THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie Santé

École doctorale Sciences Chimiques et Biologiques pour la Santé CBS2

Unité de recherche : Institut de Génomique Fonctionnelle

Etude des modulations du transcriptome des lymphocytes périphériques issus de patients atteints de sclérose en plaques traités par de fortes doses de vitamine D

Présentée par Manon GALOPPIN

Le 20 Octobre 2023

Sous la direction de Éric THOUVENOT

Devant le jury composé de

David LAPLAUD, PU-PH, CHU de Nantes, Univ. Nantes	**Rapporteur**
Laure MICHEL, PU-PH, CHU de Rennes, Univ. Rennes	**Rapportrice**
Anne ASTIER, Chargée de Recherche, INFINITy, Univ. Toulouse	**Examinatrice**
Philippe MARIN, Directeur de Recherche, IGF, Univ. Montpellier	**Examinateur**
Éric THOUVENOT, PU-PH, CHU de Nîmes, IGF, Univ. Montpellier	**Directeur de Thèse**

UNIVERSITÉ DE MONTPELLIER

À Mamie, Pépé, Christopher,

Et ceux partis trop tôt,

J'espère vous rendre fiers.

Remerciements

Merci aux membres du jury, au Dr. Anne ASTIER, Pr. David LAPLAUD, Pr. Laure MICHEL et Dr. Philippe MARIN d'avoir accepté d'évaluer mes travaux de thèse. Merci pour le temps que vous allez consacrer à la lecture de mon manuscrit de thèse. J'espère que la lecture vous sera agréable et que notre discussion sera riche.

Merci au Dr. Philippe MARIN, de m'avoir accueillie au sein de son équipe et pour les multiples échanges, toujours constructifs, au cours de ces 3 ans et demi.

Un immense merci au Pr. Éric THOUVENOT, pour m'avoir permis de réaliser cette thèse, pour m'avoir fait confiance sur un projet de cette envergure. Merci de m'avoir permis de me surpasser et de sortir looooooooin de ma zone de confort dans un projet si loin de mes compétences initiales.

Merci au Dr. Anne ASTIER et au Dr. Thomas MOORE-MORRIS, membres de mon jury de comité de suivi de thèse, pour avoir évaluer, année après année, mon travail et pour ces discussions riches et constructives.

Un grand merci au personnel du CHU de Nîmes avec qui j'ai eu l'occasion d'échanger à de multiples reprises tout au long de cette thèse. Merci à Brigitte LAFONT, Hanane AGHERBI, Céline LOISEAU et Violaine GRANDJEAN pour tout ce que vous avez fait pour mener à bien ce projet.

Merci à toutes les personnes affiliées aux diverses plateformes sans qui ce projet ne serait clairement pas ce qu'il est aujourd'hui. Merci à Amélie SARRAZIN, Myriam BOYER-CLAVEL et Stéphanie VIALA de la plateforme MRI, à Christelle REYNES, Manuela PASTORE et Léa BESNARD de la plateforme StataBio, à Anne-Laure ISCACHE et Hugo GARNIER de la plateforme de cytométrie d'INFINITY de Toulouse, à Sandra ESPEOUT-

FOIS et Aurore VERNET du CIRAD, et à Laurent JOURNOT, Dany SEVERAC, Simon GEORGE de la plateforme MGX. Un merci tout particulier pour Anaïs LOUIS de MGX, pour tout le soutien technique et surtout moral tout le long de ces interminables analyses. Merci pour ces multiples allers-retours entre ton bureau et le mien, souvent juste pour cliquer sur « lancer la ligne de script » sur R pour que ça fonctionne après mes 150 essaies infructueux.

Merci à Juliette FERRANT (CHU Rennes), Emilie DUGAST (CR2TI, Nantes), Camille KERGARAVAT (Institut Saint-Louis, Paris) et Sita SHAH (CR2TI, Nantes) pour tous ces échanges et leur aide pour les analyses. Merci d'avoir pris le temps de répondre à mes questions parfois si naïves.

Un grand merci aux ingénieurs techniques et commerciaux avec qui j'ai eu l'occasion d'échanger tout au long de ce projet et en particulier à Mathilde PERRUCHON (Thermo Fisher), Céleste Letellier et Roberta Benedetto (Standard Bio) pour leur incroyable aide et soutien technique dans les analyses et dans les situations d'urgence. La recherche ne serait pas ce qu'elle est sans des personnes comme vous.

Merci à tous les services communs de l'IGF, qui permettent d'exercer la recherche dans de bonnes conditions.

Un énooooooooooorme merci à l'équipe Marin, aux anciens comme aux nouveaux. Merci aux chercheurs, en particulier Sylvie pour ton implication au sein de l'équipe. Merci d'avoir passé toutes ces commandes au début de ma thèse quand je me retrouvais bien souvent démunie. Merci à Tomas, Thomas, Nesrine et Lucy pour m'avoir supporté, fait rire et parfois pleurer pendant toutes ces années. Merci à toutes les personnes, anciennes ou moins anciennes, du « petit bureau des tarées » en particulier Lucile, Ophélie, Alina et Manon pour la bonne ambiance quotidienne et ces discussions à n'en plus finir. J'ai adoré passer des heures ici à refaire le monde avec vous. Merci au reste de l'équipe, Alessandro, Mehdi, Sonya, Yéléna,

Candice, Rachel, Magali et Aurore, pour ces moments passés en votre compagnie, pour ces verres au bar et tous ces potins partagés. Merci aux personnes ‘de passage’, qui ont contribué à rendre ces années moins pénibles et à créer des souvenirs mémorables, en particulier Jade, Amanda et Gabriel.

Merci aux amis « de l’extérieur », en particulier Laurène et Zouhir pour leur soutien infaillible pendant ces 3 ans et demi et leur patience pendant mes moments de décharge. Vous n’imaginez pas à quel point vous m’avez aidé, que ce soit côté pro- ou perso-. Je ne vous en serai jamais assez reconnaissante.

Et enfin, un énorme merci à mes parents et à mon petit frère pour avoir su m’écouter et pour m’avoir laisser parler pendant des heures d’un sujet qui vous échappait, presque sans jamais m’interrompre. J’espère, vous aussi, vous rendre fiers. Je vous aime.

Résumé

La sclérose en plaques (SEP) est une maladie auto-immune qui affecte le système nerveux central (SNC) au niveau duquel une dysfonction du système immunitaire entraîne des lésions qui provoquent des symptômes moteurs, sensitifs et cognitifs. En France, 115.000 personnes sont atteintes de la SEP et 5.000 nouveaux cas sont diagnostiqués chaque année. La SEP est la première cause de handicap non traumatique chez le jeune adulte. Il s'agit d'une maladie aux causes indéterminées mais multiples : des facteurs propres à l'individu (génétiques, hormonaux) et des facteurs environnementaux (toxiques, infectieux, carence en vitamine D) interagissent dans le déclenchement de la SEP. La carence en vitamine D est également associée à l'activité et à la progression de la maladie. Le traitement par la vitamine D est apparu comme potentiellement protecteur, bien que les résultats des études contrôlées randomisées soient contradictoires. Dans cette étude, nous avons utilisé le single-cell RNA seq (scRNA-seq) couplé à des anticorps barcodés ciblant des marqueurs de surface (CITE-seq) pour découvrir des gènes candidats et des voies régulées dans les sous-populations de PBMC de patients atteints de SEP naïfs de traitement de fond de la SEP et traités en monothérapie par de fortes doses de vitamine D (n=5), par rapport à un placebo (n=5). Les gènes candidats ont été révélés à l'aide de plusieurs méthodes (supervisée et non supervisée) et plusieurs analyses statistiques (global ratio et DESeq2). Les meilleurs candidats ont été associés à des gènes impliqués dans la fonction immunitaire et le métabolisme de la vitamine D (178 gènes au total) pour être validés dans une nouvelle cohorte (n=8 dans chaque groupe) par qPCR à haut débit (HT-qPCR) dans les cellules CD4 naïfs, Th1, Th17, Treg, CD8 naïfs, les cellules B mémoires et naïfs, et les cellules MAIT, triées par FACS. Dans l'analyse non supervisée, l'analyse CITE-seq a montré une augmentation significative dans la proportion des Th2 chez les patients traités à la vitamine D contre une augmentation de la population Tc1 dans l'analyse supervisée. Sur les 92 gènes candidats révélés par CITE-seq, l'expression différentielle de cinq gènes (UXT, SNRPN, SUB1, GNLY et KLF6)

a été validée par HT-qPCR. De plus, l'ajout des gènes de la littérature à nos gènes candidats a permis d'observer des modulations de certains transcrits codants pour des protéines d'intérêt dans le contexte de la SEP comme une diminution de CXCL13 dans les B mémoires, une diminution de la voie IL6/JAK1/STAT3 et de CD46, CD69, CD226, ITGA4 dans les Th1, population de lymphocyte T semblant être la cible la plus importante de la vitamine D. Le CITE-seq a enfin révélé que la vitamine D régule plusieurs voies de signalisation dans les B naïfs et mémoires, y compris les voies MAPK, TLR et interleukines, et pourrait contribuer à contrecarrer la résistance à l'apoptose induite par l'EBV par l'inhibition de la voie NF-κB.

