 (q31.3)	25	1.27E-05	2.051

Table 3. Differentially expressed precursor microRNA in the CD4+ cell population. This table shows 12 precursor miRNA that were identified as more abundant (n = 9) or less abundant (n = 3) after 3 months of fingolimod treatment in comparison to baseline (p-value < 0.001 and fold-change > +2.0 or <−2.0). For each miRNA stem-loop, the table provides the miRBase database symbol, the Entrez database identifier, the genomic location, the Affymetrix HTA 2.0 probe set and the number of probes on the microarrays that were used to quantify the expression.

fingolimod therapy (FC = −2.05). CCR7 is responsible for controlling lymph node homing of naive T cells and TCM, including the Th17 population[29,43]. Fingolimod leads to the retention of these CCR7+ T cells in lymph nodes by prolonged internalisation of S1P receptors. CCR7- TEM, however, preferentially keep circulating in the peripheral blood[44,45].

Many fingolimod-responsive genes identified in this study are immune-related genes, including also the chemokine receptors CX3CR1 (FC = 3.39) and CCR2 (FC = 2.36) (Figs 3 and 5). CX3CR1 is a transmembrane receptor involved in the recruitment of leukocytes to peripheral tissues and lymphoid organs[46]. Its binding chemokine CX3CL1 is implicated in the pathogenesis of several diseases such as rheumatoid arthritis[47]. Increased CX3CL1 levels in the cerebrospinal fluid and a higher percentage of CX3CR1-expressing CD4+ T cells in the

GO accession	GO term description	Count	Size	P-value	OR
Biological processes					
GO:0002429	immune response-activating cell surface receptor signaling pathway	28	183	6.14E-16	8.68
GO:0050663	cytokine secretion	20	84	1.50E-15	14.64
GO:0050707	regulation of cytokine secretion	17	70	1.49E-13	14.87
GO:0009306	protein secretion	23	152	3.49E-13	8.41
GO:0051251	positive regulation of lymphocyte activation	25	190	8.16E-13	7.18
GO:0060333	interferon-gamma-mediated signaling pathway	16	72	3.48E-12	13.19
GO:0071346	cellular response to interferon-gamma	17	89	9.80E-12	10.92
GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	16	79	1.58E-11	11.71
GO:0034341	response to interferon-gamma	18	105	1.61E-11	9.60
GO:0030595	leukocyte chemotaxis	18	106	1.91E-11	9.49
Molecular functions					
GO:0032395	MHC class II receptor activity	8	12	2.64E-11	89.81
GO:0019864	IgG binding	6	10	2.34E-08	66.86
GO:0019865	immunoglobulin binding	7	17	4.23E-08	31.30
GO:0004896	cytokine receptor activity	11	65	1.80E-07	9.21
GO:0004982	N-formyl peptide receptor activity	5	9	6.39E-07	55.51
GO:0050664	oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor	5	13	6.06E-06	27.74
GO:0008329	signaling pattern recognition receptor activity	5	14	9.25E-06	24.66
GO:0038187	pattern recognition receptor activity	5	14	9.25E-06	24.66
GO:0003823	antigen binding	7	47	7.69E-05	7.81
GO:0019955	cytokine binding	7	51	1.31E-04	7.09

Table 4. Gene set enrichment analysis of differentially expressed genes. Listed are functional groups associated to the genes differentially expressed in peripheral blood CD4+ cells of MS patients during fingolimod treatment. The analysis was done using the GOstats R/Bioconductor software package and Gene Ontology (GO) terms of the categories "biological process" and "molecular function". Only the top 10 GO terms according to the p-value with odds ratio >7, count >4 and size <200 are shown per category. For instance, the GO term "cytokine secretion" (GO:0050663) was found to be overrepresented. In total, 84 expressed genes belonged to this term (size). Of those, more genes than expected by chance (count = 20) corresponded to the 890 filtered probe sets. According to odds ratio (OR = 14.64) and p-value (1.50E-15), "cytokine secretion" genes are thus enriched in the list of genes, which are expressed at significantly higher or lower transcript levels after 3 months of fingolimod therapy.

blood were found in RRMS patients in comparison to healthy controls[48]. Peripheral CX3CR1+ CD4+ cells have been described to produce GZMA and PRF1[47]. CX3CR1 and GZMA (FC = 3.20) both contribute to the transendothelial migration of CD4+ TEM[49,50]. The relative increase of TEM in the blood during fingolimod therapy[28,30] likely explains the higher mRNA levels of these two genes in our data. CCR2 is also known to be required for lymphocyte migration and monocyte chemotaxis during inflammatory conditions[51]. It is a surface marker of long-term CD4+ TEM prepared for mounting rapid recall responses[52]. Recent studies postulate CCR2 inhibition in MS as a therapeutic approach, but there is also evidence for protective effects of CCR2 during CNS inflammation[51]. Yopp *et al.* demonstrated that fingolimod impacts CCR2-driven migration by activating ABC transporters and ALOX5[53]. In our data, both CCR2 and ALOX5 (FC = 2.32) showed increased transcript levels during fingolimod therapy. Future studies may investigate the protein levels of these genes for confirmation.

We found that several toll-like receptor (TLR) genes (e.g., TLR1, TLR2 and TLR4) were up-regulated in expression in CD4+ cells after 3 months of fingolimod treatment. TLRs play a crucial role in the immune response to pathogen-associated and damage-associated molecular patterns, leading to the activation of specific transcription factors[54,55]. As reviewed by Jin *et al.*, TLR agonists exert diverse direct effects on T cells[56]. TLR2 (FC = 4.17) has been discussed to modulate the suppressive activity of Treg[57,58]. TLR2 stimulation also promotes the proliferation and IL-17 production of Th17 cells[59], which in turn have been linked to the pathogenesis of MS and other autoimmunity disorders[10]. Notably, loss of TLR4 (FC = 2.94) in CD4+ T cells was found to abrogate disease symptoms in the animal model of MS, mainly through blunted Th17 and Th1 responses[60]. The precise mechanisms governing T cell survival following TLR4 stimulation thus deserve further investigation.

Another gene that presented increased mRNA levels in blood CD4+ cells of fingolimod-treated patients was CD58 (LFA-3) (FC = 2.01), a cell adhesion molecule that binds to CD2 (LFA-2). This interaction provides a co-stimulatory signal for T cell activation[61], which is controlled by epigenetic changes at the chromatin level[62]. Elevated expression of CD58 in T cells was also documented after one month of natalizumab treatment[63]. The expression of CD58 was found to be reduced in the cerebrospinal fluid of MS patients compared to controls[64]. Moreover, previous studies reported single nucleotide polymorphisms (SNPs) in the CD58 gene locus to be associated with the risk of MS[65]. These SNPs are suspected to affect the processing of the miRNA hsa-mir-548ac, which

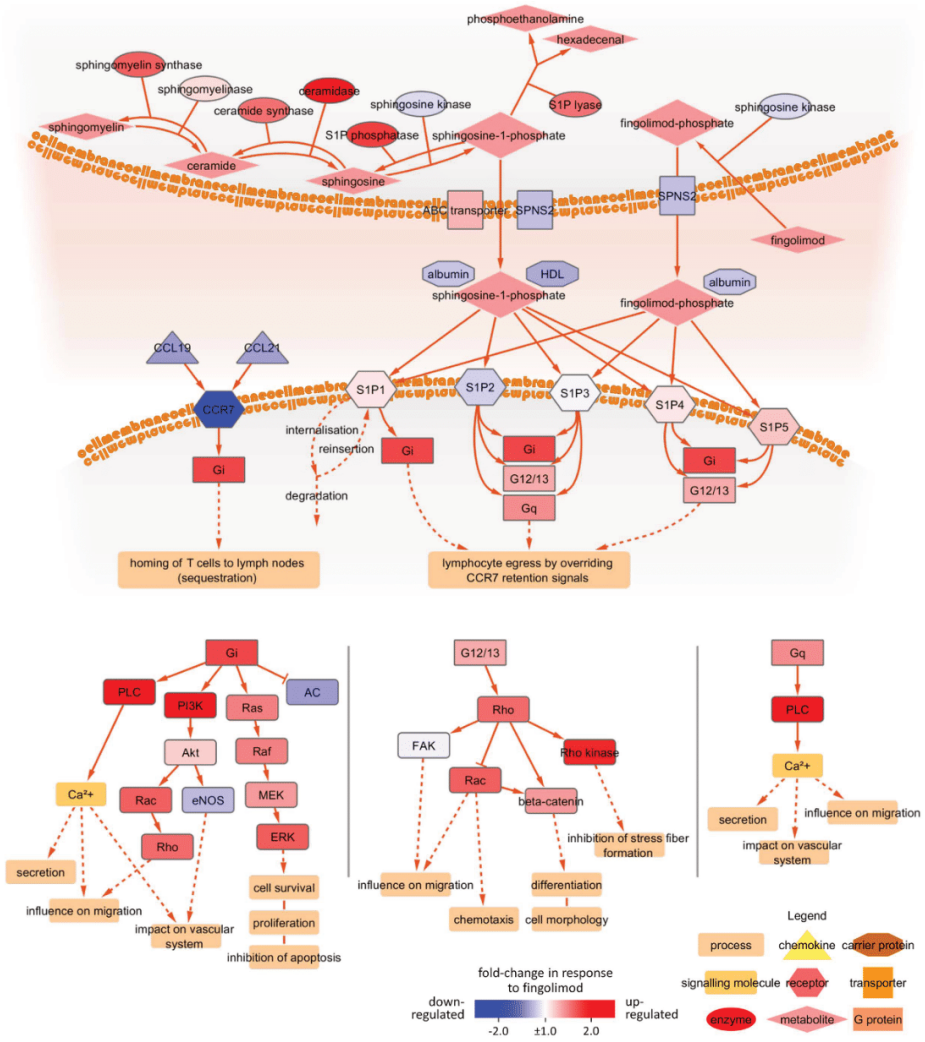


Figure 4. The molecular pathway modulated by fingolimod. Sphingosine-1-phosphate (S1P), a metabolite formed from sphingosine, is a major regulator of lymphocyte trafficking. As a structural analogue of sphingosine, fingolimod can be phosphorylated to fingolimod-phosphate, which binds to S1P receptors expressed by lymphocytes. However, while S1P leads to short-term internalisation of the S1P1 receptor, followed by reinsertion into the cell membrane, fingolimod-phosphate initiates a long-term internalisation, which leads to degradation of S1P1. This alters the chemotactic events in secondary lymphoid tissues. The S1P receptors usually associate with G proteins, which regulate a range of biological processes as presented in the three cascades in the lower part of this figure. Some pathway protein-coding genes were differentially expressed in CD4+ cells after 3 months of fingolimod treatment compared to baseline. The mRNA expression fold-change of each gene in the pathway is visualised in colour with up-regulated genes shown in red and down-regulated genes shown in blue. Different types of proteins (e.g., receptors and enzymes) are highlighted in different shapes.

is located in the first intron of CD58[66]. The stem-loop of hsa-mir-548ac was not represented on the microarrays, but we detected elevated levels of hsa-mir-548c, another member of this miRNA family, after 3 months of therapy.

Altogether, when comparing the levels at baseline with the levels after 3 months of fingolimod treatment, 12 probe sets matching to miRNA stem-loops were filtered (Table 3). The precursor miRNA hsa-mir-4668 (FC = 2.05) and its host gene UGCG (FC = 2.16) were both increased in expression. DLEU2 (FC = 2.59), the host gene of hsa-mir-16–1, was also up-regulated. Prior studies showed hsa-mir-16-1 to be down-regulated in CD4+ cells of MS patients[67] and to be up-regulated during IFN-beta therapy[68]. During GA treatment and in our study during fingolimod treatment, hsa-mir-142 was found to be differentially expressed[69]. These aforementioned miRNA are thus promising biomarkers of disease and therapy[16]. So far there has been no study performed in MS investigating explicitly the expression changes of mature miRNA in blood cells during fingolimod treatment. Our

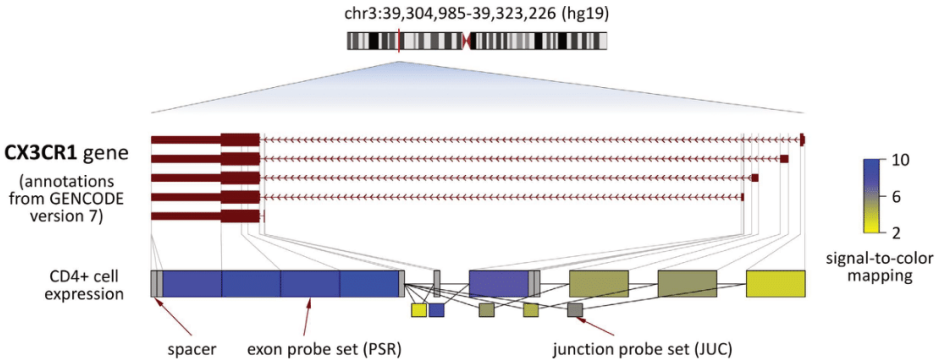


Figure 5. Exon level analysis for the CX3CR1 gene. In the upper part, the exon-intron structures of the five possible CX3CR1 mRNA variants generated by alternative promoter usage are visualised in red. Arrows in introns indicate transcription orientation. Below, the Affymetrix microarray data are visualised for CD4+ cells separated from a patient's blood sample (Pat07) obtained at study onset. For each of the eight exon fragments there were ten 25mer oligonucleotide probes, and for each of the five probe sets for exon-exon splice junctions there were four probes. The processed probe set signals are in log2 scale, and blue colours indicate high expression while yellow colours indicate low expression. Spacers refer to sequences for which no corresponding probe exists. The longest CX3CR1 transcript, which results when the most distal 5′ transcription start site is used, is expressed at much lower levels than shorter variants.

findings require further validation using real-time polymerase chain reaction analyses with higher sensitivity than oligonucleotide-based measurements.

The gene set enrichment analysis (Table 4) revealed genes involved in diverse biological processes such as "cytokine secretion" and "positive regulation of lymphocyte activation" to be significantly enriched in the list of genes differentially expressed in circulating CD4+ cells after 3 months of fingolimod therapy. These functional groups included LYN (FC = 5.16) and PAK1 (FC = 2.67). LYN encodes a Src tyrosine kinase, which is involved in regulating the activation of PI3K and MAPK signalling cascades[70]. The serine/threonine p21-activated kinase encoded by PAK1 links Rho-related GTPases to the MAPK pathway and influences cell adhesion, migration and proliferation[71,72]. PIK3CG (FC = 2.13), MAPK (ERK) and Rho kinases are downstream components in the S1P-associated signalling network (Fig. 4)[39]. Further studies are needed to investigate the precise implications of the molecular interactions of LYN and PAK1 in the context of immunomodulatory treatments for MS.

Most of the genes identified as differentially expressed in response to fingolimod treatment are evidently related to the peripheral shift in the frequencies of CD4+ cell subpopulations as a consequence of the reduced numbers of naive T cells and TCM. On closer inspection, we focused on typical markers for Th1 cells (CCR5 and CXCR3), Th17 cells (CCR6 and CD161) and Treg (CD25 and FOXP3)[73–75]. In our data set, we could not detect significant expression changes for any of those markers within the first 3 months of therapy. However, we found increased ENTPD1 (CD39) (FC = 2.42) transcript levels. This is consistent with a previous study demonstrating that CD4+ T cells from the blood of fingolimod-treated MS patients are enriched in CD39-expressing Treg[76]. It has been reported that CD39+ Treg have suppressive effects on Th17 cells[77]. Our transcriptome data did not show evidence of a preferential reduction in the number of peripheral blood Th17 cells within the CD4+ subset during therapy, which has been controversially discussed in the literature[29,43]. We did not further characterise the isolated CD4+ cells using flow cytometry, but it should be recalled that a subset of monocytes also slightly express CD4[78]. Therefore, not only CD3+ CD4+ T helper cells but also a variable proportion of CD4+ CD14+ monocytes was contained in the isolated cell population (Supplementary Fig. S1). Elevated levels of CD14 mRNA after 3 months of fingolimod therapy (Supplementary Table S1) are thus indicative of an increased frequency of monocytes and a lower level of enrichment of T cells, when only CCR7- TEM are spared in the circulation. This, in part, affected the microarray data of our study. Subsequent studies may combine flow cytometric sorting and subpopulation-specific (or single-cell) transcriptome analysis to better address this issue.

The identification of molecular biomarkers that allow prediction of disease progression may enable physicians to estimate the course of MS, to identify patients more likely to be responsive to a certain treatment and to distinguish risk patients requiring more frequent monitoring. With regard to our study cohort, it is conceivable that the two patients with ongoing disease activity during fingolimod therapy (Pat06 and Pat08, Table 1) may have an altered individual gene expression signature, but our data did not clearly indicate this. Two recent studies suggested that flow cytometric analysis of lymphocyte subpopulations in peripheral blood of MS patients might be useful for predicting clinical response to fingolimod[28,79]. However, larger patient cohorts and longer clinical follow-up periods are needed to define and establish prognostic biomarkers.

In conclusion, 890 genes were identified to be expressed at significantly higher or lower transcript levels in response to fingolimod therapy within the CD4+ immune cell population. The filtered genes comprise genes involved in functional pathways of T cells as well as less characterised genes whose role in the immunology and treatment of MS merits further investigation.