Abstract

Multiple sclerosis (MS) is an autoimmune disease affecting the central nervous system (CNS), where immune system dysfunction leads to lesions that cause motor, sensory and cognitive disturbances. In France, 115,000 people suffer from MS, and 5,000 new cases are diagnosed every year. MS is the leading cause of non-traumatic disability in young adults. It is a disease with indeterminate but multiple causes. Factors specific to the individual (genetic, hormonal) and environmental factors (toxic, infectious, vitamin D deficiency) interact to trigger MS. Vitamin D deficiency is associated with disease activity and progression. Vitamin D treatment has emerged as potentially protective, although the results of randomized controlled trials are contradictory. In this study, we used single-cell RNA seq (scRNA-seq) coupled to barcoded antibodies targeting surface markers (CITE-seq) to discover candidate genes and regulated pathways in PBMC subpopulations of MS patients naïve to background MS therapy but treated with high-dose vitamin D (n=5), versus placebo (n=5). Candidate genes were identified using several methods (supervised and unsupervised) and statistical analyses (global ratio and DESeq2). The best candidates were linked to genes involved in immune function and vitamin D metabolism (178 genes in total) for validation in a new cohort (n=8 in each group) by high-throughput qPCR (HT-qPCR) in naive CD4, Th1, Th17, Treg, naive CD8, memory and naive B cells, and MAIT cells, sorted by FACS. In the unsupervised analysis, CITE-seq showed a significant increase in the proportion of Th2 in vitamin D-treated patients versus an increase in the Tc1 population in the supervised analysis. Of the 92 candidate genes revealed by CITE-seq, the differential expression of five genes (UXT, SNRPN, SUB1, GNLY and KLF6) was validated by HT-qPCR. Moreover, the addition of genes from the literature to our candidate genes enabled us to observe modulations of certain transcripts encoding proteins of interest in the context of MS, such as a decrease in CXCL13 in memory B cells, a decrease in the IL6/JAK1/STAT3 pathway and in CD46, CD69, CD226, ITGA4 in Th1 cells, the T cell

population appearing to be the most important target of vitamin D. CITE-seq also revealed the regulation of several pathways by vitamin D in naive and memory Bs, including MAPK, TLR and interleukin pathways, which contribute to counteracting EBV-induced resistance to apoptosis through inhibition of the NF-κB pathway.

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Abréviations

ANR : Agence Nationale de la Recherche

ARSEP: Fondation pour la recherche sur la sclérose en plaques

BHE : Barrière Hémato-Encéphalique

BOC : Bandes Oligo-Clonales

BTKi: Inhibiteur de la tyrosine kinase de Bruton

CD-X : Cluster de différentiation -X

CITE-seq: *Cellular Indexing of Transcriptomes and Epitopes by sequencing*

CM: Central mémoires

CYP24A1: Cytochrome P450 24A1

CYP27B1: Cytochrome P450 27B1

DC : Cellules dendritiques

DEGs : Gènes différentiellement exprimés

EAE : Encéphalomyélite Auto-immune Expérimentale

EBV: Virus Epstein-Barr

EDSS: *Expanded Disability Status Scale*

EM: Effecteurs mémoires

EMRA+ : Effecteurs ($CD45RA^{+}$)

FACS: *Fluorescence-Activated Cell Sorting*

HLA: Antigène d'histocompatibilité

HT-qPCR: *High-Througput quantitative Polymerase Chain Reaction*

IFN-γ : Interféron Gamma

Ig-X : Immunoglobuline -X

IL-X-RA : Interleukine-X récepteur sous unité alpha

IL-X : Interleukine-X

IMC : Indice de Masse Corporelle

IRM: Imagerie par résonance magnétique

LCR : Liquide Céphalo-Rachidien

MAIT: *Mucosal-Associated Invariant T cells*

MBP : *Myelin Basic Protein*

MOG : *Myelin Oligodendrocyte Glycoprotein*

NfL : *Neurofilament Light Chain*

NK-T: *Natural Killers - T*

NORB : Névrite Optique RétroBulbaire

PBMC: *Peripheral Blood Mononuclear Cells* – Cellules mononuclées du sang

PPMS : Sclérose en plaques primaire progressive

PSMS : Sclérose en plaques progressive secondaire

RRMS Sclérose en plaques récurrente rémittente

SCI: Syndrome cliniquement isolé

scRNA-seq: *Single-cell RNA sequencing*

SEP: Sclérose en plaques

SNC : Système Nerveux central

Tc-1/2/17 : Lymphocyte cytototoxique-1/2/17

TCR : Récepteur des lymphocytes T

Tcreg : Lymphocyte cytotoxique régulateur

TGF-β : *Transforming Growth Factor Bêta*

Th-1/2/17: Lymphocyte Helper-1/2/17

TNFα : *Tumor Necrosis Factor Alpha*

Treg: Lymphocyte Régulateur

UMAP: *Uniform Manifold Approximation and Projection*

UMI: Unique Molecular Identifier

Partie I – Introduction

Chapitre I : La Sclérose en plaques

La sclérose en plaques (SEP) est une maladie auto-immune qui affecte le système nerveux central (SNC) au niveau duquel une dysfonction du système immunitaire entraîne des lésions qui provoquent des symptômes moteurs, sensitifs, cognitifs, visuels ou encore sphinctériens (le plus souvent urinaires). A plus ou moins long terme, ces troubles peuvent progresser vers un handicap irréversible.

I.I Épidémiologie

I.I.a. En quelques chiffres

En France, environ 115 000 personnes sont atteintes de sclérose en plaques et 5 000 nouveaux cas sont diagnostiqués chaque année. Elle touche majoritairement les femmes avec un sex-ratio de 3 : 1. Le diagnostic est généralement établi entre 20 et 40 ans, soit à une période de la vie cruciale sur le plan personnel, familial, professionnel et social. La SEP est la première cause de handicap non traumatique chez le jeune adulte, elle conduit à un handicap permanent, plus ou moins sévère, mais n'influence que peu l'espérance de vie. Il s'agit d'une maladie aux causes indéterminées mais multiples : des facteurs propres à l'individu (génétiques, hormonaux) et des facteurs environnementaux (toxiques, infectieux, carence en vitamine D) interagissent dans le déclenchement de la SEP.

I.II.b. Les facteurs de risques

Plusieurs facteurs de risque ont été impliqués dans le développement de la SEP. Une revue rédigée par Ramagopalan et al.[1] a fait un résumé didactique des facteurs génétiques, environnementaux et hormonaux qui jouent tous un rôle dans la susceptibilité à la maladie. Les

antécédents familiaux de SEP, l'exposition au tabagisme, la carence en vitamine D et les infections virales, tels que le virus d'Epstein-Barr (EBV), ont été associés à un risque accru de développer la maladie.

I.II.b.1. Les Facteurs génétiques

I.II.b.1.a. Génétiques et système HLA

La SEP n'est pas une maladie héréditaire puisqu'elle est non transmissible génétiquement. Cependant, il existe des facteurs génétiques favorables à son développement, sous l'influence d'autres facteurs (notamment environnementaux). Les formes familiales ne sont pas rares, et si un membre de la fratrie est atteint, le risque est multiplié par 9[2]. Depuis les années 1970, comme pour de nombreuses maladies auto-immunes, des gènes associés au développement de cette maladie ont été progressivement découverts, notamment au niveau du groupe HLA. En 2007, deux variants génétiques ont été identifiés, *IL2RA* et *IL7RA*. Ils codent tous les deux pour des récepteurs d'interleukine impliqués dans l'immunité et leur présence augmente (faiblement) le risque de maladie[3]. Des études d'association génétique ont identifié et associé plusieurs variants génétiques aux risques de développer la SEP. Sawcer et ses collaborateurs en 2011, ont identifié une cinquantaine de gènes qui influencent le risque de développer la SEP, quoique très faiblement pour la plupart des gènes[4]. Fait intéressant, deux d'entre eux codent pour des protéines qui jouent un rôle important dans le métabolisme de la vitamine D (*CYP27B1*, *CYP24A1*). Des études récentes suggèrent que le *CYP27B1* pourrait jouer un rôle critique dans le développement de la SEP chez certains individus mutants homozygotes[5]. De plus, les analyses ciblées sur les gènes de l'immunité ont révélé de nombreux loci supplémentaires constituant des facteurs de risques de SEP[6].