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Acknowledgements

We thank Christa Tiffert for coordinating patient care, Nele Retzlaff for clinical documentation, and Ildikó Tóth and Annegret Zettl for laboratory assistance. Many thanks are also directed to Anne-Kathrin Adloff for supporting our research. The microarray experiments were partly funded by Novartis. The funder had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author Contributions

U.K.Z. designed and coordinated the study. A.W., I.S. and U.K.Z. were responsible for patient care and clinical documentation. B.F., D.K., I.S. and K.F. processed the blood samples and performed the microarray analysis. J.F. and M.H. analysed the data, drafted the paper and prepared all tables and figures. B.F., L.R., I.C.A. and H.J.T. contributed to the interpretation of the results and corrected and improved the paper. All authors read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: M.H. received speaking fees and travel funds from Bayer HealthCare, Biogen, Novartis and Teva. A.W. received speaking fees and travel funds from Bayer HealthCare, Genzyme, Merck Serono and Novartis. U.K.Z. received research support as well as speaking fees and travel funds from Almirall, Bayer HealthCare, Biogen, Merck Serono, Novartis, Sanofi and Teva. J.F., L.R., D.K., B.F., I.C.A., I.S., K.F. and H.J.T. declare that they have no competing interests.

How to cite this article: Friess, J. *et al.* Fingolimod alters the transcriptome profile of circulating CD4+ cells in multiple sclerosis. *Sci. Rep.* **7**, 42087; doi: 10.1038/srep42087 (2017).

5.2. Manuskript 2

Roch L, Hecker M, Friess J, Angerer IC, Koczan D, Fitzner B, Schröder I, Flechtner K, Thiesen HJ, Meister S, Winkelmann A, Zettl UK.

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Mol Neurobiol. 2017 Sep;54(7):5511-5525. doi:10.1007/s12035-016-0075-0.

Journal Impact Factor 2019: 4,500

Mol Neurobiol (2017) 54:5511–5525
DOI 10.1007/s12035-016-0075-0

High-Resolution Expression Profiling of Peripheral Blood CD8$^+$ Cells in Patients with Multiple Sclerosis Displays Fingolimod-Induced Immune Cell Redistribution

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Received: 19 January 2016 / Accepted: 23 August 2016 / Published online: 8 September 2016

Abstract Fingolimod, a sphingosine-1-phosphate (S1P) receptor modulator, is an oral drug approved for the treatment of active relapsing-remitting multiple sclerosis (RRMS). It selectively inhibits the egress of lymphocytes from lymph nodes. We studied the changes in the transcriptome of peripheral blood CD8$^+$ cells to unravel the effects at the molecular level during fingolimod therapy. We separated CD8$^+$ cells from the blood of RRMS patients before the first dose of fingolimod as well as 24 h and 3 months after the start of therapy. Changes in the expression of coding and non-coding genes were measured with high-density Affymetrix Human Transcriptome Array (HTA) 2.0 microarrays. Differentially expressed genes in response to therapy were identified by *t* test and fold change and analyzed for their functions and molecular interactions. No gene was expressed at significantly higher or lower levels 24 h after the first administration of fingolimod compared to baseline. However, after 3 months of therapy, 861 transcripts were found to be differentially expressed, including interleukin and chemokine receptors. Some of the genes are associated to the S1P pathway, such as the receptor S1P5 and the kinase MAPK1, which were significantly increased in expression. The fingolimod-induced transcriptome changes reflect a shift in the proportions of CD8$^+$ T cell subsets, with CCR7$^-$ effector memory T cells being relatively increased in frequency in the blood of fingolimod-treated patients. In consequence, CCR7 mRNA levels were reduced by >80 % and genes involved in T cell activation and lymphocyte cytotoxicity were increased in expression. Gene regulatory programs caused by downstream S1P signaling had only minor effects.

Keywords Relapsing-remitting multiple sclerosis · Peripheral blood · CD8$^+$ T cells · Fingolimod · Sphingosine-1-phosphate · Transcriptome profiling

Abbreviations

AGCC	Affymetrix GeneChip Command Console
CNS	Central nervous system
CSF	Cerebrospinal fluid
EDSS	Expanded Disability Status Scale
EDTA	Ethylenediaminetetraacetic acid
FC	Fold change
GEO	Gene Expression Omnibus
HTA	Human Transcriptome Array
IFN	Interferon
im	Intramuscular
JUC	Junction probe set
MACS	Magnetic-activated cell sorting
MS	Multiple sclerosis
NK cell	Natural killer cell
OR	Odds ratio
Pat	Patient

Luisa Roch and Michael Hecker contributed equally to this work

Electronic supplementary material The online version of this article (doi:10.1007/s12035-016-0075-0) contains supplementary material, which is available to authorized users.

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PSR	Probe selection region probe set
RIN	RNA integrity number
RMA	Robust multi-array average
RRMS	Relapsing-remitting multiple sclerosis
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
sc	Subcutaneous
TAC	Transcriptome Analysis Console
TC	Transcript cluster
TCM	Central memory T cells
TEM	Effector memory T cells
TEMRA	$CD45RA^+$ effector memory T cells

Introduction

Multiple sclerosis (MS) is an inflammatory disorder of the brain and spinal cord, where autoaggressive lymphocyte infiltration through the blood-brain barrier causes damage and lesions [1]. On the basis of demyelination, astrogliosis, and loss of oligodendrocytes and neurons, neurodegeneration and, consequently, neurological dysfunctions result [2, 3]. The disease is characterized by episodes of relapses and recovery as well as progressive accumulation of disability. The etiology of MS is unclear, but environmental factors and genetic risk play an important role [4, 5]. Different courses of MS are distinguished [6]. Most patients (~85 %) present a relapsing-remitting MS (RRMS), which after several years often leads to secondary progressive MS [7, 8].

The main aims of current treatment are managing the symptoms and reducing the frequency of relapses and the development of new lesions. Several disease-modifying therapies are available for RRMS [9, 10]. They reduce relapse rate and inflammatory activity. In recent years, oral immunomodulatory drugs like fingolimod have been developed to supply more comfortable and more effective options for therapy [11–13]. Fingolimod is a sphingosine-1-phosphate (S1P) receptor modulator approved for patients with rapidly evolving RRMS. Fingolimod has been demonstrated not only to affect the immune system but also to have direct effects on the central nervous system (CNS) [14–17].

Fingolimod is a functional antagonist of S1P receptors (S1PRs) and a structural analog of sphingosine [18]. Sphingosine is mainly produced by platelets and erythrocytes and is responsible for the egress of lymphocytes from lymph nodes to the peripheral blood [19]. Phosphorylated sphingosine and fingolimod bind to S1PRs, which are expressed at the surface of T and B cells, macrophages, natural killer (NK) cells, dendritic cells, oligodendrocytes, astrocytes, and neurons. The interaction of fingolimod phosphate and the S1PR S1P1 leads to prolonged internalization and subsequent degradation of S1P1 [20–22]. In the absence of S1P1, the egress of certain immune cell populations from secondary lymphoid tissues is inhibited, thereby preventing their migration into the CNS and hence slowing the progression of neurodegeneration [23]. A reduction of peripheral blood and cerebrospinal fluid (CSF) lymphocyte counts is the result of the mechanism of action of fingolimod [24].

Fingolimod treatment reduces the peripheral lymphocyte count to ~30 % of baseline within a few weeks [25]. $CD4^+$ T cells are affected more than $CD8^+$ T cells, decreasing the blood $CD4^+/CD8^+$ cell ratio [26]. $CD8^+$ T cells are known for their cytotoxic activity, and they play a role in the control of viral replication. A number of studies have been performed to understand their role in the pathogenesis of MS [27]. $CD8^+$ T cells outnumber $CD4^+$ T cells in actively demyelinating CNS lesions [28, 29]. Histological analyses of MS lesions showed a significant correlation between the extent of acute axon damage and the numbers of $CD8^+$ T cells [30]. There is also a selective enrichment of $CD8^+$ T cells in the CSF of MS patients [29, 31], and activated cytokine-producing $CD8^+$ T cells in the blood of patients were shown to correlate with CNS tissue destruction [32].

$CD8^+$ T cell subsets in the blood are differentially affected by fingolimod. Fingolimod mainly modulates the recirculation of T cells expressing lymph node-homing receptors such as CCR7. The interaction of the chemokines CCL19 and CCL21 with the receptor CCR7 promotes the movement of T cells from the blood through the wall of high endothelial venules into the T cell zone of lymph nodes [33]. S1P1 signaling again ensures that $CCR7^+$ T cells, which are not activated by antigen, exit the lymph nodes via efferent lymphatics and flow back into the blood, where the levels of S1P are high [34]. Sensing the S1P concentration gradient is altered in response to fingolimod treatment; thus, naïve T cells ($CCR7^+CD45RA^+$) and central memory T cells (TCM; $CCR7^+CD45RA^-$), but not effector memory T cells (TEM; $CCR7^-CD45RA^-$) and CD45RA-expressing TEM (TEMRA; $CCR7^-CD45RA^+$), are trapped in lymph nodes. This leads to a proportional increase of TEM and TEMRA in the peripheral blood, whereas the numbers of naïve T cells and TCM are reduced [35–37]. However, so far, no study investigated via microarray analysis the genome-wide changes in transcript levels in $CD8^+$ cells during the therapy with fingolimod.

In the context of our research on the pathophysiology of MS, we examined the RNA levels of all human coding and non-coding genes in $CD8^+$ cells before and during fingolimod therapy and investigated the functions of differentially expressed genes. The aim of this study was to better understand the molecular mechanisms and to identify transcripts, which may be relevant as biomarkers for monitoring disease activity and therapeutic response. This is the first study which investigated the transcriptome dynamics in peripheral blood cells of MS patients longitudinally over the course of fingolimod therapy.

Materials and Methods

Study Population

For this study, 10 patients (Pat01–Pat10) of Western European descent with diagnosed RRMS according to the revised McDonald criteria [38] were asked to participate at the Department of Neurology at the University of Rostock. The average age of those six women and four men was 38.6 years at the beginning of the study (range 29–46), and their mean disease duration was 8.2 years (range 1–20). The average score in the Expanded Disability Status Scale (EDSS) was 3.55 at baseline (range 2.5–5.5). In the year before the study, the patients had between one and three relapses (mean 1.6; Table 1). It was decided to start a therapy with fingolimod (marketed as Gilenya by Novartis), because the patients showed persistent disease activity under first-line immunomodulatory therapy. The approved standard dose of fingolimod (0.5 mg administered orally once daily) was prescribed. The patients were treated and monitored according to the European Medicines Agency labels, following the guidelines and recommendations of the German Society of Neurology. Routine medical care was provided, and no specific study protocol was used for diagnosis and treatment.

The study was approved by the local ethics committee of the University of Rostock and conducted in line with Good Clinical Practice guidelines in accordance with the ethical principles of the Declaration of Helsinki. All patients gave written informed consent to participate in this study.

Blood Sampling and Isolation of RNA from $CD8^+$ Cells

From all patients, 20 mL of peripheral venous ethylenediaminetetraacetic acid (EDTA) blood was routinely obtained at the following three different time points: at baseline (that is, before the first dose of fingolimod), after 24 h (that is, immediately before the second dose of fingolimod), and after 3 months of treatment. To separate $CD8^+$ cells from others with magnetic-activated cell sorting (MACS), cells were labeled with Whole Blood CD8 MicroBeads (Miltenyi Biotec) and positively selected using the autoMACS separator (Miltenyi Biotec). After centrifugation and addition of lysis buffer, the samples were stored until further use at −20 °C. RNA was isolated from the cells using the mirVana Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions, and RNA concentrations were measured by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The RNA integrity was assessed with an Agilent 2100 Bioanalyzer using RNA 6000 Pico LabChips (Agilent Technologies).

Gene Expression Profiling Using Microarrays

To quantify transcript levels, the latest generation of Affymetrix microarrays was used. The high-resolution arrays from Affymetrix, GeneChip Human Transcriptome Array (HTA) 2.0, contain more than 6 million oligonucleotide probes with 25 bases per probe [39, 40]. Each human gene is interrogated by many different oligonucleotide probes, which are complementary in sequence to the respective transcript based on the current gene annotation. For protein-

Table 1 Clinical and demographic data of the patients

Patient	Gender	Age in years	Disease duration in years	Previous treatment	Treatment gap in months	EDSS at baseline	EDSS after 12 months	Relapses during 12 months prior to fingolimod	Relapses during 12-month follow-up
Pat01	Female	43	20	IFN-beta-1a sc	38	4.0	3.0	1	0
Pat02	Female	29	5	Glatiramer acetate	<1	2.5	1.5	3	0
Pat03	Female	43	1	IFN-beta-1a im	1	4.0	3.5	2	0
Pat04	Female	45	12	IFN-beta-1a im	2	3.0	3.0	3	0
Pat05	Male	31	7	Glatiramer acetate	<1	3.5	2.0	1	0
Pat06	Female	33	2	Glatiramer acetate	<1	2.5	5.5	2	2
Pat07	Female	46	9	IFN-beta-1b sc	<1	4.0	4.0	1	0
Pat08	Male	33	4	IFN-beta-1a sc	<1	3.0	6.0	1	3
Pat09	Male	46	15	IFN-beta-1b sc	<1	3.5	2.0	1	0
Pat10	Male	37	9	Glatiramer acetate	1	5.5	4.0	1	0
Median (range)		40 (29–46)	8 (1–20)		<1 (0–38)	3.5 (2.5–5.5)	3.3 (1.5–6.0)	1 (1–3)	0 (0–3)

The table provides the information for each of the 10 RRMS patients (Pat01–Pat10), including the EDSS and the number of relapses before and during therapy with fingolimod. Disease duration was quite variable with a median of 8 years. The time span between the last injection of the previous (first-line) immunomodulatory treatment and the first oral administration of fingolimod (treatment gap) was in most cases less than 1 month. Two patients (Pat06 and Pat08) showed an increase in the EDSS and experienced relapses despite fingolimod treatment

EDSS Expanded Disability Status Scale, *IFN* interferon, *im* intramuscular, *Pat* patient, *sc* subcutaneous

coding transcripts, 70 % of the probes on these microarrays are available and the remaining 30 % are for exon-exon splice junctions and non-coding transcripts, including microRNA precursors. To cover all transcript variants at a given gene locus (called transcript cluster; TC), there are 10 probes for each exon fragment and four probes for each exon-exon splice junction. The probes are disposed in probe sets, which summarize the data into gene-level, exon-level, and splice junction probe sets. Gene-level probe sets condense the data of all probes matching a TC into one expression value, realizing a one-to-one assignment of probe sets and genes.

For each array, complementary RNA (cRNA) was prepared from 200 ng total RNA according to the Affymetrix Whole Transcript (WT) protocol. The cRNA was used to generate single-stranded DNA, which was fragmented and biotinylated. The labeled single-stranded DNA was hybridized for 16 h at 45 °C on the Affymetrix HTA 2.0 microarrays. The microarrays were then washed and stained with a streptavidin-phycoerythrin conjugate in an Affymetrix Fluidics Station 450. Signal amplification with antibodies was applied. The microarrays were scanned with a GeneChip Scanner 3000 7G (Affymetrix). All procedures were performed according to the protocols by the manufacturer.

Data Analysis and Gene Filtering

The processing of the microarray scans was performed using Affymetrix GeneChip Command Console (AGCC) software version 4.0. The extracted data were then further processed using Expression Console 1.3.1. This software was also used for quality control by assuring that all hybridization controls were within bounds. The robust multi-array average (RMA) algorithm was then applied with the default configuration, which includes a log2 data transformation and quantile normalization. In this step, the measured signal intensities of the 6 million probes were summarized into probe sets. With HTA 2.0 microarrays, two workflows are possible. By gene-level analysis, the signals of all probes, which belong to one TC, were averaged to one expression value. By exon-level analysis, signal intensities were calculated for each set of 10 probes per exon fragment (probe selection region probe set (PSR)) and each set of 4 probes per exon-exon junction (junction probe set (JUC)). The latter allows examining the levels of transcript variants (isoforms) resulting from alternative transcription and splicing.

The Transcriptome Analysis Console (TAC) software version 1.0 was utilized to identify differentially expressed genes in the gene-level data set. Since all human coding and non-coding transcripts were measured, a global gene expression analysis was possible. We defined a *t* test significance threshold (p value <0.001) and a fold change (FC) cutoff (FC < −1.5 or >1.5) to filter genes expressed at higher or lower levels in response to fingolimod therapy. The fold change is the ratio of the expression levels during therapy and the expression levels at baseline. This ratio was calculated by the TAC software on linearly scaled data. A positive FC means up-regulation and a negative FC means down-regulation (in that case, the ratio is inverted and multiplied by −1).

Analysis of Gene Functions and Cell Type-Specific Expression

For examining the functions of differentially expressed genes, a gene set enrichment analysis was performed using SABiosciences categories, which define groups of genes having similar functions or participating in the same biological processes or pathways. The gene groups were retrieved from the array portfolio of the SABiosciences company (http://www.sabiosciences.com/ArrayList.php). Each of the 122 SABiosciences categories was then tested for overrepresentation in the set of filtered genes using the software GOstats [41]. GOstats calculates probabilities (p values) indicating whether the number of filtered genes associated with a gene set is larger than expected by chance. As reference gene set, all measured genes were used, which showed a probe set signal intensity of >4 for at least one sample. This analysis resulted in a table of SABiosciences categories with p values and odds ratios (OR) specifying the enrichment in the list of differentially expressed genes compared to the reference gene set. We finally extracted the categories with p value <0.001.

To investigate to which extent the transcriptome dynamics during fingolimod therapy reflect the shifts in the peripheral repertoire of T cell subpopulations, we used the data by Novershtern et al. who profiled the gene expression in 38 distinct purified populations of human hematopoietic cells using the older Affymetrix Human Genome U133 GeneChips [42]. This data set provided transcript levels in naïve $CD8^+$ T cells, $CD8^+$ TCM, $CD8^+$ TEM, and $CD8^+$ TEMRA, which were separated from blood samples of healthy volunteers. The data of these 4 T cell subtypes were compared to the data of each of the 30 microarrays (10 patients and 3 time points) in our data set. This was done by calculating p values for Spearman's rank correlation based on the levels of the subset of genes that were differentially expressed during therapy and measured in both data sets. The lower the p value was, the more the data were positively correlated. Finally, for each time point and each cell type, the average of the 10 computed p values was visualized as bar plot.