I.II.b.1.b. Hormones féminines et obésité

Comme pour la plupart des pathologies auto-immunes comme la polyarthrite rhumatoïde, les femmes sont plus touchées que les hommes, avec un sex-ratio d'environ 3 :1. Cette différence est principalement expliquée par l'influence des hormones sexuelles sur l'immunité. La survenue des poussées est également plus rare chez les femmes enceintes et plus fréquente au cours des trois mois suivant l'accouchement[7].

Plusieurs travaux ont montré une association significative entre un indice de masse corporelle (IMC) élevé pendant l'enfance (obésité) et un risque accru de développer une SEP ultérieurement[8,9]. Néanmoins, une étude a aussi montré que si l'IMC est élevé pendant l'enfance mais ne l'est plus à 20 ans, alors le risque de SEP n'est plus augmenté[10,11]. L'adolescence semble être la période critique dans le développement de la SEP.

I.II.b.2. Les Facteurs environnementaux

I.II.b.2.a. Facteurs toxiques : tabac et alcool

Plusieurs études épidémiologiques ont mis en évidence une association entre le tabagisme et un risque accru de développer la SEP[12]. Des études prospectives ont montré que les fumeurs actuels et anciens présentaient un risque plus élevé de développer la maladie par rapport aux non-fumeurs[13]. De plus, il a été observé que le tabagisme était associé à une progression plus rapide de la SEP et à une augmentation de la sévérité de symptômes[14].

Les données concernant la consommation d'alcool et la SEP sont moins concluantes que celles concernant le tabagisme. Certaines études suggèrent une association entre une consommation modérée d'alcool et un risque réduit de développer la SEP[15–18]. Cependant, d'autres études ont rapporté des résultats contradictoires[19]. Il est important de noter que la consommation excessive d'alcool peut avoir des effets néfastes sur la santé en général et sur le système immunitaire en particulier, ce qui peut aggraver les symptômes de la SEP.

Les mécanismes par lesquels la consommation de tabac peut influencer le développement et la progression de la SEP ne sont pas entièrement compris. Cependant, des études suggèrent que le tabagisme peut induire des changements dans la réponse immunitaire, favoriser l'inflammation et altérer la barrière hémato-encéphalique, ce qui peut contribuer à la pathogenèse de la maladie[14].

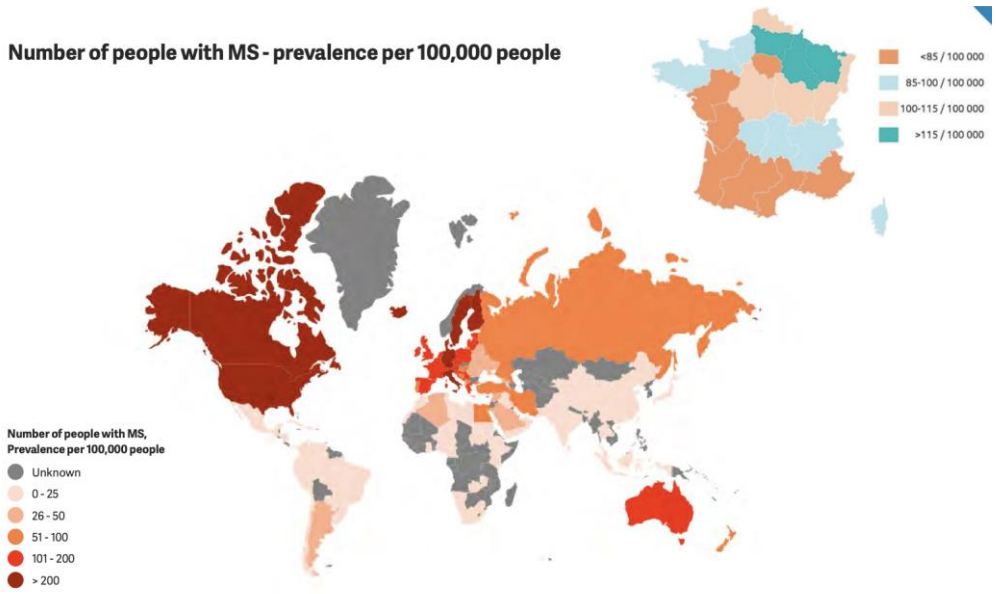
I.II.b.2.b. Facteurs infectieux : EBV

Le virus d'Epstein-Barr, l'agent causal de la mononucléose infectieuse, a été identifié comme un facteur de risque potentiel pour la SEP[20,21]. L'EBV est un virus de l'herpès qui infecte la plupart des individus pendant l'enfance. Une infection primaire avec l'EBV à l'adolescence ou à l'âge adulte peut entraîner la mononucléose infectieuse, une maladie caractérisée par une fatigue intense, une fièvre, une pharyngite et des adénopathies[22]. Plusieurs études épidémiologiques ont montré une association entre une infection par l'EBV, en particulier la mononucléose infectieuse, et un risque accru de SEP. Une méta-analyse de 18 études a révélé que les individus ayant eu une mononucléose infectieuse avaient un risque presque deux fois plus élevé de développer une SEP par rapport à ceux qui n'avaient pas eu cette maladie[23]. En outre, des études sérologiques ont montré que les individus atteints de SEP ont des titres d'anticorps anti-EBV plus élevés que les individus en bonne santé, suggérant une réponse immunitaire anormale à l'EBV chez les personnes atteintes de SEP[24]. Cependant, le mécanisme exact par lequel l'EBV pourrait contribuer à la pathogenèse de la SEP reste incertain. Une hypothèse est que l'EBV pourrait altérer la régulation du système immunitaire, conduisant à une auto-immunité[25].

I.II.b.2.c. Soleil et Vitamine D

L'exposition au soleil est la principale source de vitamine D pour la plupart des individus. La vitamine D est synthétisée dans la peau en réponse à l'exposition aux rayons ultraviolets B (UVB) du soleil. Une fois activée, la vitamine D joue un rôle crucial dans la régulation du

système immunitaire, ce qui pourrait expliquer son association avec la SEP[26]. Plusieurs études épidémiologiques ont montré une association inverse entre l'exposition au soleil et le risque de SEP. Par exemple, une étude a révélé que les individus ayant une exposition au soleil plus élevée pendant l'enfance et l'adolescence avaient un risque plus faible de développer une SEP à l'âge adulte[27]. Le niveau d'ensoleillement pourrait jouer un rôle dans la répartition de la maladie à travers le monde. En effet, celle-ci n'est pas uniforme : il existe un gradient de latitude Nord-Sud dans l'hémisphère Nord, et un gradient Sud–Nord dans l'hémisphère Sud, c'est-à-dire que la maladie est plus fréquente lorsqu'on s'éloigne de l'équateur. On trouve ainsi des zones de haute prévalence de la maladie (supérieure à 100 pour 100 000 habitants) en Amérique du Nord et en Europe du Nord, et des zones de basse prévalence (inférieure à 20) dans les régions équatoriales d'Amérique du sud, d'Afrique ou d'Asie[28,29]. Ce gradient est également observable en France où la prévalence de la maladie est plus faible dans les régions sud que dans les régions nord.



https://www.msif.org/wp-content/uploads/2020/12/Atlas-3rd-Edition-Epidemiology-report-EN-updated-30-9-20.pdf – 10.1136/jnnp-2018-320189

De même, des études ont montré une association inverse entre les niveaux de vitamine D et le risque de SEP. Une étude de cohorte a révélé que les individus avec des niveaux plus élevés de vitamine D avaient un risque plus faible de développer une SEP[30].

Cependant, le mécanisme exact par lequel l'exposition au soleil et la vitamine D pourraient protéger contre la SEP reste incertain. Une hypothèse est que la vitamine D pourrait moduler la réponse immunitaire, réduisant ainsi l'inflammation et la démyélinisation dans la SEP[31].

Le rôle et l'effet de la vitamine D dans la SEP est décrit dans une revue de littérature qui fait l'objet du second chapitre de cette thèse.

I.II Clinique

I.II.a. Les formes évolutives

La majorité des patients atteints de SEP (80 à 85 %) débutent leur maladie par un syndrome cliniquement isolé (SCI)[32] qui se manifeste le plus souvent par une névrite optique, des troubles de l'équilibre et de la marche ou par des troubles sensitifs.

La forme récurrente-rémittente de SEP, est caractérisée cliniquement par l'apparition de nouvelles poussées ou de nouvelles lésions démyélinisantes focales, caractéristiques de la forme active de SEP, qui témoignent de l'activité inflammatoire de la SEP. Les poussées sont des troubles neurologiques s'installant en quelques heures à quelques jours et généralement régressifs en quelques jours à quelques semaines, voire moins (au moins en début de maladie). Les poussées peuvent toucher toutes les fonctions neurologiques (vision, motricité, sensibilité, etc.), selon la localisation de la(des) nouvelle(s) lésion(s) inflammatoire(s). La maladie est définie par la répétition de poussées différentes, témoignant de la dissémination spatio-temporelle de l'inflammation.

Environ 15% des patients entrent dans la maladie par une forme progressive, caractérisée par une aggravation lente et progressive de troubles neurologiques associant troubles de la marche et de l'équilibre, troubles sphinctériens et troubles cognitifs.

Les formes classiques de la sclérose en plaques associent ces deux modes évolutifs : activité et progression.

- La forme **récurrente-rémittente** (RRMS) : exacerbations alternant avec des rémissions lors desquelles une reprise partielle ou totale ou une stabilité des symptômes est observée. Les rémissions peuvent durer des mois ou des années. Les exacerbations peuvent survenir spontanément ou être déclenchées par une infection comme la grippe.
- La forme **progressive primaire** (PPMS) : la maladie évolue progressivement sans rémissions, mais il peut y avoir des plateaux temporaires pendant lesquels la maladie ne progresse pas.
- La forme **secondairement progressive** (SPMS) : cette tendance commence avec des rechutes alternant avec des rémissions, suivies par une progression graduelle de la maladie.

I.II.b. Critères diagnostiques

I.II.b.1. Cliniques

Les symptômes de la SEP ainsi que leur intensité sont différents d'un patient à un autre. Ils peuvent être nombreux, visibles ou invisibles.

I.II.b.1.a Les symptômes invisibles

Il s'agit d'un type de symptômes extrêmement fréquents, souvent difficile à décrire et impacte directement la qualité de vie des patients. L'un des premiers symptômes invisibles est la présence d'une asthénie (fatigue) importante. Elle devient anormale lorsqu'elle perdure malgré le sommeil et le repos.

Les troubles de la mémoire, souvent dus à un déficit attentionnel et à la fatigabilité sont également des symptômes invisibles fréquents de la SEP. Le diagnostic est possible grâce à des tests cognitifs qui permettent de distinguer une véritable atteinte cognitive d'une fatigabilité. Le ralentissement du traitement de l'information, les difficultés à réaliser des tâches complexes et multiples sont les signes d'une atteinte cognitive.

D'autres symptômes fonctionnels extrêmement fréquents sont les troubles urinaires (urgenturie ou difficultés à la vidange vésicale) et les douleurs neuropathiques.

Les troubles de l'humeur font également partie des symptômes invisibles. Ils sont variés, allant de l'anxiété au symptôme dépressif. Ce ne sont pas des symptômes directs de la maladie mais des conséquences du diagnostic, de l'inquiétude de l'avenir et des contraintes que la SEP impose.

Un autre symptôme invisible fréquent mais peu rapporté par les patients est la présence de troubles sexuels, qui restent trop souvent tabous. Ils sont souvent sous-estimés mais peuvent affecter plus d'un patient sur deux, femme comme homme et ont un impact considérable sur la qualité de vie. Ce trouble est souvent associé à une baisse de l'estime de soi ainsi qu'à un sentiment de dépréciation et à un syndrome dépressif. Ils sont parfois associés à une baisse de libido.

I.II.b.1.b Les symptômes visibles

L'un des symptômes visibles les plus évocateurs du diagnostic de SEP est la névrite optique rétro-bulbaire (NORB)secondaire à l'atteinte du nerf optique. Près de 25 à 30% des patients sont diagnostiqués à la suite de l'apparition de ce symptôme. Le patient rapporte généralement une baisse rapide progressive de la vue d'un œil, associée à une diminution de la vision des couleurs et des contrastes et le plus souvent accompagné d'une douleur rétro-orbitaire.

Des troubles de la sensibilité sont également fréquents dans le contexte de SEP avec une diminution de la sensibilité ou des douleurs : par exemple, la névralgie du trijumeau (douleur du visage) est un signe fréquent et extrêmement douloureux.

Les troubles moteurs sont représentés en premier par une faiblesse des membres inférieurs (uni ou bilatérale) et une gêne dans la marche. Ils s'expriment aussi par une fatigabilité et une diminution de l'endurance et sont dus à une atteinte des faisceaux pyramidaux.

Les troubles de l'équilibre peuvent s'exprimer avec ou sans la présence de vertiges. Ils peuvent être dus à différentes origines : une origine vestibulaire, une origine cérébelleuse ou une origine médullaire, par atteinte cordonale postérieure. Dans certains cas, il est également possible de voir apparaître des troubles de la parole et de la déglutition.

Il existe des traitements symptomatiques pour essayer de réduire l'apparition de l'ensemble de ces symptômes (visibles et invisibles) et ainsi améliorer la qualité de vie des patients.

I.II.b.2. Radiologiques

En 2001, un panel international a recommandé la mise en place de nouveaux critères diagnostiques pour la SEP[33]. Ces critères ont été révisés en 2005[34] et en 2010[35]. Pour établir le diagnostic de SEP cliniquement définie, les présentations cliniques (poussées et lésions) doivent remplir des critères de dissémination dans le temps et dans l'espace (symptômes différents espacés d'au moins 30 jours).

L'IRM est l'examen d'imagerie de référence pour le diagnostic et le suivi des patients atteints de SEP. En mettant en évidence la dissémination des plaques dans plusieurs régions caractéristiques du cerveau et de la moelle épinière, il va contribuer à poser le diagnostic et ce, dès la première poussée. Si un patient présente au moins une lésion dans 2 des 4 territoires suivants, il réunit les critères de dissémination spatiale nécessaires au diagnostic de SEP[36] : périvasculaire, juxta-corticale, sous-tentorielle (sous la tente du cervelet) et médullaire.

L'IRM peut également aider à réunir les critères de dissémination temporelle. C'est le cas quand sur une même IRM, il existe des lésions dites « actives » (positives à la prise de contraste, lésions gadolinium positives) et des lésions dites « non actives » (lésions gadolinium négatives), mettant ainsi en évidence la présence de lésions d'âge différent.

Les plaques sont principalement visualisées au niveau de la substance blanche mais peuvent être présentes dans la substance grise, au niveau du cortex, dès les premiers stades de la maladie[37–39].

I.II.b.3. Biologiques

Le liquide céphalorachidien (LCR) baigne le SNC, entraînant avec lui des molécules et des cellules produites lors d'infections, d'inflammations, de lésions cellulaires et par la perte d'intégrité de la barrière hémato-encéphalique (BHE)[40]. L'analyse du LCR est donc cruciale pour comprendre les mécanismes pathologiques qui se produisent dans le SNC. Cette analyse est rendue possible grâce à la ponction lombaire.

La présence de cellules inflammatoires et une augmentation de la synthèse intrathécale d'IgG, de cytokines et de molécules d'adhésion indiquent la présence d'une inflammation. En ce qui concerne les processus neurodégénératifs, ils peuvent être associés à la présence ou à l'augmentation de protéines spécifiques aux cellules du SNC (comme *neurofilament light chain* (NFL) et *glial fibrillary acidic protein* (GFAP)) qui sont corrélés à la progression du handicap dans le cas de la SEP[40–42].

L'analyse du LCR permet de détecter un profil caractéristique de la SEP qui, bien qu'il ne soit pas spécifique à la maladie, possède une valeur diagnostique et pronostique[36]. Ce profil se caractérise par une augmentation des leucocytes de 5 à 50/µL, ainsi que la présence d'une synthèse intrathécale d'IgG[41]. Cette dernière peut être mise en évidence soit par une augmentation de l'index IgG, soit de manière plus sensible par la présence de bandes

oligoclonales (BOC) qui sont absentes ou présentes en quantité moindre dans le sérum[43] ou par une augmentation de l'index des chaines légères libres kappa (index kappa).

I.III Physiopathologie

I.III.a. Une maladie auto-immune et neurodégénérative

Bien que l'origine de la SEP ne soit pas connue, la majorité des hypothèses suggèrent que l'élément initiateur soit un phénomène d'activation de lymphocytes T auto-réactifs contre la myéline du SNC conjointement associé à une dérégulation de la balance immunitaire entre les différentes populations lymphocytaires. Plusieurs populations de lymphocytes jouent un rôle crucial dans la pathogenèse de la SEP, y compris les lymphocytes T $CD4^+$ auxiliaires (Th1, Th2, Th17), les lymphocytes T régulateurs (Tregs), les lymphocytes $CD8^+$ et les lymphocytes B[2].