S1P Pathway Analysis

After oral administration, fingolimod is phosphorylated by sphingosine kinases to its active form, fingolimod phosphate, which binds as an analog of S1P to four of the five S1PRs

(S1P1 and S1P3–5). In particular, the interaction with S1P1 results in the prolonged internalization of this receptor, which leads to sequestration of lymphocytes in lymph nodes [11, 15]. To investigate how much the associated molecular pathway is modulated at the transcript level in response to therapy, a model of the pathway was constructed. This was done based on a literature search in the PubMed database. By this means, genes involved in S1P signaling were gathered from review articles published within the last few years. In total, 11 different review articles were chosen to extract a consensus S1P pathway [33, 43–52]. The pathway includes the synthesis of natural S1P, the phosphorylation of fingolimod, the interactions with S1PRs, and the processes resulting from downstream signaling events. Moreover, the chemokine receptor CCR7 was integrated, which plays a critical role for the homing of lymphocytes. The S1P pathway was then visualized with the software Cytoscape 3.1.0 [53], with nodes (e.g., genes) and edges (e.g., interactions) forming the molecular network. Changes in expression during treatment were visualized in color in the pathway.

Results

Patients and Samples

Most of the RRMS patients showed a benefit from fingolimod treatment with reduced disease activity and progression. Within a clinical follow-up period of 1 year, only two patients (Pat06 and Pat08) still had relapses. These two patients also showed an increase in the EDSS of 3.0 points 12 months after treatment initiation, whereas the other patients did not show a worsening of disability according to the EDSS. Overall, the mean EDSS of the patients slightly decreased to 3.45 (range 1.5–6.0) after 12 months of fingolimod therapy (Table 1). Most patients continuously received the treatment for at least 1 year. Only Pat08 discontinued the treatment with fingolimod after 8 months and switched to alemtuzumab later on. Pat01 and Pat05 showed temporarily elevated liver enzymes, which did not led to discontinuation of fingolimod treatment. All the other patients reported no side effects in the first year after the start of therapy.

With MACS separation, $CD8^+$ cells were positively selected from each EDTA blood sample. The number of collected $CD8^+$ cells per milliliter of blood was, on average, ~250,000 cells at baseline, ~300,000 cells after 24 h (+20 %), and ~100,000 cells after 3 months (−60 %). Hence, after 3 months of fingolimod treatment, the number of separated $CD8^+$ cells was significantly lower. Accordingly, all patients showed markedly decreased lymphocyte counts during therapy.

The quality of the RNA isolated from the $CD8^+$ cells was estimated using a Bioanalyzer by determining RNA integrity numbers (RINs). RIN values range from 10 to 1, with a value of 10 for a perfect RNA sample without degradation products. For the 30 samples, the average RIN value was 9.4. Thus, all samples were accepted for the high-resolution microarray experiments.

Differentially Expressed Genes

Affymetrix microarrays were used to measure the transcriptome in all 30 $CD8^+$ cell samples. Each resulting expression profile consisted of 70,523 gene-level probe sets and 914,585 exon-level probe sets. The gene-level profiles included 44,699 probe sets for coding transcripts, 22,829 probe sets for non-coding transcripts, and 2995 other probe sets. The latter category was neglected in the following analysis. The raw and preprocessed HTA 2.0 microarray data are publicly available at the Gene Expression Omnibus (GEO) database (accession numbers GSE73081 and GSE73172).

The expression matrix generated by the processing of the data according to the gene-level analysis workflow was used to filter differentially expressed genes. This was done by comparing the gene expression at the two time points during therapy with the gene expression at baseline. No probe set showed significantly higher or lower signal intensities 24 h after the first oral administration of fingolimod. However, 3 months after treatment initiation, remarkable changes in expression were observed. In total, 861 probe sets passed the stringent filtering criteria (p value <0.001 and fold change <−1.5 or >1.5) at this time point (Online Resource 1). Of these, 562 probe sets were assigned to protein-coding genes (438 up-regulated and 124 down-regulated) and 299 probe sets were assigned to non-coding genes (252 up-regulated and 47 down-regulated; Fig. 1). Hence, in total, 861 genes were filtered as differentially expressed, and the majority of these genes (n = 690) was expressed at higher levels in response to therapy (Fig. 2). However, the most significant expression changes with p values <1.00E-09 were calculated for down-regulated genes (n = 14), including CCR7 (p value = 4.55E−12, FC = −6.52), LEF1 (p value = 1.20E−10, FC = −3.96), IL6ST (p value = 6.78E−11, FC = −2.77), and IFNGR2 (p value = 1.47E−10, FC = −1.58; Online Resource 1). In Fig. 3, the time course profiles of selected genes were visualized.

On closer inspection, the genes tended to be modulated in expression already early in fingolimod therapy. Most of the 690 genes with elevated expression after 3 months (n = 605) had a FC >1.0 at the 24-h time point, and most of the 171 genes with lowered expression after 3 months (n = 160) had a FC < −1.0 at the 24-h time point. While this shows that the $CD8^+$ cell transcriptome shift starts early, the effects after the first dose of fingolimod were modest as no gene passed the filtering criteria when comparing the levels after 24 h with the pretreatment levels.

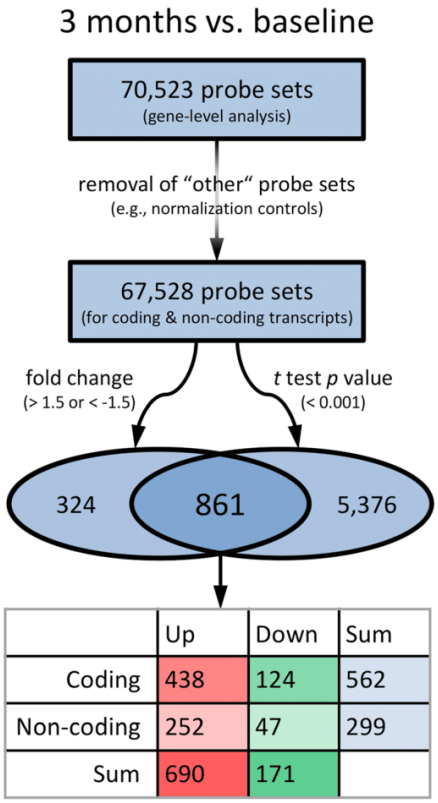

Fig. 1 Filtering of probe sets to identify differentially expressed genes. Peripheral blood $CD8^+$ cells were obtained from 10 RRMS patients before the first oral administration of fingolimod (baseline) as well as after 24 h and 3 months. We investigated the transcriptome changes with Affymetrix HTA 2.0 microarrays containing 70,523 gene-level probe sets. The subset of 2995 probe sets of the category "others" was neglected. Genes were considered differentially expressed, if they showed a *t* test *p* value <0.001 and a fold change >1.5 or <−1.5. With these criteria, no probe set was filtered after 24 h compared to baseline (not shown in this scheme). However, 861 probe sets were filtered after 3 months, of which 562 and 299 are representing coding and non-coding transcripts, respectively. The table is giving the numbers of up-regulated (*green*) and down-regulated (*red*) transcripts. *HTA* Human Transcriptome Array, *RRMS* relapsing-remitting multiple sclerosis

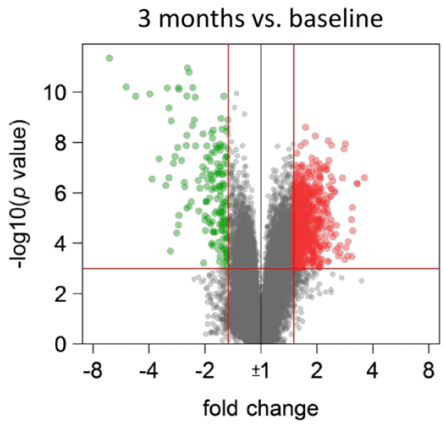

Fig. 2 Volcano plot of gene expression changes during fingolimod therapy. The transcriptome of circulating $CD8^+$ cells from RRMS patients receiving fingolimod therapy for 3 months was compared to baseline. The *x* axis specifies the fold changes (FCs), and the *y* axis specifies the negative logarithm to the base 10 of the *t* test *p* values. The *red vertical and horizontal lines* indicate the filtering criteria (FC = ±1.5 and *p* value = 0.001). The *red and green dots* represent the probe sets for transcripts expressed at significantly higher (n = 690) or lower (n = 171) levels during therapy, respectively. The *gray dots* are shown for probe sets that did not pass the filtering criteria

Functional Annotation of the Genes

We investigated the association of differentially expressed genes to functional categories and molecular pathways using the GOstats software [41] and SABiosciences categories. The genes were found to be overrepresented in 16 different gene groups (Table 2). The category with the most filtered genes (count = 25) was "inflammatory response and autoimmunity 384HT." The category "T cell anergy and immune tolerance" had the lowest *p* value (3.68E−10) and a count of n = 14. These two categories overlapped with five genes (CD27 and TNFSF8, which were down-regulated, and FASLG, IFNG, and IL2RB, which were up-regulated). Genes involved in "T cell and B cell activation" were enriched with 10 genes, including CCR1 and CCL4, which were expressed at higher levels during therapy. CCR7, as a member of the group "G protein-coupled receptors," was the most significantly differentially expressed gene (FC = −6.52, *p* value = 4.55E−12). Interestingly, distinct effects were seen in the group "interferon (IFN) and receptor" (n = 8, *p* value = 5.32E−05), with increased transcript levels of IFNG (FC = 1.91) and IFNGR1 (FC = 1.67) and lower transcript levels of IFNGR2 (FC = −1.58) after 3 months of treatment. There was also an elevated expression of genes coding for proteins involved in cytotoxic processes in T cell-mediated immune responses (e.g., GZMA, GZMB, and PRF1). The functional analysis further revealed overrepresented helper T cell-associated gene sets, which are also expressed in $CD8^+$ cells. For instance, the down-regulated genes IL23A (FC = −1.55) and IL6R (FC = −1.70) as well as five up-regulated genes belonged to the category "Th17 for autoimmunity and inflammation" (*p* value = 2.96E−04).

Transcriptome Profiles Relate to Shifts in T Cell Populations

Of the 861 differentially expressed genes, 299 genes were also contained in the data set by Novershtern et al. [42]. These data allowed to explore to which extent the transcriptome dynamics reflect changes in the frequency of $CD8^+$ naïve T cells, TCM, TEM, and TEMRA in the blood of fingolimod-treated patients. Spearman's correlation coefficients and respective

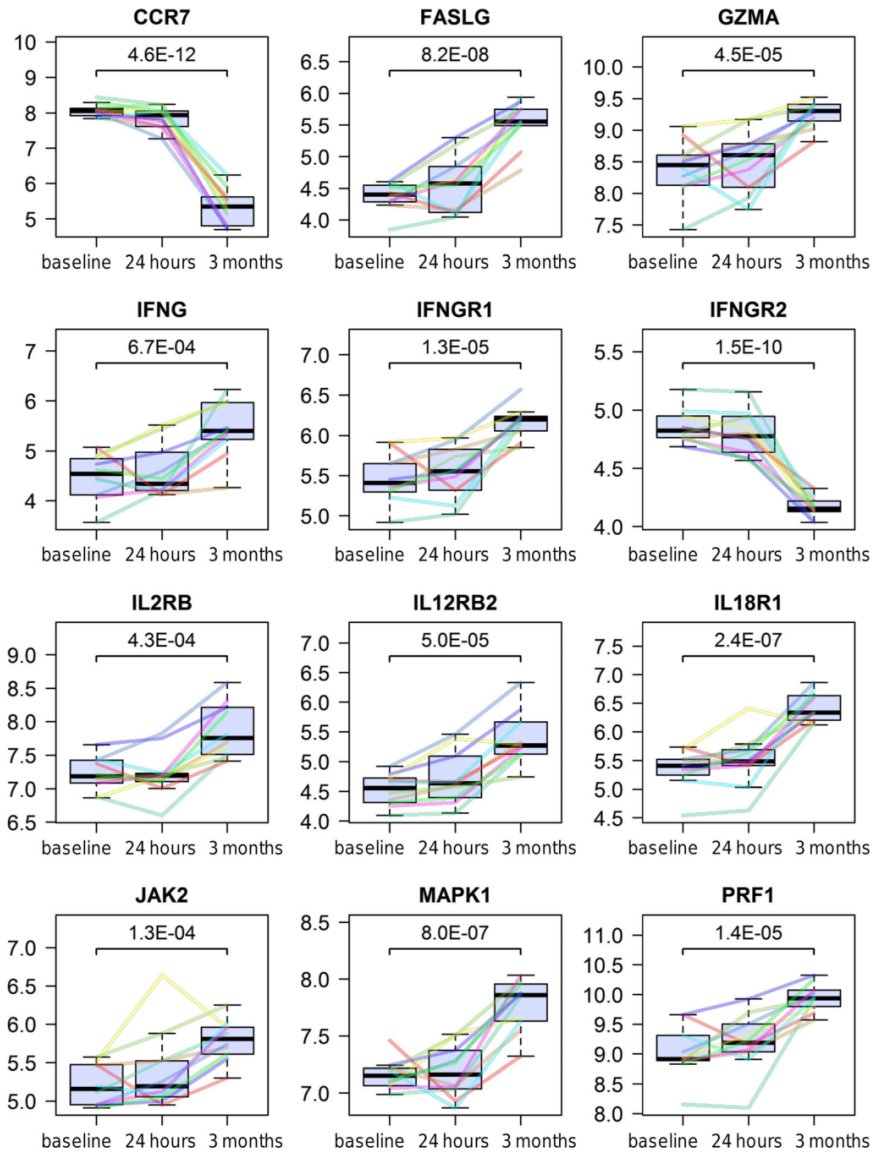

Fig. 3 Time course profiles of 12 selected differentially expressed genes. Gene expression dynamics were measured longitudinally using microarrays. Here, the transcript levels (in log2 scale) at baseline as well as 24 h and 3 months after the start of fingolimod therapy are shown for 12 genes. The *differently colored lines* refer to each of the 10 individual patients. The *p* values were calculated by *t* test. In particular, the signal intensities for CCR7 were strongly decreased in $CD8^+$ cells for all patients

p values were computed for the comparison of our data with the data of the four T cell subtypes. As shown in Fig. 4, the $CD8^+$ cell transcriptome profiles at baseline and after 24 h correlated strongly with the expression signature of $CD8^+$ naïve T cells and $CD8^+$ TCM. However, after 3 months, this correlation was less pronounced. In contrast, the transcriptome data of all 10 patients correlated strongly with the gene expression patterns of TEM and TEMRA at all three time points, with even increased similarities (*p* values <1.00E−15) after 3 months of fingolimod therapy. This clearly demonstrates that after continued administration of fingolimod, when $CCR7^+$ naïve T cells and TCM are trapped in lymph nodes, the frequency of $CCR7^-$ TEM and TEMRA within the $CD8^+$ cell population of the blood is increased during therapy and that this is also seen at the transcript level in a genome-wide manner. The shift in $CD8^+$ T cell subpopulations was only weakly seen already 24 h after the first dose of fingolimod (Fig. 4).

S1P Pathway Analysis

We studied the interactions within the S1P-associated molecular network. Therefore, we constructed a model as a compilation of the physiological pathways for S1P synthesis and

Table 2 Analysis of gene functions with SABiosciences categories

SABiosciences array	Count	*P* value	OR	Differentially expressed genes
Inflammatory response and autoimmunity 384HT	25	5.27E−10	5.05	C3AR1, CCL4/L2, CCR1/7, CD27/97, CX3CR1, CXCR1, EPHX2, FASLG, IFNG/R1/R2, IL2RB, IL6R/ST, IL12RB2, IL18R1/RAP, IL23A, PREX1, TLR3, TNFSF8, TTN
G protein-coupled receptors	16	2.13E−04	3.00	ADRB2, C3AR1, CCR1/7, CD97, CMKLR1, CX3CR1, CXCR1, GPR56/65/114/141, NMUR1, PTGDR, S1PR5, VIPR1
T cell anergy and immune tolerance	14	3.68E−10	11.30	BTLA, CD27, DGKA, EOMES, FASLG, GZMB, IFNG, IL2RB, ITCH, NFATC2, PRF1, SELL, TBX21, TNFSF8
T helper cell differentiation	12	2.13E−08	9.99	CCL4, EOMES, FASLG, IFNG, IKZF2, IL12RB2, IL18R1/RAP, MAF, NFATC2, TBX21, TOX
Inflammatory response and autoimmunity	11	5.36E−08	10.47	C3AR1, CCL4, CCR1/7, CXCR1, FASLG, IFNG, IL6R, IL18RAP, IL23A, TLR3
Th1-Th2-Th3	11	1.09E−07	9.67	CD27, FASLG, IFNG, IL6R, IL12RB2, IL18R1, IL23A, JAK2, MAF, NFATC2, TBX21
T cell and B cell activation	10	1.21E−06	8.44	CCL4, CCR1, CD27, FASLG, IFNG/R1/R2, IL12RB2, IL18R1, KIF13B
Stem cell signaling	10	6.05E−06	6.90	BCL9, IL6ST, LEF1, NFATC2, PTCH1, SMAD7, TCF7, TGFBR1/3, ZEB2
Cell surface markers	9	1.49E−05	7.05	CD38/79A/160/244, KLRB1/C1/D1, NCAM1, NT5E
Glycosylation	9	4.64E−05	6.01	B4GALT5, FUT11, GALNT3, GCNT4, GNPTAB, MAN1A1/C1, ST8SIA4/6
Interferon (IFN) and receptor	8	5.32E−05	6.85	IFI16, IFNG/R1/R2, IL2RB, IL6R, PYHIN1, TTN
Dendritic and antigen-presenting cell	8	6.73E−05	6.60	CCL4, CCR1, CXCR1, IFNG/R1, ITGAM, LYN, VCL
Inflammatory responses	7	1.02E−04	7.37	CCL4, CX3CR1, FASLG, IFNG, IL6R, IL18RAP, TNFRSF1B
Th17 for autoimmunity and inflammation	7	2.96E−04	6.09	IFNG, IL6R, IL12RB2, IL23A, JAK2, NFATC2, TBX21
GPCR signaling PathwayFinder	7	5.39E−04	5.46	ADRB2, CCL4, CFLAR, MYC, PIK3CG, PRKCA, PTGDR
NFkB signaling pathway	7	7.75E−04	5.11	CD27, CFLAR, FASLG, IFNG, SLC20A1, TBK1, TLR3

Categories were overrepresented for the 861 differentially expressed genes. To be part of the list, the gene groups had to be enriched with p value <0.001. As an example, 16 of the filtered genes ("count") belonged to the category "G protein-coupled receptors." This is proportionally more than in the reference gene set as specified by odds ratio (OR; 3.00) and p value (2.13E−04)

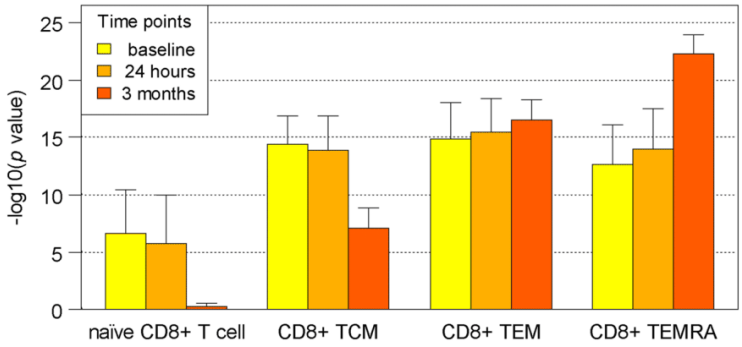

Fig. 4 Fingolimod-induced shift of $CD8^+$ T cell subtypes in peripheral blood. Treatment with fingolimod inhibits the egress of naïve T cells and central memory T cells (TCM) from lymph nodes by modulating S1P1 receptor signaling. Fingolimod does not affect effector memory T cells (TEM) and $CD45RA^+$ TEM cells (TEMRA) that do not express lymph node-homing receptors. The selective sequestration of naïve T cells and TCM within lymphoid tissues results in a relative increase of TEM and TEMRA in the $CD8^+$ cell population of the peripheral blood. This is also reflected in the $CD8^+$ cell transcriptome profiles of fingolimod-treated patients. Spearman's rank correlation was used to compare the expression of the differentially expressed genes in our data with their expression in naïve T cells, TCM, TEM, and TEMRA as measured in healthy subjects by Novershtern et al. [42]. The p values for correlation, averaged over the 10 patients in our study, are shown as *bars*, with *lines* representing the standard deviations. The microarray data of samples obtained after 3 months of treatment correlated very strongly with the gene expression signatures of TEM/TEMRA (p values <1.00E−15) but not any longer with those of naïve T cells

signaling as well as the therapeutic pathway with fingolimod resulting in functional antagonism and thus altered cellular responses (Fig. 5 and Online Resources 2 and 3). The S1P pathway is complex, comprising various protein-protein interactions and enzymatic reactions, and not all steps have already been elucidated in detail. For instance, it remains somewhat unclear how fingolimod actually enters the cells and whether other receptors than SPNS2 also play a role in the transport of fingolimod phosphate out of cells [54]. Extracellular S1P and phosphorylated fingolimod are bound mainly by albumin and other plasma proteins [55]. Their binding to S1P1 leads to internalization of this receptor. However, while S1P binding results in short-term internalization followed by reinsertion in the membrane, fingolimod phosphate initiates a long-term internalization, which can lead to ubiquitination and degradation of S1P1. The network model includes the competition between the CCR7 axis and the S1P axis, which have different chemotactic consequences [34]. Binding of the chemokine ligands CCL19 and CCL21 on CCR7 facilitates the homing of T cells to lymph nodes, and the interaction between S1P and S1PRs overrides this retention signal as a requirement for lymphocyte egress. Fingolimod terminates the signaling by receptor internalization, resulting in altered G protein-mediated intracellular signaling cascades and the sequestration of lymphocytes in lymphoid tissues. Ultimately, fingolimod is metabolized in the liver, specifically by the cytochrome P450 enzyme CYP4F [56].

To better understand the mutual regulatory interactions in the network, we analyzed the changes in expression of the coding genes participating in the S1P pathway. The average messenger RNA (mRNA) fold changes, 3 months versus baseline, were visualized in the pathway (Fig. 5). There were seven genes, which are part of the network and which were significantly differentially expressed in $CD8^+$ cells from the peripheral blood of RRMS patients receiving fingolimod. These included the up-regulated signaling transducers MAPK1, PIK3CG, PLCB1, and PLCG2. Moreover, CCR7 (FC = −6.52), S1P5 (FC = 2.07), and Rho kinase ROCK2 (FC = 1.60) are part of the molecular pathway, possibly implicating feedback mechanisms. In addition, there were modest gene expression changes not satisfying the filtering criteria (FC < −1.5 or >1.5 and p value <0.001), such as lower levels of ABC transporters and SPNS2 and higher levels of genes coding for Gi and G12/13 proteins and S1P1.

Exon-Level Analysis of IFNGR1

Due to the design of Affymetrix HTA 2.0 microarrays, deep insights into all human transcripts are possible. The processing of the data with the exon-level analysis workflow resulted in 914,585 probe sets for exon fragments (PSR) and junctions (JUC). To demonstrate the high resolution of these data, we visualized as an example the baseline data of patient Pat08 for the gene IFNGR1 at the exon level (Fig. 6). This facilitated the examination of different IFNGR1 transcript variants resulting, e.g., from exon skipping and alternative transcription start sites. In case of IFNGR1, which is localized on chromosome 6, there are five known transcript variants according to the annotation from the GENCODE database. In Fig. 6, the signal intensities summarized from probes matching to all distinct exon fragments and exon-exon junctions are shown. If the exons of a certain alternative splice variant have high signal intensities, it can be inferred that this transcript variant is expressed. Exon fragments contained only in the two shorter transcript variants of IFNGR1 showed low signal intensities. The data thus indicate that the three longer variants are preferentially expressed in $CD8^+$ cells.

Discussion

In this study, we investigated for the first time the transcriptome profiles in peripheral blood $CD8^+$ cells of RRMS patients longitudinally before and during treatment with fingolimod. The high-resolution Affymetrix HTA 2.0 microarrays provided deep insights into all human transcripts down to the exon level. The data revealed 861 genes to be differentially expressed 3 months after the start of therapy. Of them, 690 genes were up-regulated and 171 genes were down-regulated. To evaluate in which molecular functions, biological processes, and pathways the 861 differentially expressed genes participate, a gene set enrichment analysis was performed. Overrepresented gene categories were related to, e.g., inflammatory responses, immune tolerance mechanisms, T cell activation, IFN signaling, cytotoxicity, and chemotaxis.

The fingolimod-induced decrease in peripheral lymphocyte counts is a consequence of the reversible immune cell redistribution. Not only homing of lymphocytes into lymph nodes leads to a reduction of T cells in the blood but also blocking of egress from the thymus [57–59]. However, the overall functionality of T cells is not affected by fingolimod, which is believed to be important for the maintenance of immune surveillance under therapy [36]. The transcriptome profiles of $CD8^+$ cells confirmed that fingolimod leads to a selective reduction of naïve T cells and TCM in blood but to a relative increase of TEM. This cell population shift has been described earlier based on flow cytometry experiments with cell surface proteins [35–37]. In our data, genes preferentially expressed in naïve T cells and TCM were expressed at lower levels, and genes preferentially expressed in TEM and TEMRA were expressed at higher levels during therapy. The transcript levels of the differentially expressed genes after 3 months of fingolimod treatment thus strongly correlated with reference gene expression signatures of $CD8^+$ TEM and $CD8^+$ TEMRA [42] (Fig. 4). Circulating TEM are not affected by the inhibition of lymphocyte egress by fingolimod, because they lack

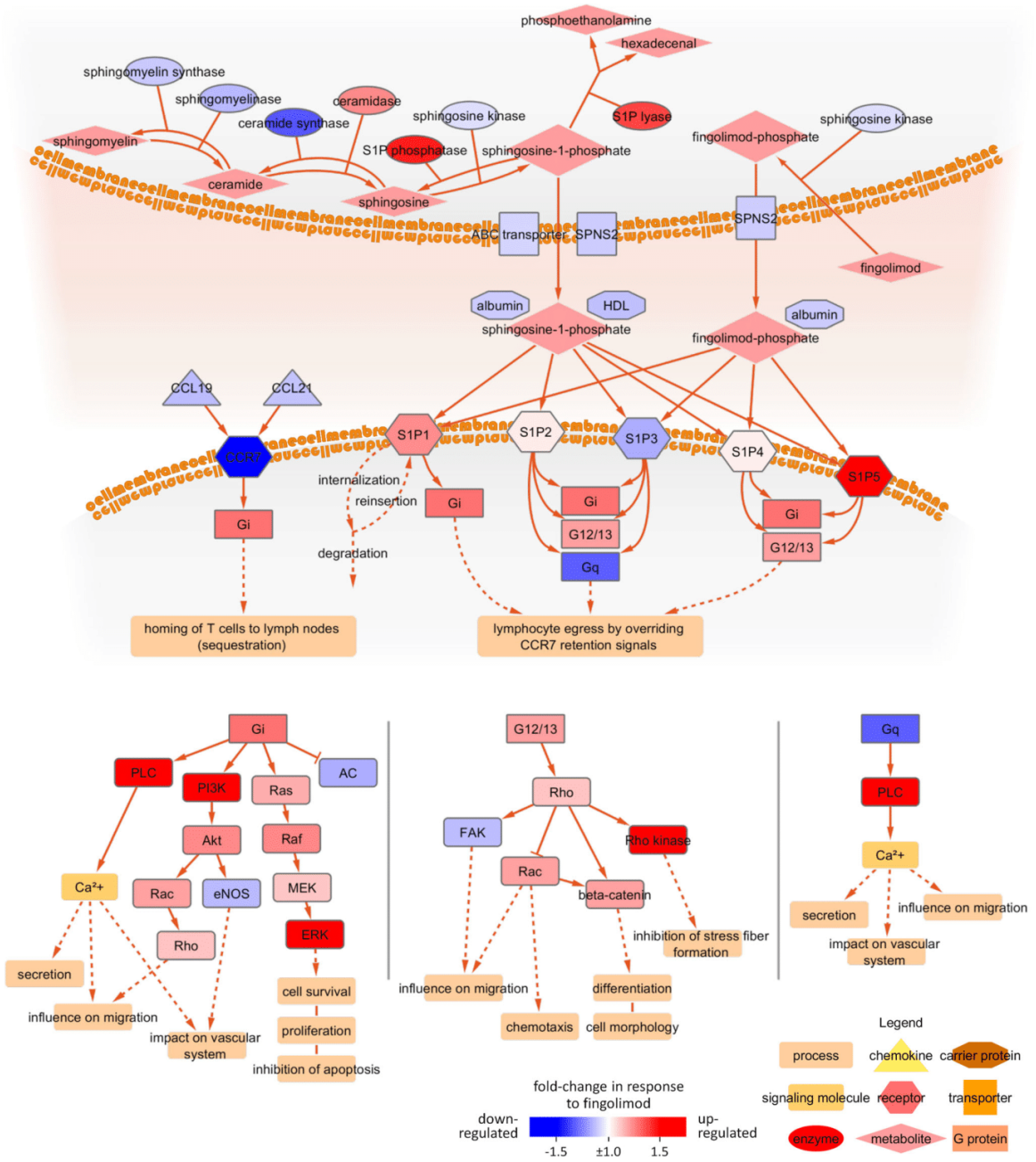

Fig. 5 S1P pathway and the changes in CD8$^+$ cell expression during fingolimod therapy. The network of the molecular pathway modulated by fingolimod was compiled based on literature information and visualized in the Cytoscape software (Online Resources 2 and 3). Sphingosine-1-phosphate (S1P), a bioactive metabolite formed from sphingosine, is a major regulator of immune cell trafficking. Functional antagonism of S1P receptors by fingolimod favors lymphocyte retention in lymph nodes. S1P receptors are coupled with G proteins, which modulate a range of biological processes. Some protein-coding genes in the pathway were differentially expressed in the CD8$^+$ cell population within the first 3 months of fingolimod treatment. The fold change of each gene in the pathway is visualized in *color*. The *nodes in blue and red* refer to the genes expressed at lower and higher transcript levels in response to therapy, respectively. Different types of proteins (e.g., receptors and enzymes) are highlighted in *different shapes*

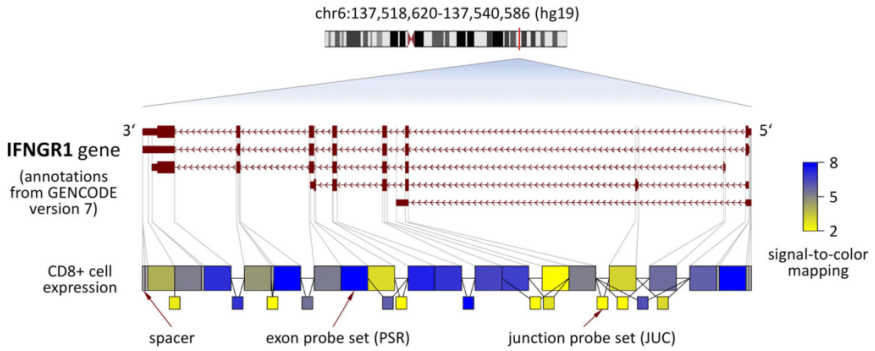

Fig. 6 Analysis of alternative splicing variants of IFNGR1 in $CD8^+$ cells. The gene IFNGR1 is located on chromosome 6. Five transcriptional variants are described for this gene, and the respective exon-intron structures are visualized in *red*. The *arrows* in introns indicate the transcription orientation. IFNGR1 was measured by 178 probes (25mer oligonucleotides), which were arranged into exon-level probe sets summarizing the data for exon fragments (PSR; $n = 10$ probes) and exon-exon splice junctions (JUC; $n = 4$ probes). Signal intensities were visualized exemplary for the baseline data of Pat08. The signals are in log2 scale, and high signals are shown in *blue*. Spacers refer to the sequences for which no corresponding probe exists on the Affymetrix HTA 2.0 microarrays. As can be seen, the two shorter transcript variants of IFNGR1 have exon fragments, which showed low signals (*yellow color*), suggesting that these variants are expressed at low levels. In contrast, particularly the longest variant can be considered as abundantly expressed in $CD8^+$ cells

expression of lymph node-homing receptors. In contrast, cell populations like TCM and naïve T cells, which express CCR7 and SELL (also referred to as CD62L), are trapped in lymphoid tissues in response to fingolimod therapy. Accordingly, both CCR7 and SELL were significantly reduced in expression after 3 months of treatment. The observed changes in RNA levels of $CD8^+$ cells mainly reflected the modulated migration of $CD8^+$ cell subtypes. Our data did not provide clear evidence of additional significant gene regulatory effects caused by S1P pathway downstream signaling events and the mutual interactions between immune cells.

CCR7 is a main marker of the effects of fingolimod. Three months after the start of therapy, CCR7 was, as expected, the most significantly differentially expressed gene in peripheral blood $CD8^+$ cells with a 84.7 % decrease in mRNA levels (FC = −6.52, p value = 4.55E−12). The homing of $CCR7^+$ lymphocytes into lymph nodes is guided by local chemokine gradients (CCL19 and CCL21) [33, 34, 52]. During fingolimod therapy, these cells lose the ability to override the retention signals by S1P signaling. $CD8^+$ cells expressing SELL, which is another receptor mediating the migration of lymphocytes into lymph nodes, were also previously described to be preferentially reduced in the blood after administration of fingolimod [60]. Consistent with this, in our data, SELL was significantly decreased in expression (FC = −2.13, p value = 6.60E−07) after 3 months of treatment with fingolimod. CD27, another cell surface receptor [61], was down-regulated (FC = −1.81) as well. Johnson et al. demonstrated that this indicates a relative increase in late effector memory cells ($CD27^-$) and a decrease in early effector memory cells ($CD27^+$) within the $CD8^+CCR7^-$ population of MS patients receiving fingolimod [62]. Differences in the migratory capacity of $CD8^+$ subpopulations thus result in an altered distribution of functionally distinct subsets of cytotoxic T cells in the circulation. Other cell membrane receptors such as CD97 (FC = 1.52), CCR1 (FC = 1.59), and CX3CR1 (FC = 1.81) were expressed at higher transcript levels during therapy. CX3CR1 is the sole receptor for CX3CL1, which was found to be increased in CSF and serum from patients with RRMS [63], and the G protein-coupled receptor CD97 might contribute to chronic inflammation in the brain of MS patients through interaction with CD55 [64].