I.III.a.1. Les différents acteurs de l'inflammation périphérique

I.III.a.1.a. Les Th1

Parmi les sous-populations de lymphocytes T, les lymphocytes T de type 1 (Th1) ont été largement étudiés en relation avec la SEP[44]. Ils sont dérivés des lymphocytes T $CD4^+$ naïfs sous l'effet des cytokines IL-12 (composée des sous unités IL-12p35 et IL-12p40) et de l'interféron-gamma (IFN-γ). Caractérisés par l'expression du facteur de transcription *T-bet* et par la production de Tumor Necrosis Factor (TNFα), d'IFN-γ, d'interleukine 2 (IL-2) (qui servent à l'activation des macrophages, des natural killers (NK) et des lymphocytes $CD8^+$), ils sont impliqués dans la réponse immunitaire cellulaire et dans la destruction des myélines observée dans la SEP[45]. L'implication des Th1 dans la SEP a été étudiée dans le modèle animal de la sclérose en plaques : l'encéphalomyélite auto-immune expérimentale (EAE). Par exemple, dans un modèle de souris KO pour l'IL-12p40 ou T-bet, la maladie ne se développe pas[46]. Chez l'Homme, une corrélation positive entre le taux sérique de l'IFN-γ et le score de handicap de la

maladie (EDSS : Expanded Disability Status Scale) a été mis en évidence[47,48]. L'IL-12 est également détectée dans le LCR des patients SEP et son taux est corrélé à l'activité de la maladie[49]. Les lymphocytes Th1 jouent un rôle important dans la pathogenèse de la sclérose en plaques en induisant une réponse immunitaire cellulaire et en contribuant à la destruction de la myéline. La modulation des réponses des lymphocytes Th1 pourrait représenter une stratégie thérapeutique prometteuse pour le traitement de la SEP.

I.III.a.1.b. Les Th17

Les lymphocytes T helper 17 (Th17) jouent un rôle crucial dans la pathogenèse de la SEP. Les Th17 sont une sous-population de lymphocytes T $CD4^+$ qui produisent l'interleukine-17 (IL-17) et d'autres cytokines pro-inflammatoires. Ils sont définis par l'expression du facteur de transcription *RORγT* et par la sécrétion de cytokines pro-inflammatoires comme IL-17, IL-21, IL-22 ou encore IL-26. Ils sont décrits comme une population cellulaire très impliquée dans la physiopathologie de la SEP, notamment à cause de leur importance dans le développement des modèles EAE[50]. Les lymphocytes Th17 sont capables de traverser la barrière hémato-encéphalique (BHE) et d'induire une inflammation dans le SNC[51,52], ce qui contribue à la progression de la SEP. L'équipe de Komiyama a mis en évidence la contribution des Th17 dans la pathogenèse de l'EAE[53]. Leur implication est renforcée par le fait que les souris KO pour RORγT sont résistantes à l'induction de la maladie[54]. Chez l'Homme, l'ARNm de l'IL-17 ainsi que la protéine elle-même sont augmentés dans le LCR et le sang de patient SEP comparativement à des témoins sains, en particulier lors des poussées[55,56].

Une équipe a récemment montré que les patients SCI et les patients ayant différentes formes de SEP présentaient un profil cellulaire et cytokinique lié au Th1- Th17- distincts, ce qui permettrait d'établir une discrimination entre les différentes formes cliniques. La fréquence des cellules Th1 est plus élevée dans le SCI et la RRMS que dans la SEP progressive, et la RRMS présente une fréquence de cellules Th17 plus élevée que le SCI. Ils ont également suggéré que

ces paramètres pourraient être utiles comme biomarqueurs pour prédire la progression de la maladie[57]. Cette étude souligne l'importance de l'équilibre entre les sous populations de lymphocytes T dans la SEP.

I.III.a.1.c. Les Th1.17

Les lymphocytes Th1-like Th17 (appelés parfois Th1/Th17) sont un sous-type particulier de lymphocytes T $CD4^+$ qui montrent des caractéristiques à la fois des lymphocytes Th1 (production d'IFN-γ) et des Th17 (production d'IL-17) [58] . Ils contribuent à l'attaque du système nerveux central et à la démyélinisation en interagissant avec d'autres cellules immunitaires, telles que les macrophages et les cellules gliales, pour amplifier la réponse inflammatoire dans le SNC[51]. Les lymphocytes Th17 peuvent se transformer en lymphocytes Th1-like Th17 en réponse à des signaux spécifiques dans le microenvironnement inflammatoire, ce qui peut contribuer à la diversité des réponses immunitaires observées dans la SEP[59].

I.III.a.1.d. Les Treg

Les lymphocytes T régulateurs (Tregs) ont émergé comme des acteurs essentiels dans la régulation de la réponse immunitaire et de la tolérance immunitaire. Ils constituent également une sous population des lymphocytes $CD4^+$, caractérisée par l'expression du facteur de transcription *FoxP3* et jouent un rôle clé dans le maintien de la tolérance immunitaire en inhibant les réponses des lymphocytes T effecteurs via différents mécanismes encore mal connus : en sécrétant des cytokines anti-inflammatoires (IL-10, TGF-β ou IL-35), en consommant l'IL-2 ce qui limite la prolifération des autres lymphocytes, par cytolyse directe ou via l'expression à leur surface de molécules inhibitrice (Galectin-1)[60]. Dans le contexte de la SEP, il est possible que les effets des Tregs soient modulés. En effet, il a été montré une réduction de l'effet suppressif des Tregs sur la réponse immunitaire dirigée contre la glycoprotéine oligodendrocytaire myélinique (MOG) chez les patients atteints de SEP[61]. L'équipe de David Hafleur et celle de Piet Stinissen ont démontré une altération de la fonction

des Tregs chez les patients SEP, associée à une fréquence réduite de cellules positives pour *FoxP3* et une expression réduite de *FoxP3* au niveau cellulaire[62,63]. Des altérations dans le développement et la fonction des Tregs chez les patients atteints de SEP, avec une restauration de l'homéostasie des Tregs à mesure que la maladie progresse ont également été mises en évidence[64]. Les altérations dans le nombre, la fonction et l'homéostasie des Tregs peuvent contribuer à la dysrégulation immunitaire observée dans la SEP. Toutefois, l'équipe de David Laplaud a au contraire montré que la fonction des Tregs dans la SEP n'est pas altérée lorsqu'on exclut du tri par FACS des Tregs ($CD4^+CD25^{high}$) les lymphocytes exprimant fortement CD127, un marqueur d'activation T[65].

I.III.a.1.e. Les Th2

Les lymphocytes T auxiliaires de type 2 (Th2) sont une sous-population de lymphocytes T $CD4^+$ dérivés des lymphocytes T immatures sous l'effet de l'IL-4, spécialisée dans la régulation des réponses immunitaires de type allergique et dans la promotion de l'immunité protectrice contre les infections parasitaires. Ils sont caractérisés par l'expression du facteur de transcription *GATA3* et par la production d'IL-4, d'IL-5 et d'IL-13. Ils sont souvent décrits comme ayant un rôle neutre, voire bénéfique, dans la maladie avec un potentiel anti-inflammatoire. Chez la souris, les Th2 spécifiques de la MBP (myelin basic protein) sont capables d'inhiber la réponse inflammatoire par la réduction de la production d'IL-1 et d'oxyde nitrique (NO) par la microglie[66]. Des clones Th2 sont retrouvés dans le LCR de patients SEP *post-mortem* et sont capables d'induire une réponse lymphocytaire B (production d'anticorps et induction de prolifération)[67]. Fernando et ses collaborateurs ont montré qu'un biais préexistant en faveur d'une réponse immunitaire Th2 peut réduire la gravité des maladies inflammatoires démyélinisantes, y compris la SEP[68].

I.III.a.1.f. Les CD8

Les lymphocytes T $CD8^+$ sont une sous-population de lymphocytes T cytotoxiques qui jouent un rôle central dans la réponse immunitaire cellulaire. Dans le contexte de la SEP, les lymphocytes $CD8^+$ sont impliqués dans divers aspects de la pathogenèse de la maladie. En effet, l'argument majeur est leur présence prédominante au sein des lésions SEP en comparaisons des $CD4^+$, phénomène observé indépendamment de la durée et de l'évolution de la maladie ainsi que de la thérapeutique appliquée[69–71]. Les lymphocytes $CD8^+$ ont été décrits au sein des plaques corticales, associées à la progression de la maladie et aux dysfonctions cognitives précoces[39]. On retrouve des sous populations $CD8^+$ ayant des caractéristiques similaires à celles des $CD4^+$: les Tc1, les Tc2, les Tc17 et les TcReg. En réponse aux antigènes, les lymphocytes $CD8^+$ sont capables de produire des cytokines pro-inflammatoires comme l'IFN-γ et/ou l'IL-17. Ils sont retrouvés dans le LCR des patients SEP et semblent être corrélés avec le niveau de handicap[72]. Leur activation, leur cytotoxicité et leur capacité régulatrice peuvent influencer l'inflammation, la destruction de la myéline et la progression de la maladie.