Several cytokines were differentially expressed during therapy, including up-regulated CCL4 (FC = 1.90), IFNG (FC = 1.91), and XCL2 (FC = 1.71) and down-regulated IL23A (FC = −1.55). The significantly increased levels of IFNG are in line with earlier studies. The IFNG production by stimulated blood T cells was shown to be greater for fingolimod-treated patients compared to untreated patients [35, 62]. As IFNG secretion is characteristic of TEM cells [65], this again reflects the overrepresentation of TEM in peripheral $CD8^+$ T cells during therapy. Conversely, as shown in experiments with mice, fingolimod reduces IFNG production of $CD8^+$ T cells in the spleen [66]. The magnitude of $CD8^+$ T cell responses and memory differentiation, in turn, is controlled by IFNG receptor signaling [67]. IFNGR1 (FC = 1.67) and IFNGR2 (FC = −1.58), though not always present together on the surface of cells, are both required for full activity of IFNG [68]. Putative transcript variants of the receptor-coding genes are listed in databases but not well explored (Fig. 6) [69]. Our data further revealed a significantly lower expression of the receptor and the signal transducer for interleukin IL6, IL6R (FC = −1.70) and IL6ST (FC = −2.77), compared to pretreatment levels. IL6-mediated signaling has been demonstrated to be crucial for the maintenance of immune homeostasis and the suppression of autoreactive T cell

responses. Enhanced levels of IL6R were detected during active RRMS and shown to promote resistance of TEM toward regulatory T cells [70, 71]. However, further research is needed to elucidate to which extent the modulation of the IL6 pathway may contribute to therapeutic effects of fingolimod.

GZMA, GZMB, and PRF1 were expressed at higher mRNA levels within 3 months of fingolimod therapy compared to baseline. They are key effector molecules for T cell-mediated cell death. PRF1 promotes the cytolysis of target cells by facilitating the uptake of cytotoxic granzymes. This again points to a relatively larger proportion of granzyme-producing $CD8^+$ TEM that remain in the blood during fingolimod therapy. The increased proportion of GZMB-producing effector $CD8^+$ T cells in the blood was previously also demonstrated in fingolimod-treated mice [66].

Of the genes involved in S1P synthesis and signaling, seven genes were differentially expressed after 3 months of fingolimod therapy (Fig. 5). Down-regulated CCR7 (FC = −6.52) and up-regulated S1P5 (FC = 2.07) are important lymphocyte receptors in the pathway. The expression of S1P5 in $CD8^+$ effector T cells has been shown to be promoted by TBX21 (FC = 2.28) [72]. In addition, MAPK1 (FC = 1.61), which regulates $CD8^+$ T cell proliferation and survival [73, 74], as well as PIK3CG, PLCB1, PLCG2, and ROCK2, which are all part of G protein-activated cascades, were increased in expression, possibly indicating molecular feedback mechanisms. Gi and G12/13 protein-coding genes were slightly up-regulated, and Gq was slightly down-regulated, but these changes did not reach significance. Future studies may investigate not only the transcript levels of the genes in the network but also their protein levels. It would be also interesting to analyze whether the effects of fingolimod therapy in the S1P pathway are different in other cell types of the blood or CNS [75]. Moreover, it is possible to incorporate the interactions between microRNAs and their target mRNAs in the network. In this regard, it should be noted that for the 3-month samples, significantly increased signal intensities were measured for probe sets matching the stem-loop sequences of the microRNA precursors hsa-mir-548j, hsa-mir-644a, and hsa-mir-4295 (Online Resource 1). The mature forms of these microRNAs are thus worth to be further analyzed by real-time PCR.

In our patient cohort, two patients (Pat06 and Pat08) showed relapses and a worsening of disability despite the therapy with fingolimod (Table 1). It is tempting to speculate whether this insufficient therapeutic response is related to different gene expression profiles in these individual patients. Therefore, we studied the microarray data to search for $CD8^+$ cell transcripts, which could be used as prognostic biomarkers. However, the two patients showed no obvious differences in the gene expression levels at baseline or in the gene expression changes in response to fingolimod. Strong effects, such as the down-regulation of CCR7 and IFNGR2, were consistently observed for all 10 MS patients (Fig. 3). A recent study by Song et al. also aimed to establish biomarkers to predict relapses in MS patients receiving fingolimod. They confirmed that the percentages of $CD8^+$ naïve T cells and $CD8^+$ TCM are significantly reduced in the peripheral blood during therapy. However, the percentages of $CD8^+$ subpopulations did not differ significantly between patients with and without relapses, but patients with relapses had proportionally more $CD4^+$ TCM, in particular, after 3 months [37]. Yokoseki et al. provided evidence that the expression of CCR7 on $CD4^+$ T cells in CSF may be associated with disease activity under fingolimod [76]. Fingolimod influences lymphocyte counts in blood and CSF differently [24, 76]. Future studies with larger cohorts are needed to validate whether molecular and cellular CSF parameters correlate with clinical effects.

A limitation of the study is the limited number of patients and time points analyzed. There was a trend showing that transcriptome changes were already initiated after 24 h, but at this time point, the effects were modest and no gene passed the strict filtering criteria (FC < −1.5 or >1.5 and p value <0.001). Only with continued daily dosing, the peripheral blood lymphocyte counts continue to decrease over a 2-week period [37]. Therefore, first significant gene expression changes are expected to be seen after the first few doses of fingolimod. Afterward, the biological effects of the treatment remain stable as long as the medication is taken. It is thus likely that similar changes in gene expression can be observed after several months of therapy as we have determined after 3 months (Online Resource 1). The filtering results were quite robust. All 861 filtered probe sets also had a false discovery rate <0.05 when correcting for multiple testing. $CD8^+$ cells were positively selected in our data. This is an issue as a subset of NK cells is also part of the $CD8^+$ cell population [77]. Only a minor population of NK cells has been shown to express CCR7; thus, absolute numbers of NK cells are only slightly altered during therapy [78]. Future studies may investigate explicitly the transcriptome dynamics in NK cells and other cells of the blood to better understand the drug's mechanisms of action. Furthermore, $CD8^+$ T cell subpopulations should be studied in more detail, and in this case, one may focus on the list of differentially expressed genes attained in our study. More research is also needed to functionally characterize the non-coding genes expressed at higher or lower levels in response to fingolimod (n = 299). Several long non-coding RNAs were already described to be associated with neurological diseases [79]. For instance, NEAT1, which was differentially expressed in our data (FC = 1.59), was found to be up-regulated in the brain of patients with Huntington's disease [80].

To conclude, we used high-resolution gene expression microarrays to analyze $CD8^+$ cells from the blood of RRMS patients receiving fingolimod. After 3 months of therapy, significant transcriptome changes were observed along with

clearly reduced peripheral lymphocyte counts. In total, 861 genes were identified to be differentially expressed during treatment compared to baseline. Previously described shifts in the composition of $CD8^+$ cell subsets were confirmed at the RNA level, in particular by the reduced expression of lymph node-homing receptors. Genes coding for cytokines (e.g., IFNG and CCL4) and proteases (e.g., GZMA and GZMB) were increased in expression, corresponding to a higher percentage of effector memory T cells in the blood of fingolimod-treated patients. The changes in expression were mainly attributable to this cellular shift, whereas potential secondary effects independent of S1PR binding or due to intracellular S1P pathway signaling were not clearly visible. Subsequent studies may inspect the differentially expressed genes in specific $CD8^+$ T cell subsets, evaluate their use as biomarkers for disease prognosis and monitoring, and elucidate the roles of non-coding transcripts.

Acknowledgments We thank Christa Tiffert for coordinating patient care, Nele Retzlaff for the clinical documentation, Ildikó Tóth and Annegret Zettl for their laboratory assistance, and Anne-Kathrin Adloff for the supportive feedback throughout the study.

Compliance with Ethical Standards This study was approved by the local ethics committee of the University of Rostock and conducted in line with Good Clinical Practice guidelines in accordance with the ethical principles of the Declaration of Helsinki. All patients gave written informed consent to participate in this study.

Funding The microarray experiments were partially funded by Novartis. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of Interest MH received speaking fees and travel funds from Bayer HealthCare, Biogen, Novartis, and Teva. AW received speaking fees and travel funds from Bayer HealthCare, Novartis, Genzyme, and Merck Serono. UKZ received speaking fees, travel funds, and financial support for research activities from Bayer HealthCare, Biogen, Merck Serono, Novartis, Sanofi-Aventis, Almirall, and Teva. LR, JF, ICA, DK, BF, IS, KF, HJT, and SM declare that they have no conflict of interest.

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5.3. Manuskript 3

Angerer IC, Hecker M, Koczan D, Roch L, Friess J, Rüge A, Fitzner B, Boxberger N, Schröder I, Flechtner K, Thiesen HJ, Winkelmann A, Meister S, Zettl UK.

Transcriptome profiling of peripheral blood immune cell populations in multiple sclerosis patients before and during treatment with a sphingosine-1-phosphate receptor modulator.

CNS Neurosci Ther. 2018 Mar;24(3):193-201. doi:10.1111/cns.12793.

Journal Impact Factor 2019: 4,074

Received: 26 September 2017 | Revised: 7 December 2017 | Accepted: 7 December 2017

DOI: 10.1111/cns.12793

ORIGINAL ARTICLE

WILEY CNS Neuroscience & Therapeutics

Transcriptome profiling of peripheral blood immune cell populations in multiple sclerosis patients before and during treatment with a sphingosine-1-phosphate receptor modulator

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Funding information
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Summary

Aims: Fingolimod is a sphingosine-1-phosphate (S1P) receptor modulator approved for the treatment of the relapsing form of multiple sclerosis (MS). It prevents the egress of lymphocyte subpopulations from lymphoid tissues into the circulation. Here, we explored the broad effects of fingolimod on gene expression in different immune cell subsets.

Methods: Utilizing 150 high-resolution microarrays from Affymetrix, we obtained the transcriptome profiles of 5 cell populations, which were separated from the peripheral blood of MS patients prior to and following oral administration of fingolimod.

Results: After 3 months of treatment, significant transcriptome shifts were seen in CD4+ and CD8+ cells, which is mainly attributable to the selective homing of naive T cells and central memory T cells. Although the number of B cells was greatly reduced in the blood of fingolimod-treated MS patients, the analysis of differential expression in CD19+ cells identified only a small set of 42 genes, which indicated a slightly higher frequency of transitional B cells. The transcriptome signatures of CD14+ monocytes and CD56+ natural killer cells were not affected.

Conclusion: Our study corroborates changes in the composition of circulating immune cells in response to fingolimod and delineates the respective implications at the RNA level. Our data may be valuable for comparing the effects of novel S1P receptor modulating agents, which may be a therapeutic option for patients with secondary progressive MS as well.

KEYWORDS

CD19+ B cells, fingolimod therapy, peripheral blood, relapsing-remitting multiple sclerosis, transcriptome microarray analysis

The first two authors contributed equally to this work.

1 | INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS), which is driven by autoreactive immune cells.[1,2] Distinct B cell and T cell subpopulations play different roles in the initiation and progression of this clinically heterogeneous disease. Activated cytotoxic T cells provoke inflammation and neurodegeneration in the CNS by targeting autoantigenic structures.[3] T helper cells and memory B cells are mediating the destructive processes by presenting antigens and secreting proinflammatory cytokines to costimulate immune responses.[4] The intrathecal formation of antibody-producing plasma cells and plasmablasts is another hallmark of the pathophysiology of MS.[5]

Fingolimod is an oral drug approved for the treatment of relapsing-remitting MS (RRMS).[6] As a sphingosine-1-phosphate (S1P) receptor modulator, it affects the migration of lymphocytes out of secondary lymphatic tissues.[7,8] During fingolimod therapy, certain immune cell subpopulations are sequestered in lymph nodes, because their S1P receptors are internalized and degraded. In consequence, there are less autoreactive cells circulating in the blood and hence less cells are able to migrate into the CNS. Immune cells expressing lymph node homing receptors such as CCR7 and SELL are preferentially trapped. Continued administration of fingolimod thus leads to a shift of cell populations in the peripheral blood. After 3 months of therapy, there is a significant decrease in the proportions of CD4+ T cells (from 46% to 6% of lymphocytes) and CD19+ B cells (from 11% to 6%) and a moderate decrease of CD8+ T cells (from 20% to 15%),[9] while frequencies of CD14+ monocytes and CD56+ natural killer (NK) cells are relatively increased.[10,11] However, although the altered trafficking of cell subsets has been well studied, the effects of fingolimod on gene expression and cellular signaling are still not fully established.

We examined the transcriptome of lymphocytes and monocytes before and during S1P receptor modulator therapy to obtain further insights on the effects on molecular signatures thought to be related to the pathophysiology of MS. To identify differentially expressed genes (DEG) in immune cells separated from the blood of treated RRMS patients, we measured all human protein-coding and noncoding transcripts at the exon level. The data allowed to analyze to which extent transcriptome changes correlate with the fingolimod-induced shift of circulating cell populations, to investigate gene regulatory programs potentially caused by downstream S1P signaling, and to define molecular markers for monitoring therapeutic responses.

Key points

- CD4+ and CD8+ cell populations demonstrated huge shifts in the transcriptome during fingolimod therapy, reflecting the selective sequestration of smaller subpopulations in lymphoid tissues (eg, naive T cells and central memory T cells)
- CD19+ cells, although reduced in number in the blood circulation by more than 80%, showed only marginal gene expression changes, indicating that fingolimod similarly affects the trafficking of the different B cell subpopulations
- CD14+ monocytes and CD56+ natural killer cells showed fairly stable cell counts and transcriptome profiles in the course of treatment, with no evidence of significant gene regulatory effects independent of the sphingosine-1-phosphate pathway

2 | METHODS

Details about study design and analysis methods were published previously.[12,13] Briefly, a large dataset comprising 150 high-resolution microarrays was obtained for specific cell populations from blood samples of MS patients. The patients were aged between 26 and 46 and diagnosed with RRMS according to the revised McDonald criteria.[14] Disease duration was, on average, 8 years. All patients were previously treated with either glatiramer acetate or interferon-beta, and none of them received a S1P receptor modulator before. In the 12 months prior to the study, each patient experienced 1-3 relapses. For this reason, the therapy was switched to fingolimod (standard dose of 0.5 mg orally once daily), following the treatment guidelines and recommendations for routine medical care of the German Society of Neurology. As part of our research on MS, the study was approved by the local ethics committee of the University of Rostock and conducted in accordance with the ethical principles of the Declaration of Helsinki. All patients gave prior written informed consent to participate in this study.

Blood samples (~20 mL) were always taken immediately before the start of fingolimod therapy (baseline) as well as after 24 hours (before the second dose of fingolimod) and after 3 months. Five different cell subsets were magnetically labeled based on the cell surface markers CD4, CD8, CD14, CD19, and CD56 using Whole Blood MicroBeads from Miltenyi Biotec (Bergisch Gladbach, Germany). Afterward, the positively selected cell fractions were collected and then counted under a conventional microscope. Total RNA was isolated from the cells using the mirVana Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). For each cell population, samples of ten MS patients were prepared to analyze the gene expression in the course of therapy. In doing so, the 3 longitudinal samples for each patient and cell subset were handled at the same day by the same laboratory technician. In total, 150 high-coverage human transcriptome arrays (HTA) 2.0 from Affymetrix (Santa Clara, CA, USA) were prepared according to the manufacturer's instructions. HTA 2.0 microarrays contain over 6 million 25mer oligonucleotide probes for measuring the transcripts of all coding and noncoding genes. They are designed with 10 probes per exon fragment and 4 probes per exon-exon splice junction.[15] The probes were summarized in the Expression Console 1.3.1 software (Affymetrix) to 70523 gene-level probe sets and 914585 exon-level probe sets. Quality control

and data normalization by the robust multiarray average algorithm[16] including a log2 transformation were performed as described elsewhere.[12,13] The processing of the microarray data was performed separately for each cell population. The complete raw and processed data are publicly available together with the clinico-demographic data of the patients in the Gene Expression Omnibus (GEO) database (SuperSeries GSE73174).

The Transcriptome Analysis Console (TAC) software version 1.0 (Affymetrix) was used to explore the effects of fingolimod on the transcriptome in the 5 different cell populations. For each gene-level probe set, the signal intensities of the 24-hour samples and the 3-month samples, respectively, were compared with those of the pretreatment samples. DEG were determined by filtering probe sets with Student's two-tailed unpaired *t* test *P*-value <0.001 and fold change (FC) >1.5 or <−1.5. Probe sets not mapping to genes (n = 2995; hybridization controls, etc.) were discarded. The FC was calculated by TAC in linear scale, with negative FC indicating reduced gene expression. Accordingly, a statistically significant increase of more than 50% or decrease of more than 33% in the transcript levels distinguished DEG in response to the therapy. False discovery rate (FDR) correction[17] was applied to adjust the *P*-values for multiple testing.

For DEG in CD19+ B cells, a gene regulatory network (GRN) model was constructed on the basis of the microarray data and information on predicted transcription factor (TF) binding sites (TFBS) in the promoter region of the genes. For this purpose, TFBS-integrating least angle regression (TILAR) was employed as described elsewhere.[18,19] We used transcription start sites from the GeneCards database version 3.04[20] and evolutionarily conserved TFBS from the tfbsConsSites track of the UCSC database build hg19.[21] TFBS-integrating least angle regression (TILAR) was applied with backward stepwise selection on TF-gene interactions and no additional prior knowledge on gene-TF interactions (δ = 1). All other steps of the GRN inference (eg, prevention of overfitting) were performed with the default configuration.

3 | RESULTS

Moderate differences (up to ~20%) in the numbers of cells that were separated from the individual blood samples were noted 24 hours after the first administration of fingolimod. After 3 months of therapy, however, the mean cell counts of sorted CD4-, CD8-, and CD19-expressing cells were significantly reduced by ~60%-90% compared to baseline (Table 1). The numbers of circulating CD14+ and CD56+ cells showed strong interindividual differences but, on average, remained relatively stable even after continued drug administration (Figure 1A).

The transcriptome analysis of the 5 immune cell populations revealed only a single DEG 24 hours after treatment initiation (Table 1). At this time point, solely the signal intensities of the probe set "TC10001500.hg.1" (no official gene symbol available) were significantly increased in the CD4+ cell subset. The lack of early gene regulatory effects reflects that the first dose of fingolimod does not have a lasting effect on the frequencies of blood cell populations and also indicates that S1P receptor downstream signaling does not elicit a discernible transcriptional response in these cells.

Significant alterations in the transcriptome profiles were seen after 3 months of fingolimod therapy (Table 1). The treatment effect was also well visible in a principal component analysis of the whole dataset comprising the signals of 70523 gene-level probe sets for all 150 HTA 2.0 microarrays, which were aggregated after data normalization (Figure 1B). The greatest change in gene expression was observed in the CD4+ cell subpopulation with in total 6489 DEG (890 of those even had a FC >2.0 or <−2.0). For CD8+ cells and CD19+ cells, 861 genes and 42 genes, respectively, were found to be expressed at significantly higher or lower levels relative to pretreatment levels. On the other hand, there were no significant gene expression dynamics in CD56+ cells and CD14+ cells. For the latter, only 1 probe set ("TC03002386.hg.1," no official gene symbol) fulfilled our filtering criteria (FC >1.5 or <−1.5 and *t* test *P*-value <0.001).

TABLE 1 Overview of fingolimod-induced cellular and transcriptome shifts for different blood cell populations

	24 h after the first dose		After 3 mo of therapy	
Cell population	Cell count change (%)	DEG (up/down)	Cell count change (%)	DEG (up/down)
CD4+	−21.1	1 (1/0)	−92.4	6489 (2574/3915)
CD8+	18.7	0 (0/0)	−59.5	861 (690/171)
CD14+	15.8	0 (0/0)	−5.3	1 (1/0)
CD19+	−14.2	0 (0/0)	−87.0	42 (41/1)
CD56+	15.5	0 (0/0)	−15.7	0 (0/0)

Shown are the effects of fingolimod therapy on average absolute counts of cells sorted from the peripheral blood of relapsing-remitting MS patients (see also Figure 1A). In comparison with baseline, significant changes in the number of circulating cells were only observed after 3 mo of treatment for CD4+ cells (Student's *t* test *P*-value = 0.00002), CD8+ cells (*P* = 0.027), and CD19+ cells (*P* = 0.00006). Moreover, the number of differentially expressed genes (DEG, *t* test *P*-values <0.001 and fold changes >1.5 or <−1.5) is indicated for each cell population and each time point during therapy (see also Figure 1D). There was basically no increase or decrease in expression 24 h after the first dose of fingolimod. In contrast, after 3 mo of treatment, huge transcriptome alterations were seen in CD4+ cells and CD8+ cells. For CD19+ B cells, there were 42 DEG despite a strong reduction in the respective cell count of almost 90%.

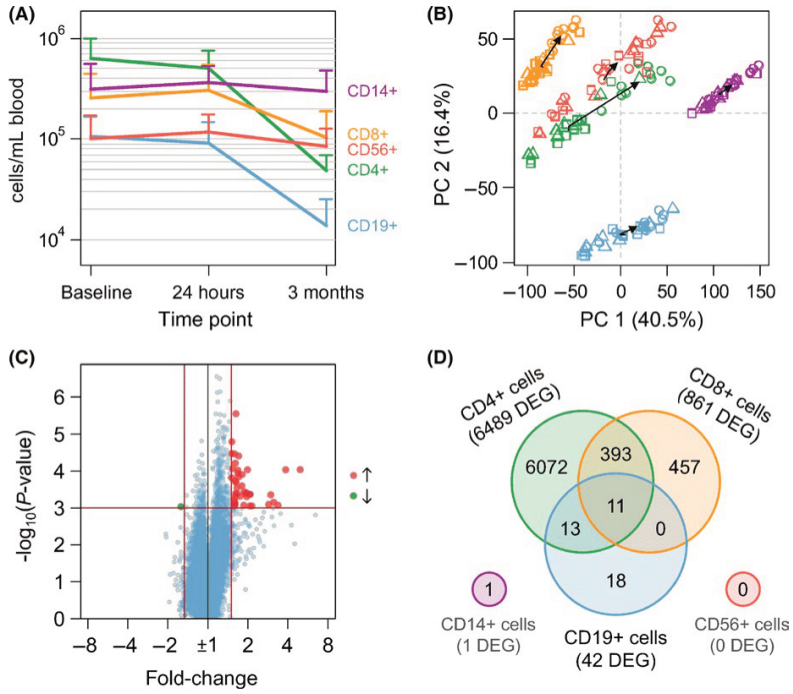

FIGURE 1 Analysis of cellular and molecular effects of fingolimod therapy in the blood of RRMS patients. Microarray-based transcriptome profiling was performed for monocytes and lymphocyte subpopulations, which are represented in the figure by different colors (CD4+ cells in green, CD8+ cells in orange, CD14+ cells in purple, CD19+ cells in blue, and CD56+ cells in red). A, Concentration of cells (mean plus standard deviation) for each of the 5 separated cell populations and each of the 3 study time points. After 3 mo of fingolimod therapy, the numbers of sorted CD4+ cells, CD8+ cells as well as CD19+ cells were significantly reduced (Student's *t* test *P*-values <0.05). B, Principal component (PC) analysis of the complete dataset comprising 150 microarrays (10 patients × 3 time points × 5 cell populations). The samples were plotted in the first two dimensions explaining most of the variation in the normalized and mean-centered gene-level probe set signal intensities (n = 70 523 per array). Dark arrows connect the centers of each time point (depicted by rectangles for baseline, triangles for "24 h," and circles for "3 mo"). C, Volcano plot of gene expression changes in CD19+ cells within 3 mo after initiation of fingolimod therapy. Red and green dots display probe sets for gene transcripts expressed at significantly higher or lower levels, respectively (n = 42). The red vertical and horizontal lines indicate the fold change and *P*-value filtering criteria. D, Venn diagram summarizing for each cell population the sets and their intersections of differentially expressed genes (DEG) after 3 mo of fingolimod therapy relative to pretreatment levels. Eleven DEG were shared among CD4+ cells, CD8+ cells, and CD19+ cells

The huge transcriptome shifts in CD4+ cells and CD8+ cells clearly display the selective homing of T cell subpopulations to lymphoid tissues in response to sustained fingolimod therapy.[12,13] On the contrary, the gene expression changes in CD19+ B cells were quite subtle considering the fact that their cell count was also remarkably reduced in the peripheral blood by almost 90% after 3 months (Table 1). As the purity of isolated B cells was high before and during therapy (Figure S1), this indicates that fingolimod similarly affects the trafficking of the different B cell subpopulations. Overall, 41 genes showed an up to 5-fold increase in expression, while just 1 transcript (probe set "TC15000493.hg.1") was expressed at lower levels in the CD19+ cell subset during therapy (Figure 1C). Five of these genes remained significant at the 5% FDR level (Table 2). Predictions of TFBS revealed 5 TF to be associated with the DEG at the significance level $\alpha = 0.05$. This information was used as template for modeling the underlying GRN using TILAR.[18,19] The resulting network linked 27 of the DEG by 15 TF-gene interactions and 42 gene-TF interactions (Figure S2). MEF2A appeared as the most highly connected TF in the network.

A comparative analysis of the sets of DEG at the 3-month time point showed marked overlaps. Almost half of the DEG of CD8+ cells (46.9%) were also modulated in expression in CD4+ cells, and 11 genes were consistently upregulated in CD4+, CD8+, and CD19+ cells (Figure 1D). Three of these 11 shared DEG encode the proteins IQGAP2, MYBL1, and PTPN12 (Figure 2), and the other ones constitute gene fragments and less well-characterized noncoding transcripts. A subset of 18 genes was significantly differentially expressed in CD19+ B cells only.

4 | DISCUSSION

We obtained a huge dataset of 150 high-resolution HTA 2.0 microarrays to profile the transcriptome of 5 different types of circulating

TABLE 2 Genes with significant changes in expression in B cells from MS patients receiving fingolimod therapy

No	Probe set	Chromosome location	FC	*P*-value	Probes	Gene symbol	2 sets	3 sets
1	TC01001498.hg.1	chr1(+):171810621-172387606	2.14	0.000883	305	DNM3		
2	TC01001619.hg.1	chr1(+):192127587-192154945	4.95	0.000092	105	RGS18	✓	
3	TC02001958.hg.1	chr2(−):70476123-70476193	2.08	0.000431	30	(tRNA Gly)	✓	
4	TC02004620.hg.1	chr2(−):145143042-145143557	1.66	0.000287	30	(ZEB2)	✓	✓
5	TC02004622.hg.1	chr2(−):145251832-145254445	1.70	0.000039*	30	(ZEB2)	✓	✓
6	TC02004623.hg.1	chr2(−):145268952-145277958	1.63	0.000003*	30	(ZEB2)	✓	✓
7	TC04002285.hg.1	chr4(+):156902136-156938403	3.13	0.000705	50	–		
8	TC05002969.hg.1	chr5(−):39383148-39393457	3.37	0.000854	70	(DAB2)	✓	✓
9	TC05000368.hg.1	chr5(+):75699074-76003957	1.62	0.000409	629	IQGAP2	✓	✓
10	TC05001839.hg.1	chr5(−):138700366-138705406	1.68	0.000117	19	(SLC23A1)	✓	
11	TC06002615.hg.1	chr6(+):21597661-21598850	1.87	0.000369	15	(SOX4)	✓	
12	TC06004064.hg.1	chr6(+):27777842-27779078	3.85	0.000093	20	HIST1H3A-J		
13	TC06000759.hg.1	chr6(+):80451636-80451669	1.96	0.000503	10	RNY4	✓	
14	TC07000495.hg.1	chr7(+):77166415-77269388	1.57	0.000616	476	PTPN12	✓	✓
15	TC08001286.hg.1	chr8(−):67474410-67525529	1.60	0.000701	242	MYBL1	✓	✓
16	TC08002364.hg.1	chr8(−):67476954-67525175	1.80	0.000253	160	(MYBL1)	✓	✓
17	TC08000531.hg.1	chr8(+):86376081-86393722	1.93	0.000411	154	CA2		
18	TC08002454.hg.1	chr8(−):102193086-102195404	1.65	0.000410	30	(ZNF706)		
19	TC08002460.hg.1	chr8(−):104395306-104396082	1.81	0.000126	30	–	✓	✓
20	TC09001206.hg.1	chr9(−):74517371-74517466	1.61	0.000062	30	(Y RNA)	✓	
21	TC09001401.hg.1	chr9(−):100689073-100707138	1.99	0.000093	90	HEMGN		
22	TC09001610.hg.1	chr9(−):130594914-130595022	1.60	0.000821	30	(Y RNA)		
23	TC10001072.hg.1	chr10(−):17256238-17271983	1.63	0.000206	60	VIM-AS1	✓	
24	TC10002487.hg.1	chr10(−):17256245-17271983	1.70	0.000146	90	(VIM-AS1)	✓	✓
25	TC10001111.hg.1	chr10(−):25270908-25351208	2.07	0.000889	120	ENKUR		
26	TC10002540.hg.1	chr10(−):31109147-31110214	2.97	0.000446	27	–		
27	TC10000448.hg.1	chr10(+):73980510-73980610	1.59	0.000035*	30	(Y RNA)		
28	TC11002664.hg.1	chr11(+):48191599-48192391	1.53	0.000087	24	(PTPRJ)	✓	
29	TC11002092.hg.1	chr11(−):74971166-75062875	1.58	0.000889	197	ARRB1	✓	
30	TC12000011.hg.1	chr12(+):890299-890424	1.61	0.000185	30	(WNK1)	✓	✓
31	TC12002795.hg.1	chr12(−):24332887-24333863	1.87	0.000852	30	(SOX5)		
32	TC12000297.hg.1	chr12(+):32552463-32798984	1.51	0.000034*	454	FGD4	✓	
33	TC12000977.hg.1	chr12(+):123252646-123252747	1.75	0.000486	30	(Y RNA)		
34	TC13001561.hg.1	chr13(−):67787977-67789866	1.68	0.000096	30	(PCDH9)		
35	TC15001162.hg.1	chr15(−):31248451-31248553	1.60	0.000778	30	(MTMR10)	✓	
36	TC15000493.hg.1	chr15(+):62533117-62533147	−1.59	0.000930	7	–		
37	TC16001729.hg.1	chr16(−):223162-223620	1.95	0.000390	30	–		
38	TC16000008.hg.1	chr16(+):226679-227521	2.87	0.000807	70	HBA1-2		
39	TC16000732.hg.1	chr16(−):686736-686806	2.08	0.000431	30	(tRNA Gly)	✓	
40	TC17000978.hg.1	chr17(−):1519486-1519589	1.51	0.000154	30	(SLC43A2)	✓	
41	TC17002022.hg.1	chr17(+):5016654-5018299	1.61	0.000503	30	–		
42	TC19002594.hg.1	chr19(−):57804451-57805837	1.52	0.000016*	30	–		

In total, 42 genes were filtered as differentially expressed with *t* test *P*-values <0.001 and fold changes (FC) >1.5 or <−1.5 in CD19+ B cells when comparing the data at baseline with the data at 3 mo of treatment. The table provides the Affymetrix microarray gene-level probe set identifiers, the chromosome positions of the genes (GRCh37/hg19 genome assembly) in sorted order, FC, raw *t* test *P*-values, the number of 25mer oligonucleotide probes for each probe set as well as official gene symbols if available. Symbols in brackets (n = 21) indicate that the respective probe set interrogates only a part of a protein-coding gene or a noncoding RNA class. We marked the genes that were also expressed at significantly higher transcript levels in CD4+ cells ("2 sets," n = 24) or in both CD4+ cells and CD8+ cells ("3 sets," n = 11) as depicted in Figure 1D.
*Significant at false discovery rate of 5%.

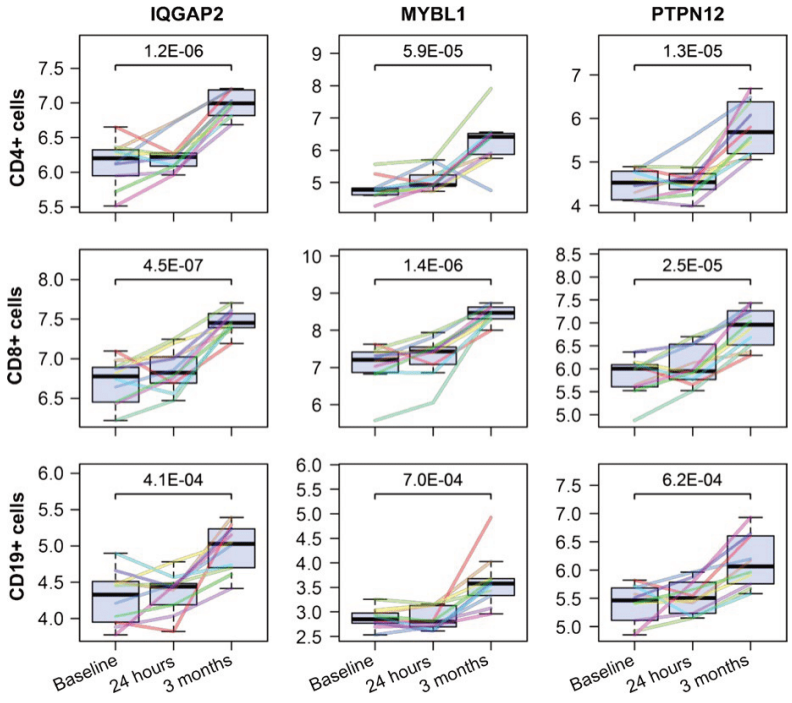

FIGURE 2 Gene expression dynamics of IQGAP2, MYBL1, and PTPN12 in the course of fingolimod therapy. Matrix of boxplots visualizing the mRNA expression of 3 selected genes (in columns) in 3 distinct immune cell populations (in rows). The cells were separated from the peripheral blood of relapsing-remitting MS patients based on cell surface markers such as CD4 and CD8 for T cells and CD19 for B cells. Pretreatment expression values as well as levels after 24 h and 3 mo of fingolimod therapy are presented in log2 scale. Differently colored lines refer to each of the 10 individual patients per cell population. The *P*-values above the brackets were calculated by Student's *t* test. In all 3 cell populations shown, IQGAP2, MYBL1, and PTPN12 were consistently expressed at significantly higher transcript levels in response to continued administration of fingolimod in comparison with baseline (*P*-values <0.001 and fold changes >1.5)

immune cells before and during fingolimod therapy. Alterations in gene expression were not yet observed after the first dose but could be ascertained in CD4+, CD8+, and CD19+ cells (but not in CD14+ and CD56+ cells) after continued drug exposure. This goes along with a significant reduction in the number of T and B cells and, consequently, a relative increase in the frequencies of CD14+ monocytes and CD56+ NK cells in the peripheral blood of treated patients. These changes in the composition of lymphocytes, as a result of the prevention of their egress from lymphoid tissues, have already been well established in the literature.[9-11,22] Preferential lymph node homing of naive T cells and central memory T cells, but not effector memory T cells, correlates with hundreds of DEG that we identified in the CD4+ and CD8+ cell subsets in response to fingolimod treatment. Additional gene regulatory effects independent of S1P receptor binding, however, could not be evidenced in these cells in our study. The transcriptome shift in T cells and its impact on biological processes and S1P-related pathways are presented in detail in our previous publications.[12,13] In the following, we will focus on the results for the subset of B cells, which recently gained increasing attention in MS research.[23,24]

Other studies have shown that the absolute numbers of naive B cells, memory B cells, and plasmablasts are significantly reduced in the peripheral blood during fingolimod treatment.[25-27] As all these subpopulations are affected, only 42 DEG passed our filtering criteria when comparing the gene expression levels in separated CD19+ cells after 3 months of therapy vs baseline, although total B cell counts were decreased by 87%. Some of the DEG such as arrestin beta-1 (ARRB1), hemoglobin alpha 1/2 (HBA1-2), SRY-box 4 (SOX4), and genes of the histone H3 family (HIST1H3A-J) are known to be expressed at higher levels in immature B cells compared to mature B cells.[28-31] Their elevated expression thus likely indicates a proportionally higher frequency of circulating transitional B cells in fingolimod-treated MS patients, which would also explain the emergence of TF families promoting early B cell development in the GRN (such as MEF2[32]), even though classical cell surface markers such as CD24 and CD38 were not identified as DEG. Consistent with this, a relative (but not absolute) increase of newly produced immature B cells during therapy has been demonstrated by flow cytometry immunophenotyping.[10,27,33,34] Interestingly, lower baseline percentages of transitional B cells in the peripheral blood might predict clinical response to fingolimod,[34] but this still has to be confirmed in larger patient cohorts.