I.III.a.1.g. Les Tfh

Les lymphocytes T folliculaires (Tfh) sont une population de lymphocytes T CD4+ spécialisés dans la régulation des réponses immunitaires au sein des follicules lymphoïdes et des ganglions lymphatiques, en particulier dans la formation des réponses immunitaires humorales[73]. Bien que leur rôle dans la SEP soit moins étudié que celui d'autres lymphocytes T, des données suggèrent qu'ils contribuent à la dysrégulation immunitaire et à la production d'autoanticorps[74] en interagissant avec les lymphocytes B[75]. Les lymphocytes Tfh expriment souvent la protéine PD-1 (programmed cell death protein 1), qui régule les réponses immunitaires et l'expression de cette protéine peut influencer leur fonction[76].

I.III.a.1.h. Les Tr1

Les lymphocytes régulateurs Tr1 (Type 1 regulatory T cells) sont une population de lymphocytes T régulateurs CD4+ qui jouent un rôle essentiel dans le maintien de la tolérance immunitaire et la suppression des réponses immunitaires excessives en produisant des cytokines anti-inflammatoires, comme l'interleukine-10 (IL-10)[77]. Des études récentes ont suggéré que les lymphocytes Tr1 pourraient être déficients chez les patients atteints de SEP, ce qui pourrait contribuer à la dysrégulation immunitaire observée dans la maladie[78]. L'activation des lymphocytes Tr1 ou l'induction de leur différenciation sont des stratégies potentielles pour le traitement de la SEP et d'autres maladies auto-immunes[79].

I.III.a.1.i. Les lymphocytes B

Bien que les lymphocytes T aient longtemps été considérés comme les principaux acteurs de la pathogenèse de la SEP, des preuves de plus en plus nombreuses suggèrent que les lymphocytes B jouent également un rôle clé dans la maladie[80]. Ils sont caractérisés par le cluster de différenciation CD19 ou CD20 et peuvent se subdiviser en plusieurs sous populations, notamment en B naïfs et en B mémoires. Les lymphocytes B mémoire sont de plus séparés en deux catégories : les lymphocytes B mémoires ayant réalisé leur commutation isotypique (lymphocytes B mémoire « switchés » IgD^{-} $CD27^{+}$) et les lymphocytes B mémoire n'ayant pas réalisé de commutation isotypique et exprimant encore l'IgD (et habituellement l'IgM : lymphocytes B mémoire « non switchés » IgD^{+} $CD27^{+}$) Ce sont des composants essentiels du système immunitaire adaptatif et ils sont responsables de la production d'anticorps. Cependant, leur rôle dans la SEP va au-delà de la simple production d'anticorps, et ils participent à plusieurs aspects de la pathogenèse de la maladie. Il a été mis en évidence des anomalies des réponses cytokiniques des lymphocytes B chez certains patients atteints de SEP, soulignant leur contribution à la dérégulation immunitaire dans la maladie[81]. Des études ont montré une dérégulation dans la production de l'IL-10 par les lymphocytes B dans la SEP[82]. Les

lymphocytes B semblent avoir un rôle temporel dans le développement de la SEP, la déplétion précoce des cellules B (jour -7 avant induction de l'EAE) chez des souris dont le système immunitaire est normal a exacerbé non seulement la phase précoce et maximale de l'EAE, mais aussi la phase de récupération de la maladie tout en augmentant le nombre de cellules T encéphalitogènes dans le SNC. De plus, le transfert d'une sous population en particulier, les B régulateurs (Breg, productrices d'IL-10) dans ce modèle animal, a normalisé la pathogenèse de l'EAE[83]. Une étude a montré une augmentation d'une sous population de lymphocytes B exprimant T-bet dans le sang des patients atteints de SEP à un stade précoce de la maladie[84]. La dysrégulation immunitaire de ces lymphocytes peut donc contribuer à la pathogenèse de la SEP.

I.III.a.1.g. Les autres populations

Outre les lymphocytes T et B, les cellules natural killer (NK), les cellules dendritiques (DC), les lymphocytes MAIT (*mucosal-associated invariant T cells)* et les cellules T γδ, ont également été étudiées dans le contexte de la SEP[85,86]. Les cellules NK peuvent avoir un rôle cytotoxique direct envers les cellules du système nerveux central, tandis que les DC sont impliquées dans la présentation de l'antigène et l'activation des lymphocytes T. Les MAIT sont une sous-population de cellules $CD3^+$, préférentiellement retrouvés dans les muqueuses, dotés de propriétés antimicrobiennes qui seraient capables de sécréter des cytokines telles que l'IL-17 ou encore l'IFN-γ[87]. Les cellules T γδ sont une sous-population de lymphocytes T avec des fonctions immunitaires distinctes : ils appartiennent à la population des lymphocytes T et plus particulièrement aux lymphocytes T non conventionnels. Ils participent à la fois à la réponse immunitaire innée et adaptative. D'un côté, les lymphocytes T γδ peuvent être considérés comme faisant partie de l'immunité adaptative, en cela qu'ils ont opéré une recombinaison V(D)J (mécanisme de recombinaison de l'ADN qui se produit uniquement au cours des premiers stades de la maturation des lymphocytes B et les lymphocytes T); de l'autre, la

diversité de recombinaison est relativement restreinte et s'apparente ainsi à un récepteur de reconnaissance de motifs moléculaires. Le TCRγδ (T-cell receptor ou récepteur des cellules T) définit cette lignée par opposition au TCR alpha-bêta (αβ) des lymphocytes T conventionnels.

I.III.a.2 L'inflammation du SNC et la démyélinisation

À l'état physiologique, l'accès des cellules immunitaires au SNC est restreint grâce à la présence de la BHE. Dans le contexte de la SEP, et principalement au niveau des plaques actives, on observe une forte infiltration de lymphocytes activés au niveau périvasculaire et du parenchyme[88–90]. Cette infiltration est rendue possible grâce à l'expression de molécules d'adhésion et de chimiokines spécifiques à la surface des différentes sous-populations lymphocytaires décrites précédemment. La sécrétion de cytokines pro-inflammatoires telles que l'IL-17, du TNFα et de l'IFN-γ par ces lymphocytes va mener à l'activation du système immunitaire propre au système nerveux central (macrophages et microglie), et provoquer une inflammation. L'infiltrat inflammatoire est associé à la démyélinisation : les macrophages activés contribuent à l'inflammation du SNC en phagocytant la myéline endommagée et en libérant des médiateurs inflammatoires. La destruction de la myéline (démyélinisation) et des cellules nerveuses provoque la formation de lésions inflammatoires (plaques) dans le cerveau et la moelle épinière, qui sont à l'origine des symptômes neurologiques de la SEP.

I.III.a.3. La remyélinisation

Une remyélinisation spontanée peut survenir dans les phases de rémission de la maladie, conduisant à une régression partielle ou totale des symptômes. Cette capacité de régénération varie entre chaque patient mais également chez un même patient au sein des différentes lésions cérébrales. Un défaut de remyélinisation a été mis en évidence au niveau des zones périventriculaires cérébrales, associées à une plus grande perte neuronale (neurodégénérescence). Ces zones périventriculaires cérébrales sont des régions du cerveau situées autour des cavités où circule le liquide céphalorachidien, principalement sécrété au

niveau des plexus choroïdes. Récemment, Tonietto et ses collaborateurs se sont intéressés à ce phénomène en suggérant que des facteurs "locaux" pouvaient interférer avec les mécanismes de remyélinisation[91]. La mise en évidence d'anomalies des plexus choroïdes visibles en IRM et associées à l'insuffisance de remyélinisation autour des ventricules a finalement suggéré que la composition du liquide céphalorachidien peut influencer l'efficacité de la remyélinisation.

I.III.a.4. La neurodégénérescence

À long terme, la dissémination et la diffusion des plaques et donc de l'inflammation et de la démyélinisation via l'activation des cellules résidentes du SNC (microglie et macrophages) conduit à une neurodégénérescence avec une atrophie corticale, un mécanisme clé dans la SEP progressive[92]. L'atrophie corticale dans les formes progressives de SEP est associée à une aggravation progressive des symptômes neurologiques et des incapacités fonctionnelles chez les patients.

I.III.b. Les traitements

I.III.b.1 Traitements des poussées et des formes actives de SEP

Il existe deux grands types de traitements dans la prise en charge de la SEP : le traitement des poussées, et le traitement de fond. Ces deux types de traitements vont avoir un effet direct sur le système immunitaire, dérégulé dans la pathologie.

Pour les poussées, de fortes doses de corticoïdes (méthylprednisolone) sont délivrées au patient dans le but de diminuer rapidement l'inflammation et de faire régresser les symptômes récents. Ce traitement n'a pas d'effet sur le long terme.