The probe sets for the protein-coding genes IQGAP2, PTPN12, and MYBL1 were consistently filtered in the transcriptome analyses of circulating CD4+, CD8+, and CD19+ cells. This implicates that some of the 42 DEG that were identified in the CD19+ cell population after 3 months of fingolimod therapy exert various functions not only in B cells but also in other immune and non-immune cells. IQGAP2 is a broadly expressed calmodulin binding protein regulating cytoskeletal dynamics.[35] PTPN12, on the other hand, is a protein tyrosine phosphatase that was shown to suppress antigen-receptor signaling in B cells and T cells.[36] The related family member PTPRJ also plays a role in lymphocyte signaling, particularly in B cell activation and B cell development.[36,37] Notably, the filtered probe set for PTPRJ ("TC11002664.hg.1") matches only the 3′ untranslated region of the last exon, whereas the designated probe set for the full-length transcript ("TC11000412.hg.1") narrowly missed the significance threshold (*t* test *P*-value = 0.004). MYBL1 is a strong transcriptional activator, which is implicated in

Burkitt's lymphoma,[38] a disease associated with Epstein-Barr virus (EBV).[39] Three probe sets measured increased RNA levels of gene fragments from ZEB2, a transcriptional repressor that contributes to maintenance of EBV latency by inhibiting lytic reactivation.[40] Latent EBV infection of B cells is one of the strongest environmental risk factors for MS.[1] However, the possible relevance of MYBL1 and ZEB2 in MS remains to be investigated. Another differentially expressed gene in B cells during fingolimod therapy was ARRB1. An earlier study demonstrated the presence of antibodies reactive with ARRB1 in sera from patients with MS.[41] Moreover, the first intron of ARRB1 harbors the precursor sequence for the microRNA hsa-miR-326, which is dysregulated in peripheral blood cells of MS patients.[42] DNM3 is also host gene of a microRNA, namely hsa-miR-3120. DNM3 is preferentially expressed in the brain and as a GTP-binding protein involved in vesicular transport,[43] while hsa-miR-3120 regulates heat shock cognate protein 70 and vesicle uncoating.[44] Furthermore, some of the genes, for example, ARRB1, FGD4, IQGAP2, and RGS18, are known to act as regulators of GTPases.[45]

Among the DEG in CD19+ cells, there were also several non-coding transcripts, which deserve further research. For instance, Ro-associated Y4 (RNY4) is affiliated with the Y RNA class, a group of small RNA acting as licensing factors for chromosomal DNA replication through interactions with chromatin and initiation proteins.[46] In addition to RNY4, we filtered 4 probe sets corresponding to paralogous Y RNA sequences in intronic regions. Moreover, 2 copies of glycine transfer RNA were found with elevated levels of expression in the B cells from patients treated with fingolimod for 3 months in comparison with baseline levels.

The strengths of our study are that we analyzed the gene expression signatures of distinct blood cell populations longitudinally in a well-characterized cohort of RRMS patients.[12,13] Moreover, we used 150 high-resolution HTA 2.0 microarrays[15] to obtain very accurate and comprehensive snapshots of the cellular transcriptomes. As limitations, our study does not give insights into RNA expression changes after the 3-month time point, and it was not designed to detect prognostic biomarkers of the long-term individual clinical response to fingolimod therapy. Subsequent studies thus may further evaluate the identified DEG in larger cohorts using focused approaches such as real-time PCR. On the other hand, a more exhaustive characterization of immune cells from treated patients is feasible as massively parallel sequencing technologies emerged that allow to measure RNA profiles at single-cell resolution.[47,48] Other possible extensions of this study include the analysis of alternative splicing events,[49] protein levels, and other cell subpopulations. A recent study demonstrated that fingolimod also stimulates gene expression in neurons, thereby affecting axonal growth and regeneration,[50] but these effects so far have not been examined in detail at the transcriptome level.

To conclude, fingolimod selectively alters the trafficking of immune cells and, consequently, changes the gene expression profiles of CD4+, CD8+, and CD19+ cells circulating in the peripheral blood. Genes induced in B cells of MS patients receiving fingolimod therapy for 3 months comprised signaling molecules and transcriptional regulators but not cell surface markers and cytokines. However, in comparison with T cells, the transcriptome shift in B cells was quite subtle. This reflects that the B cell subpopulations are sequestered to similar extents in lymphoid tissues. Basically, all treatments for MS modulate B cell subsets.[51,52] Thus, further research on their roles in the pathogenesis of MS is of critical importance. Finally, our data may also be useful to compare molecular effects of fingolimod with other emerging S1P receptor modulators being examined in clinical trials of MS and other brain-related diseases.[53]

ACKNOWLEDGMENT

We thank Christa Tiffert for coordinating patient care, Nele Retzlaff for clinical documentation, and Ildikó Tóth and Annegret Zettl for laboratory assistance. The microarray experiments were partly funded by Novartis. The funder had no role in the study design, data collection, and analysis, decision to publish or preparation of the manuscript.

DISCLOSURE

MH received speaking fees and travel funds from Bayer HealthCare, Biogen, Novartis, and Teva. AW received speaking fees and travel funds from Bayer HealthCare, Genzyme, Merck Serono, and Novartis. UKZ received research support as well as speaking fees and travel funds from Almirall, Bayer HealthCare, Biogen, Merck Serono, Novartis, Sanofi, and Teva. ICA, DK, LR, JF, AR, BF, NB, IS, KF, HJT, and SM declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Angerer IC, Hecker M, Koczan D, et al. Transcriptome profiling of peripheral blood immune cell populations in multiple sclerosis patients before and during treatment with a sphingosine-1-phosphate receptor modulator. *CNS Neurosci Ther*. 2018;24:193–201. https://doi.org/10.1111/cns.12793

6. Diskussion

6.1. Klinischer Verlauf

Der in der vorliegenden Studie beobachtete klinische Verlauf mit einer deutlichen Reduktion der Schubhäufigkeit stimmt mit den Ergebnissen vorangegangener Studien überein. In der Zulassungsstudie konnte bei 70,4% der Patienten eine Schubfreiheit in den ersten zwei Jahren der Therapie mit 0,5mg Fingolimod täglich erreicht werden [46]. Die beobachtete leichte Leberwerterhöhung bei 2 Patienten ist bereits als Nebenwirkung von Fingolimod beschrieben [48]. Die vorliegende Studie unterstützt die vorbeschriebene Einschätzung, dass der Nutzen in der Reduktion der Krankheitsaktivität das Risiko des Auftretens von Nebenwirkungen überwiegt [80]. Neue, bisher nicht beschriebene Nebenwirkungen traten in der untersuchten Patientengruppe nicht auf.

6.2. Änderung der Zellzahlen im Blut unter Fingolimod-Therapie

Die Reduktion der Anzahl von CD4+-, CD8+- und CD19+-Zellen im peripheren Blut von RRMS-Patienten nach 3 Monaten Therapie mit Fingolimod um 60% und mehr stimmt mit den Ergebnissen anderer Studien überein [81]. Die Anzahl im Blut zirkulierender Immunzellen wird initial bereits in den ersten Stunden nach der 1. Gabe von Fingolimod reduziert, erreicht allerdings nach 24 Stunden wieder in etwa das ursprüngliche Niveau. Erst bei wiederholter Gabe von Fingolimod ist eine anhaltende Absenkung der Zellzahlen zu sehen [82, 83] Ein Zusammenhang zwischen der effektiven Reduktion der peripheren Immunzellzahl und dem Therapieerfolg ließ sich nicht nachweisen [84]. Durch die erstmalige komplette Transkriptomanalyse der wichtigsten Immunzellpopulationen im Zeitverlauf konnten in der vorliegenden Arbeit neue Erkenntnisse über die molekularen Effekte der Therapie gewonnen werden, auf die ich im Weiteren näher eingehen werde.

6.3. Änderungen im Genexpressionsprofil von Immunzellen

Besonders bei den CD4+- und CD8+-Zellen ging die signifikante Reduktion der Zellzahlen im peripheren Blut mit einer deutlichen Veränderung des Genexpressionsprofils einher. Bei den CD14+-Zellen und den CD56+-Zellen zeigte sich hingegen keine Änderung auf Transkriptomebene. Dies korreliert mit der allgemein geringeren Expression von Homing-Rezeptoren wie CCR7 durch diese Zellen [85]. Durch diese wird eine Rezirkulation der exprimierenden Zellen durch das Lymphsystem stimuliert [86].

Bei den T-Zellen zeigte sich eine deutliche Assoziation der differentiell exprimierten Gene mit funktionellen Gruppen, die Immunreaktionen regulieren, ausführen oder initiieren. Beispielsweise

war sowohl bei den CD4+- als auch bei den CD8+-Zellen der Oberflächenrezeptor CX3CR1 unter der Therapie stärker exprimiert. Dieser bindet den Liganden CX3CL1, dessen Konzentration im Serum und im Liquor von RRMS-Patienten als erhöht beschrieben wurde [87]. Bei den CD4+-Zellen gehörten zu den stärker exprimierten Genen verschiedene Toll-Like-Rezeptor (TLR)-Transkripte (z.B. TLR1, TLR2, TLR4). Diese sind relevant für die Regulation der Immunzellaktivierung aufgrund von Pathogenen sowie Zell- und Gewebeschädigungen [88]. Bei den CD8+-Zellen zeigte sich eine signifikant verringerte Expression der Transkripte für IL6R und IL6ST im Verlauf der Therapie. Dabei handelt es sich um den Rezeptor (IL6R) sowie ein Signaltransduktionsmolekül (IL6ST) für das Interleukin IL6. Dieses Zytokin spielt eine Rolle bei der Interaktion verschiedener Immunzellen und der Entwicklung von autoreaktiven B- und T-Zell-Antworten [89, 90]. Unter den Transkripten, die bei den CD4+-, CD8+ und CD19+-Zellen differentiell exprimiert waren, befand sich unter anderem das Gen MYBL1, welches kodierend ist für einen Transkriptionsfaktor, der zuvor in B-Zellen von Patienten mit Burkitt-Lymphom verstärkt exprimiert nachgewiesen wurde [91]. Dabei handelt es sich um ein B-Zell-Lymphom, dass mit einer EBV-Infektion in Zusammenhang gebracht wird [92]. Eine Infektion mit EBV stellt einen starken Risikofaktor für MS dar [8]. Diese Parallele könnte bedeuten, dass überlappende Pathomechanismen sowohl im Rahmen eines Burkitt-Lymphoms als auch im Rahmen der MS auftreten und durch Fingolimod moduliert werden.

Unter den T-Zell-Subpopulationen ergab sich ein deutlich anderes Genexpressionsprofil der verbleibenden zirkulierenden Zellen. Der Chemokin-Rezeptor CCR7 war sowohl bei CD4+-Zellen (FC=-2,05) als auch bei CD8+-Zellen (FC=-6,52) nach 3 Monaten Therapie mit Fingolimod in seiner Expression deutlich reduziert. Bei den CD19+-Zellen war CCR7 in geringem Maße ebenfalls verringert exprimiert (FC=-1,28, *p*-Wert= 0,105). Die Signalweganalyse zeigte, dass unter der Therapie mit Fingolimod die Signale der S1P-Rezeptoren nicht mehr gegen die CCR7-vermittelten Homing-Signale konkurrieren können, was die vorbeschriebene Retention vom Lymphozyten in Lymphknoten bewirkt [53]. Die vorliegenden Expressionsprofile bestätigten, dass unter den T-Zellen einige Subpopulationen schwächer in den Lymphknoten zurückgehalten werden als andere. So zirkulieren beispielsweise die CD4+-CCR7– -Effektor-Gedächtnis-T-Zellen auch unter der Therapie mit Fingolimod im Blut [93]. Bei den CD8+-Zellen zeigte sich in der vorliegenden Studie eine Verringerung der Expression von CD27 auf mRNA-Ebene (FC=-1,81). Vorherige Studien legen nahe, dass dies einen relativen Anstieg der späten Effektor-Gedächtnis-T-Zellen widerspiegelt. Es ist kritisch anzumerken, dass bei der vorgenommenen Positivselektion der T-Zellen ebenfalls einige Monozyten und NK-Zellen, die zu einem geringen Anteil auch CD4 bzw. CD8 auf der Zelloberfläche tragen, mitselektiert wurden [85]. Dadurch sind in diesen Zellpopulationen auch einige Zellen, welche die sonstigen Charakteristika von T-Zellen nicht vorweisen.

Im Vergleich zu den T-Zell-Subpopulationen konnte bei den B-Zellen eine geringere Veränderung des Expressionsprofils unter der Therapie mit Fingolimod festgestellt werden. Dies lässt in Kombination mit der deutlichen Zellzahlreduktion von 87% im Blut der Patienten nach 3 Monaten der Fingolimod-Therapie darauf schließen, dass die verschiedenen B-Zell-Subpopulationen etwa gleich stark durch Fingolimod in den Lymphknoten zurückgehalten werden. Vorherige Studien haben bereits beschrieben, dass die Anzahl von B-Gedächtniszellen, naiven B-Zellen sowie Plasmablasten durch die Therapie mit Fingolimod ähnlich effektiv im Blut reduziert wird [94,95]. Die 42 gefilterten Probesets repräsentierten aber auch mehrere protein-kodierende Transkripte (CA2, HIST1H3A-J, HBA2, MYBL1), die nach 3 Monaten Therapie mit Fingolimod stärker exprimiert waren (Kap. 5.3) und die in unreifen B-Zell-Subpopulationen stärker exprimiert werden als in reifen Subpopulationen [96]. Dies könnte ein Indiz dafür sein, dass der Anteil ausgereifter B-Zellen durch die Therapie mit Fingolimod effektiver im Blut reduziert wird, wobei unreife Zellen auch vermehrt aus dem Knochenmark freigesetzt werden, wenn die Zahl der B-Zellen im Blut sinkt [97]. Eine ähnliche moderate Verschiebung der Subpopulationen der B-Zellen hin zu unreifen Subtypen wurde ebenfalls in anderen Studien festgestellt [98,99,100].

Zwei der Patienten erlitten auch unter der Therapie mit Fingolimod Schübe. Hierbei war es interessant zu untersuchen, ob sich bei diesen Patienten ein spezielles Genexpressionsprofil oder eine besondere Expression bestimmter Gene auf mRNA-Ebene feststellen lässt. So könnte ein potenzieller prognostischer Marker für den Krankheitsverlauf gefunden werden. Allerdings zeigte sich bei diesen beiden Patienten keine signifikante Abweichung in den Genexpressionsdynamiken als Zeichen des unzureichenden Ansprechens auf die Therapie. Für die Identifikation von zuverlässigen prädiktiven Biomarkern war die Studiengröße (n=10) jedoch auch nicht ausreichend.

6.4. Veränderte Expression von bestimmten microRNA-Vorläufern

Die Transkriptomanalyse der CD4+- und CD8+-Zellen zeigte zumindest 13 Vorläufertranskripte von miRNA (d.h. Probesets, die spezifisch sind für sogenannte stem-loop-Sequenzen) mit differentieller Expression nach 3 Monaten Therapie mit Fingolimod. Es konnten dabei 2 miRNA-Vorläufer (für hsa-mir-644a und hsa-mir-4295) identifiziert werden, die sowohl bei den CD4+- als auch bei den CD8+-Zellen infolge der Therapie verstärkt exprimiert waren (Kap. 5.1, 5.2). Die Funktion dieser miRNA im Rahmen der MS ist allerdings noch nicht ausreichend untersucht. Das Gen DLEU2, das unter Therapie mit Fingolimod in den T-Zellen stärker exprimiert war, enthält die stem-loop-Sequenz für die miRNA hsa-mir-16-1. Bisherige Studien haben gezeigt, dass diese miRNA in CD4+-Zellen bei MS-Patienten schwächer exprimiert ist [101], unter Therapie mit Interferon-beta jedoch hochreguliert wird [102]. Bei den CD19+-Zellen zeigten sich ebenfalls 2 Gene mit höherem Expressionsniveau unter der Therapie mit Fingolimod, die in intronischen Bereichen miRNA kodieren. Es handelt sich zum einen um das Gen für Dynamin 3 [103]. Die in

der DNM3 prä-mRNA kodierte miRNA ist hsa-mir-3120. Diese interagiert unter anderem mit dem Hitzeschock-verwandten Protein 70 (heat shock cognate protein 70) [104], welches vor allem in neuronalen Zellen exprimiert ist [85]. Das zweite Gen (ARRB1) enthält den Vorläufer von hsa-mir-326, welche bei MS im Vergleich zu Gesunden in Blutzellen als verschieden exprimiert beschrieben wurde [105]. Ergänzend zu den Microarray-Messungen wäre es interessant, die reifen Formen der miRNA mittels rt-PCR in künftigen Studien weiter zu analysieren.