Les traitements de fond la SEP sont des traitements qui ciblent le ou les mécanismes impliqués dans la maladie. Leur rôle est donc d'agir sur les processus responsables de la maladie, en particulier l'inflammation. Ils sont ainsi à distinguer des traitements « symptomatiques » qui

ciblent les symptômes de la SEP, mais n'empêchent pas la progression de la maladie. Parmi ces différents traitements, on distingue ceux qui sont utilisés en première ligne (ou en première intention) et ceux qui sont utilisés en deuxième intention. Ces derniers peuvent toutefois être prescrits directement en première intention lorsque la maladie est agressive. Enfin, de façon exceptionnelle, on peut être amenés à utiliser des traitements de troisième ligne.

Ces traitements se divisent en deux catégories : les immunomodulateurs et les immunosuppresseurs. Leur but est de limiter la fréquence (ou faire disparaître) les poussées, mais aussi l'apparition de nouvelles lésions à l'IRM.

Les immunomodulateurs prescrits en traitement de première ligne sont principalement l'acétate de glatiramère et les interférons β. Des revues de diverses études ont montré une diminution du nombre de poussées avec une amélioration du score EDSS[93,94]. Le teriflunomide et le diméthylfumarate sont des immunosuppresseurs largement utilisés en première ligne de traitement de la SEP. D'autres immunosuppresseurs plus puissants ayant une AMM en première ligne sont généralement utilisés en cas d'échec des traitements précédents. L'ocrélizumab est un anticorps spécifique dirigé contre le CD20, le marqueur d'expression spécifique des lymphocytes B. Comme expliqué auparavant, ces cellules ont diverses fonctions, notamment celle de produire des anticorps, d'activer les lymphocytes T et de sécréter des cytokines pro-inflammatoires. En ciblant les lymphocytes B, l'ocrélizumab va induire une diminution sanguine rapide et durable de cette population. Son utilisation par voie intraveineuse semestriellement réduit le risque de poussée de 50% et le risque de progression du handicap de 40% environ dans une étude comparée au traitement par interférons[95]. Il s'agit de l'un des rares traitements à avoir démontré une efficacité dans les formes progressives primaires de la SEP. L'ofatumumab, administré par voie sous-cutanée, est un autre anti-CD20 utilisé dans les formes actives de SEP.

Les traitements de deuxième ligne sont en principe utilisés lorsque ceux de première ligne sont insuffisants pour contrôler la maladie, ce sont tous des immunosuppresseurs, et parmi eux se trouvent le natalizumab et le fingolimod.

Le natalizumab est un inhibiteur sélectif des molécules d'adhésion. En l'occurrence, il cible VLA-4, une molécule présente sur les cellules immunitaires qui permet le passage de la BHE entre le sang et le SNC. De par son mode d'action, il va empêcher la migration des cellules immunitaires vers le système nerveux et ainsi induire une immunosuppression. Il réduit le risque de poussées de 68% et le risque de progression du handicap de 42% par rapport au placebo[96], ce qui en fait un des traitements les plus efficaces actuellement.

Le fingolimod est un inhibiteur sélectif des récepteurs de la sphingosine1-phosphate (S1P). De par son mode d'action, il va diminuer la circulation des lymphocytes dans le sang en empêchant leur sortie des ganglions lymphatiques. De fait, la diminution des lymphocytes entraîne une diminution de leur passage dans le SNC. Son efficacité a été prouvée dans les formes actives de SEP en réduisant le risque de poussées de 54% par rapport au placebo contre un traitement de référence (interféron)[97].

Ces traitements constituent des traitements bloquant l'inflammation au niveau de la BHE ou en périphérie.

Enfin, il existe des traitements déplétant certaines cellules du système immunitaire de manière assez prolongée, comme les anti-CD20 avec les lymphocytes B. C'est le cas de la mitoxantrone, de l'alemtuzumab et de la cladribine.

La mitoxantrone est une chimiothérapie immunosuppressive développée initialement dans le traitement du cancer du sein. Son mécanisme d'action est celui des chimiothérapies classiques : le traitement se lie aux molécules d'ADN des cellules prolifératives, entraînant leur mort. Son efficacité a été montrée sur des critères de poussées, de progression du handicap et d'IRM[98].

L'alemtuzumab est un anticorps dirigé contre le CD52 (anti-CD52), molécule exprimée à la fois par les lymphocytes B et par les lymphocytes T. Il provoque une mort cellulaire rapide et prolongée des lymphocytes circulants. Le traitement a montré une efficacité par rapport à un traitement actif (interféron) sur des critères de poussées, de progression du handicap et d'IRM[99].

La cladribine induit une apoptose spécifique des lymphocytes en bloquant l'enzyme impliquée dans la synthèse de l'ADN, l'adénosine désaminase (ADA), provoquant ainsi une diminution prolongée des lymphocytes circulants. Comme la mitoxantrone et l'alemtuzumab, ce traitement a montré des effets sur les poussées, la progression du handicap et d'IRM[100].

Ces traitements étant des immunosuppresseurs, leur efficacité à diminuer l'activité du système immunitaire peut entraîner des effets secondaires plus importants, notamment des risques infectieux, et ils doivent être adaptés à chaque patient.

I.III.b.2. Traitements des formes progressives

La découverte récente de l'efficacité majeure des traitement anti-CD20 a mis en avant le rôle majeur des lymphocytes B dans la SEP. De manière générale, comprendre les mécanismes inhérents aux traitements permet d'identifier de nouvelles cibles thérapeutiques potentielles. Comme préciser dans les parties précédentes, les lymphocytes ne sont pas les seuls acteurs de la réponse inflammatoire dans la maladie et les cellules du système immunitaire inné (macrophages et microglie) ont été identifiées comme des acteurs importants contribuant à l'évolution des lésions inflammatoires, mais aussi à la composante progressive de la maladie. De nouvelles pistes thérapeutiques ciblant les cellules microgliales, en particulier celles qui se différencient de façon persistante vers un profil pro-inflammatoire s'ouvrent actuellement. Un exemple prometteur est probablement celui des inhibiteurs de la *Bruton Tyrosine Kinase* (BTKi). Ces molécules ont démontré un effet sur les lymphocytes B et sur les cellules immunitaires innées, avec des résultats positifs en phase 2 sur la réduction des lésions de

SEP[101], ouvrant la voie à des traitements pour les phases progressives de la maladie (des études de phase 3 sont en cours avec evobrutinib, fenebrutinib, remibrutinib et tolebrutinib).

I.III.b.3. Traitements favorisant la remyélinisation

Aujourd'hui, les traitements contre la SEP ciblent principalement la composante immunitaire de la maladie en diminuant la formation de plaques de démyélinisation et les poussées. Cependant, ces traitements ne sont pas suffisants pour diminuer la progression du handicap associée à la perte neuronale, notamment dans les formes progressives. Plusieurs molécules ont été développées dans le but de favoriser la remyélinisation afin de limiter les pertes et dommages axonaux et neuronaux, mais très peu ont été testées sur des cohortes de patients présentant des formes rémittentes ou progressives de SEP. Cette recherche fondamentale a conduit à l'identification de cibles thérapeutiques, puis à plusieurs essais thérapeutiques de phase 2. Parmi ceux-ci, le traitement par opicinumab (anticorps monoclonal dirigé contre la molécule Lingo-1, un inhibiteur de la remyélinisation) a montré un effet partiellement positif dans les atteintes démyélinisantes du nerf optique chez des personnes atteintes de sclérose en plaques. Toutefois, il s'est révélé décevant sur le plan clinique car il n'a pas permis d'améliorer le niveau de handicap des patients[102]. La clémastine (inhibiteur de la voie des récepteurs muscariniques) a également montré un effet remyélinisant dans des atteintes chroniques démyélinisantes du nerf optique chez des patients souffrant d'une forme rémittente de sclérose en plaques. Plusieurs essais cliniques sont en cours avec d'autres molécules thérapeutiques. Au-delà de la régénération des gaines de myéline, de nouvelles stratégies de protection font actuellement l'objet de travaux expérimentaux et cliniques. Elles passent par l'utilisation de modulateurs de canaux ioniques, des statines et d'inhibiteurs des phosphodiestérases qui ont montré des résultats prometteurs sur la progression de l'atrophie cérébrale dans des formes progressives, ou encore de médicaments plus innovants ciblant le stress cellulaire[103].

Parmi les autres perspectives, les approches utilisant des neuroprotecteurs comme la biotine[104] ont été décevantes pour l'heure.