6.5. Ausblick

Es konnten in der vorliegenden Arbeit Erkenntnisse über die transkriptomweiten Effekte des S1P-Rezeptor-Modulators Fingolimod gewonnen werden. Es zeigten sich signifikante Änderungen in der Anzahl der im peripheren Blut zirkulierenden Zellen sowie den Genexpressionsprofilen der CD4+- CD8+-, und CD19+-Zellen. Die vorliegende Analyse könnte weiterführend durch Untersuchung der Effekte auf das Transkriptom über einen längeren Therapiezeitraum und eine größere Studienpopulation ausgebaut werden, auch im Hinblick auf die Identifikation von molekularen Biomarkern zur Prognose und Verlaufskontrolle. Neben Fingolimod gibt es bereits weitere S1P-Rezeptor-Modulatoren, wie Siponimod, Ponesimod und Ozanimod, welche für die Therapie der MS und anderer Autoimmunerkrankungen, wie Psoriasis oder systemischem Lupus erythematodes, erprobt werden [106]. Hierbei zeigte sich bei Siponimod über die bei Fingolimod bestehende Wirkung bei RRMS hinaus auch eine Verzögerung der Krankheitsprogredienz bei SPMS. Siponimod ist mit dieser Indikation seit 01/2020 EU-weit zugelassen [107]. Es wäre interessant zu untersuchen, inwieweit sich die Genexpressionsänderungen und Verschiebungen der Anteile zirkulierender B- und T-Zellsubpopulationen der verschiedenen S1P-Rezeptor-Modulator-Therapien unterscheiden.

Durch die Analyse des Transkriptoms konnten Erkenntnisse über den Effekt von Fingolimod auf die Signaltransduktion und funktionelle Veränderungen der verschiedenen Immunzellpopulationen gewonnen werden. Es war möglich durch die Transkriptomanalyse zu bestimmen, welche Gene durch die Medikamentenwirkung verändert transkribiert werden, allerdings ist bei vielen nicht-kodierenden Genen die Funktion und damit auch die Bedeutung einer veränderten Transkription bisher nicht bekannt. Durch alternatives Spleißen können aus einer prä-mRNA mehrere Varianten entstehen, die zu verschiedenen Proteinisoformen translatiert werden. Deshalb ist es interessant, anhand der Transkriptomdaten auch Spleißvarianten quantitativ zu bestimmen, was im Rahmen einer anderen Doktorarbeit an der Sektion Neuroimmunologie untersucht wird. Auch wäre es in Zukunft spannend, der Analyse des Proteoms, also der Gesamtheit der Proteine, näher zu kommen, was aktuell aufgrund technischer Schwierigkeiten eine sehr komplexe Untersuchung darstellt. Dabei könnte noch weitreichender analysiert werden, welche funktionellen Veränderungen der Immunzellpopulationen es im Rahmen von MS oder der Therapie mit Fingolimod gibt.

7. Zusammenfassung

Die Multiple Sklerose (MS) ist eine durch autoreaktive Immunreaktionen vermittelte chronisch-entzündliche Erkrankung des zentralen Nervensystems, die mit degenerativen Prozessen vor allem der weißen Substanz einhergeht. Die erste zugelassene orale sekundärprophylaktische Therapie zur Behandlung der schubförmigen MS war Fingolimod, ein Sphingosin-1-Phosphat (S1P)-Rezeptor-Modulator, der eine transiente Retention der im Blut zirkulierenden Immunzellen in Lymphknoten bzw. lymphoiden Geweben bewirkt.

Ziel der durchgeführten Untersuchungen war, die Wirksamkeit und Sicherheit der Therapie mit Fingolimod in einer Rostocker Kohorte zu untersuchen sowie die molekularen Effekte der Therapie mit Fingolimod auf Transkriptomebene besser zu verstehen.

Zu diesem Zweck wurde 11 Patienten mit schubförmig verlaufender MS, die vorher noch nicht mit Fingolimod behandelt wurden, zu 3 Zeitpunkten Blut abgenommen, jeweils vor der ersten Dosis von täglich 0,5 mg sowie 24 Stunden und 3 Monate nach der ersten Gabe von Fingolimod. Eine klinische Verlaufsbeurteilung mit Erfassung der Schubhäufigkeit sowie des Grades der Behinderung im Alltag erfolgte über 1 Jahr. In den entnommenen Blutproben wurden sowohl die Zellzahlen der CD4+-, CD8+-, CD14+-, CD19+- und CD56+- Immunzellen bestimmt als auch mittels hochauflösender Microarrays mit über 6 Millionen Gensonden eine vollständige Transkriptomanalyse der verschiedenen Zellpopulationen zu allen 3 Zeitpunkten für jeweils 10 Proben durchgeführt.

Durch die Therapie mit Fingolimod konnte bei der Mehrheit der Patienten eine Reduktion der Schubhäufigkeit ohne das Auftreten von beeinträchtigenden Nebenwirkungen erreicht werden. Im Verlauf der Therapie mit Fingolimod zeigten sich nach 24 Stunden kaum Veränderungen in der Anzahl der verschiedenen Zellpopulationen im Blut, nach 3 Monaten konnte allerdings eine 60-90%ige Reduktion in der Anzahl der CD4+-, CD8+- und CD19+-Zellen im peripheren Blut nachgewiesen werden. Bei diesen Zellpopulationen konnte zudem eine signifikante Veränderung des Genexpressionsprofils festgestellt werden. Bei den CD4+- und CD8+-Zellen, die die stärksten Veränderungen der Expressionsprofile zeigten, ergab eine Zuordnung der differentiell exprimierten Transkripte zu funktionellen Gruppen eine deutliche Assoziation zu Gengruppen, die bei der Regulation von Immunreaktionen und der Migration von Immunzellen wichtige Rollen spielen. Bei den CD19+-Zellen waren nach 3 Monaten Therapie mit Fingolimod 41 Gene stärker und 1 Gen schwächer exprimiert als zu Therapiebeginn. Die CD14+- und CD56+-Zellen zeigten nur eine geringe Absenkung der Zellzahlen im Blut sowie keine signifikanten Veränderungen der Genexpression nach 3 Monaten Therapie mit Fingolimod.

Durch die Analyse der Transkriptomveränderungen im Therapieverlauf mit Fingolimod konnten neue Erkenntnisse über die molekularen Effekte des Medikaments gewonnen werden. Es wäre

interessant weiterführend zu untersuchen, inwieweit sich diese Veränderungen auch im Proteom niederschlagen. Des Weiteren gibt es neue klinische Studien zu weiteren S1P-Rezeptor-Modulatoren, welche etwas selektiver einzelne S1P-Rezeptoren binden als Fingolimod. Durch vergleichende Transkriptomanalysen könnte man Gemeinsamkeiten und Unterschiede dieser Klasse von Medikamenten herausarbeiten.

8. Abkürzungsverzeichnis

Abb.	Abbildung
AGCC	Affymetrix GeneChip Console
autoMACS	automatischer magnetic-activated cell separator (Eigenname)
CIS	Klinisch isoliertes Syndrom (clinically isolated syndrome)
cRNA	Komplementäre RNA
DNA	Desoxyribonukleinsäure
EBV	Epstein-Barr Virus
EC	Expression Console
EDSS	Expanded Disability Status Scale
EDTA	Ethylendiamintetraessigsäure
FC	Fold change
GA	Glatirameracetat
GO	Gene Ontology
HLA	Humanes Leukozytenantigen
HTA	Human Transcriptome Array
IFN	Interferon
Ig	Immunglobulin
Kap.	Kapitel
miRNA	microRNA
mRNA	Messenger-RNA
MRT	Magnetresonanztomografie
MS	Multiple Sklerose
NK-Zellen	Natürliche Killerzellen
OKB	Oligoklonale Banden
Pat.	Patient
PPMS	Primär progrediente Multiple Sklerose
RIN	RNA integrity number
RMA	Robust Multi-array Average
RNA	Ribonukleinsäure
RRMS	Schubförmig remittierende Multiple Sklerose (relapsing-remitting multiple sclerosis)
rt-PCR	Echtzeit-Polymerase-Kettenreaktion (real time polymerase chain reaction)
S1P	Sphingosin-1-Phosphat
S1PR	Sphingosin-1-Phosphat-Rezeptor
SPMS	Sekundär progrediente Multiple Sklerose

TH	T-Helferzellen
TLR	Toll-like-Rezeptor
tRNA	Transfer-RNA
ZNS	Zentrales Nervensystem

9. Literaturverzeichnis

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10. Thesen

1. Die Multiple Sklerose (MS) ist eine chronisch-entzündliche Erkrankung des zentralen Nervensystems, welche sich typischerweise im Alter zwischen 20 und 40 Jahren klinisch manifestiert und zu verschiedenen neurologischen Defiziten führen kann. Die MS verläuft bei den meisten Patienten schubförmig.
2. Die sekundärprophylaktische Therapie der MS basiert auf immunmodulatorischen Medikamenten, welche die entzündliche Krankheitsaktivität hemmen und so die Behinderungsprogression der Patienten verringern. Eines dieser Medikamente ist Fingolimod, welches durch Modulation des Sphingosin-1-Phophat (S1P)-Rezeptors wirkt, welcher normalerweise durch Bindung von S1P eine Migration der Immunzellen durch Lymphknoten bewirkt. Durch Modulation des Rezeptors unter Fingolimod-Therapie werden die Immunzellen in den Lymphknoten zurückgehalten.
3. Durch Transkriptomanalysen, also die quantitative Bestimmung aller Transkripte bestimmter Zellen, können Veränderungen in der Genexpression verschiedener Zellpopulationen im zeitlichen Verlauf einer Therapie untersucht werden.
4. Ziel der vorliegenden Untersuchungen war es, die Effekte der Therapie mit Fingolimod auf das Transkriptom von Immunzellen im Blut von MS-Patienten zu analysieren sowie die funktionelle Bedeutung der Veränderungen für die einzelnen Zellpopulationen auszuwerten. Ferner war es Ziel, das klinische Ansprechen der Patienten auf die Therapie zu evaluieren.
5. Bei 11 MS-Patienten, bei denen eine Umstellung der immunmodulatorischen Therapie auf Fingolimod geplant war, wurde je vor der 1. Dosis sowie 24 Stunden und 3 Monate danach Blut entnommen. Hieraus wurden CD4+-, CD8+-, CD14+-, CD19+- und CD56+-Zellen isoliert, gezählt und deren RNA extrahiert. Eine klinische Beobachtung der Patienten erfolgte über ein Jahr.
6. Es erfolgte dann zu jedem Zeitpunkt von je 10 Proben pro Zellpopulation ein Transkriptomprofiling mittels high-densitiy Microarrays, welche alle derzeit bekannten protein-kodierenden und nicht-kodierenden Transkripte abdecken. Anschließend erfolgten vergleichende Analysen der Genexpressionsveränderungen, Assoziationsanalysen differentiell exprimierter Gene zu funktionellen Gengruppen sowie Untersuchungen der Effekte innerhalb der S1P-assoziierten Signalwege.
7. Bei 9 von 11 Patienten konnte eine Reduktion der Schubhäufigkeit ohne das Auftreten von beeinträchtigenden Nebenwirkungen erreicht werden. Es konnte bei allen untersuchten Zellpopulationen nach 3 Monaten Therapie mit Fingolimod eine Reduktion

der im Blut zirkulierenden Zellen festgestellt werden, am stärksten bei den CD4+-, CD8+- und CD19+-Zellen. Bei den 3 letztgenannten Zellpopulationen ließen sich auch signifikante Transkriptomveränderungen nachweisen. Nach 3 Monaten Therapie mit Fingolimod waren bei CD4+-Zellen 6489 Gene, bei CD8+-Zellen 861 Gene und bei CD19+-Zellen 42 Gene in ihrem Expressionslevel verändert ($p<0,001$, Fold change >1,5 oder <-1,5). 24 Stunden nach der ersten oralen Einnahme von Fingolimod zeigten sich bei allen 5 Zellpopulationen keine nennenswerten Transkriptomveränderungen.

8. Die vergleichende Analyse zeigte 11 Gene, welche bei CD4+-, CD8+- und CD19+-Zellen verstärkt exprimiert waren. Hierzu gehören auch die 3 protein-kodierenden Transkripte MYBL1, IQGAP2 und PTPN12. Der für das Homing in Lymphknoten wichtige Rezeptor CCR7 war bei den T-Zellen unter Therapie mit Fingolimod verringert exprimiert. Die Analyse der Effekte auf den S1P-Signalweg zeigte eine verminderte Wirkung von S1P.

9. Es zeigten sich bei den T-Zellen viele unter Therapie mit Fingolimod differentiell exprimierte Gene, die Verschiebungen von T-Zellubpopulationen aufzeigen und die bei der Regulation von verschiedenen Immunprozessen wirken. Bei den CD8+-Zellen war beispielsweise das Transkript für den Rezeptor für Interleukin 6, ein Zytokin, dessen fehlerhafte Regulation bei verschiedenen autoreaktiven T-Zell-Reaktionen nachgewiesen wurde, unter Therapie niedriger exprimiert

10. Unter den differentiell exprimierten Genen zeigte sich bei den T-Zellen eine Überrepräsentation bestimmter funktioneller Gengruppen. Bei den CD4+-Zellen waren es Gruppen der „Biologischen Prozesse“ wie beispielsweise „Regulation der Zytokinsekretion“ und „Positive Regulation der Lymphozyten-Aktivierung“. Bei den CD8+-Zellen war es z.B. die Gruppe der „G-Protein-gekoppelten Rezeptoren“.

11. Des Weiteren zeigten sich bei CD4+-, CD8+- und CD19+-Zellen verschiedene Vorläufersequenzen von miRNA in ihrem Expressionslevel verändert. Zwei solcher Vorläufertranskripte (für hsa-mir-644a und hsa-mir-4295) waren sowohl bei den CD4+- als auch bei den CD8+-Zellen unter Therapie verstärkt exprimiert. Deren Bedeutung bei MS ist jedoch noch nicht abschließend analysiert. Zur Validierung dieser Beobachtungen wäre eine Messung der reifen miRNA, z.B. mittels PCR-Untersuchungen, in der Zukunft sinnvoll.

12. Zusammenfassend lässt sich sagen, dass es unter der Therapie mit Fingolimod zu einer signifikanten Reduktion der Anzahl zirkulierender T- und B-Lymphozyten kommt. Die CD4+-, CD8+- und CD19+-Zellen zeigten außerdem signifikante Veränderungen des Genexpressionsprofils, insbesondere auch eine differentielle Expression von Genen, welche bei der Regulation von Immunprozessen mitwirken.

11. Danksagung

Ein großer Dank meinerseits geht an Prof. Dr. U. K. Zettl für die Überlassung der Themen und die Unterstützung bei der Erarbeitung und dem Perfektionieren der Texte.

Ich möchte mich bei allen Bedanken, die bei der Durchführung der Studie und der Veröffentlichung der Publikationen mitgewirkt haben. Insbesondere möchte ich mich bei Dr. D. Koczan für die Durchführung der Microarray-Experimente bedanken. J. Friess danke ich für die Vorverarbeitung der Daten. Mein Dank gilt auch L. Roch für die Analyse des S1P-Signalweges. Außerdem möchte ich mich bei N. Retzlaff und I. Schröder für die Erhebung der klinischen Daten und die Koordinierung der Blutprobensammlung bedanken.

Ein besonderer Dank gilt meinem Betreuer Dr. M. Hecker, der mich jedes Mal überrascht hat, wie schnell er es schafft, meine Fragen und Probleme zu lösen und durch wiederholtes Nachhaken und Zusprechen maßgeblich dazu beigetragen hat, dass nicht nur die Publikationen, sondern auch diese Dissertation wahr wurden.

Ich danke außerdem meinen Freunden und Mitbewohnern, insbesondere all den Menschen, denen ich meine teils festgefahrenen Gedanken vortragen und dabei selbst besser verstehen konnte, was oft ganz andere Fragen aufgeworfen hat, als ich dachte. Außerdem möchte ich mich bei meiner Mutter bedanken, da sie mich immer daran erinnert hat, dass ich meine Pläne und Träume umsetzen sollte, für mich. Ein besonderer Dank geht auch an meinen aktuellen Chef Dr. A. Kauert und meine Kollegen am Evangelischen Krankenhaus Königin Elisabeth Herzberge, Berlin, die mir durch die Möglichkeit, in Teilzeit zu arbeiten, den Rücken freigeräumt haben, damit ich diese Arbeit dann auch zu Ende bringen konnte.

12. Eidesstattliche Erklärung

Hiermit erkläre ich, Ines Charlotte Angerer, eidesstattlich, dass ich die vorliegende Dissertation selbstständig und ohne fremde Hilfe verfasst, keine anderen als die genannten Hilfsmittel genutzt und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Die vorgelegte Dissertation wurde bisher weder im Ausland noch im Inland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Rostock, den 22.01.2021 Ines Charlotte Angerer

13. Lebenslauf

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Publikationen

- **Angerer IC**, Hecker M, Koczan D, Roch L, Friess J, Rüge A, Fitzner B, Boxberger N, Schröder I, Flechtner K, Thiesen HJ, Winkelmann A, Meister S, Zettl UK:
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  Mol Neurobiol. 2017 Sep;54(7):5511-5525. doi:10.1007/s12035-016-0075-0.

Fremdsprachen

- Englisch mündlich und schriftlich fließend (Level C2) inkl. Fachwortschatz Medizin
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