Chapitre II : La Vitamine D dans la SEP

II.I. Contexte et résumé

Les principaux facteurs de risque environnementaux dans l'apparition d'une SEP sont le tabac, l'obésité, l'infection au virus de la mononucléose (EBV), l'exposition au soleil et une déficience en vitamine D[105]. Les taux circulants de vitamine D sont modulés par des variants génétiques spécifiques, la couleur de peau ou encore l'exposition au soleil, en particulier au cours des premières années de vie[106]. De nombreuses études ont mis en évidence des corrélations entre les taux de vitamine D dans la SEP et l'activité clinique de la maladie[107,108] et la progression du handicap[109,110]. Les concentrations de vitamine D sont plus faibles chez les femmes que chez les hommes[111] et le manque d'exposition au soleil et la carence en vitamine D semblent augmenter davantage le risque de SEP chez les femmes que chez les hommes[112]. De nombreuses études *in vitro* et sur l'EAE ont mis en évidence l'action pléiotrope de la vitamine D sur le système immunitaire. Globalement, la vitamine D semble avoir un effet immunomodulateur en diminuant la différenciation des lymphocytes B et T effecteurs et en augmentant les sous populations régulatrices. Ces effets immunomodulateurs découverts *in vitro* et chez l'animal sont des pistes intéressantes pour un éventuel potentiel thérapeutique de la vitamine D dans le contexte de la SEP.

Dans cette revue de la littérature, écrite en collaboration avec Saniya Kari (INFINITY, Toulouse) et Sasha Soldati (TKI, Bern), nous avons résumé les effets biologiques de la vitamine D sur les cellules immunitaires dans les modèles animaux, sur la BHE, sur l'activation des cellules gliales et son potentiel effet neuroprotecteur. Nous avons aussi discuté de l'optimisation de l'utilisation thérapeutique de la vitamine D pour les patients SEP ainsi que les nouvelles

techniques permettant une analyse en profondeur des régulations des cellules immunitaires par la vitamine D.

II.II Article de Revue « Full spectrum of vitamin D immunomodulation in multiple sclerosis : mechanisms and therapeutic implications" Galoppin, Kari & Soldati et al., 2022

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BRAIN COMMUNICATIONS

REVIEW ARTICLE

Full spectrum of vitamin D immunomodulation in multiple sclerosis: mechanisms and therapeutic implications

Manon Galoppin,[1,*] Saniya Kari,[2,*] Sasha Soldati,[3,*] Arindam Pal,[3] Manon Rival,[1,4] Britta Engelhardt,[3,†] Anne Astier[2,†] and Eric Thouvenot[1,4†]

* These authors contributed equally to this work.
† These authors contributed equally to this work.

Vitamin D deficiency has been associated with the risk of multiple sclerosis, disease activity and progression. Results from *in vitro* experiments, animal models and analysis of human samples from randomized controlled trials provide comprehensive data illustrating the pleiotropic actions of Vitamin D on the immune system. They globally result in immunomodulation by decreasing differentiation of effector T and B cells while promoting regulatory subsets. Vitamin D also modulates innate immune cells such as macrophages, monocytes and dendritic cells, and acts at the level of the blood–brain barrier reducing immune cell trafficking. Vitamin D exerts additional activity within the central nervous system reducing microglial and astrocytic activation. The immunomodulatory role of Vitamin D detected in animal models of multiple sclerosis has suggested its potential therapeutic use for treating multiple sclerosis. In this review, we focus on recent published data describing the biological effects of Vitamin D in animal models of multiple sclerosis on immune cells, blood–brain barrier function, activation of glial cells and its potential neuroprotective effects. Based on the current knowledge, we also discuss optimization of therapeutic interventions with Vitamin D in patients with multiple sclerosis, as well as new technologies allowing in-depth analysis of immune cell regulations by vitamin D.

1 IGF, University Montpellier, CNRS, INSERM, Montpellier, France
2 Toulouse Institute for Infectious and Inflammatory Diseases (Infinity), INSERM UMR1291 – CNRS UMR5051 – Université Toulouse III, 31024 Toulouse cedex 3, France
3 Theodor Kocher Institute, University of Bern, Bern, Switzerland
4 Department of Neurology, Nîmes University Hospital, University Montpellier, Nîmes, France

Correspondence to: Eric Thouvenot
Service de Neurologie, Hôpital Caremeau
4 place du Professeur Robert Debré
30029 Nîmes cedex 9, France.
E-mail: eric.thouvenot@chu-nimes.fr

Keywords: vitamin D; multiple sclerosis; cytokines; lymphocytes; immunomodulation

Abbreviations: BBB = blood–brain barrier; CITE-seq = Cellular Indexing of Transcriptomes and Epitopes by sequencing CyTOF = cytometry by time of flight; DCs = dendritic cells; EAE = experimental autoimmune encephalomyelitis; FACS = fluorescence activated cell sorting; ICAM-1 = intercellular adhesion molecule-1; IFN-γ = interferon gamma; IgG = immunoglobulin G; IL = interleukin; KO = knockout; MBP = myelin basic protein; MHC = major histocompatibility complex; MMP9 = matrix metalloproteinase-9; MOG = myelin oligodendrocyte glycoprotein; NFAT = nuclear factor of activated T cells; NF-kB = nuclear factor-κB; OPCs = oligodendrocyte precursor cells; PBMC = peripheral blood mononuclear cells; PDGF = platelet derived growth factor; P-gp = P-glycoprotein; PLP = proteolipid protein; RCTs = randomized clinical trials; RNAseq = RNA

Received March 10, 2022. Revised May 03, 2022. Accepted June 28, 2022. Advance access publication June 30, 2022
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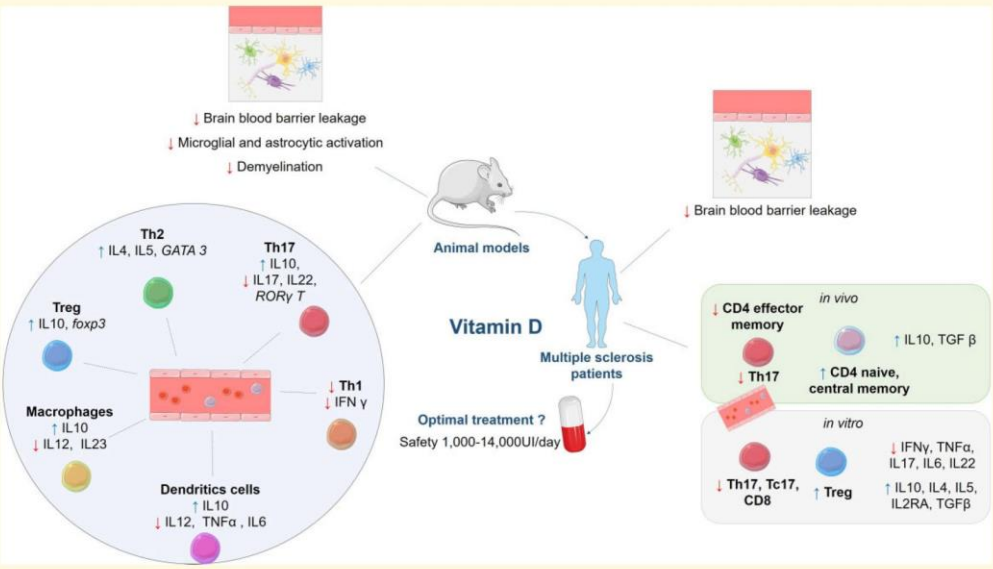
sequencing; RRMS = relapsing-remitting multiple sclerosis; RXR = retinoid-X-receptor; SAEs = severe adverse events; scRNAseq = single cell RNA sequencing; SPMS = secondary progressive multiple sclerosis; TEER = trans-endothelial electrical resistance; TJs = tight junctions; TGF-β = transforming growth factor beta; TNFα = tumour nuclear factor alpha; VCAM-1 = vascular cell-adhesion molecule-1; VDR = vitamin D receptor; VDRE = vitamin D receptor element; VitD = vitamin D; WT = wild type

Graphical Abstract



Introduction

Multiple sclerosis is a chronic inflammatory and neurodegenerative disease of the CNS especially affecting women (3:1 sex ratio). Eighty-five per cent of patients develop multiple sclerosis with a clinically isolated syndrome corresponding to an acute demyelination of the optic nerves, brainstem or spinal cord, followed by recovery. Most multiple sclerosis patients experience subsequent relapses (relapsing-remitting multiple sclerosis—RRMS) with some later converting to secondary progressive multiple sclerosis (SPMS), characterized by gradual neurological deterioration, with or without superimposed relapses. In 15% of cases, neurological symptoms progress from onset (primary progressive multiple sclerosis), with equal frequency in males and females.

Epidemiological studies have highlighted a familial aggregation of multiple sclerosis potentially explained by several environmental and genetic risk factors.[1] Family studies assessing risks to relatives of multiple sclerosis patients showed that first-degree relatives are generally at 10–25 times greater risk of developing multiple sclerosis than the general population, but this risk could be lower.[2] Several major histocompatibility complex (MHC) loci—especially HLA DRB1*15:01—are the most important genetic risk factors for developing multiple sclerosis with odd ratios around three. Moreover, genome wide association studies (GWAS) studies have identified more than 200 susceptibility variants other than MHC that implicate multiple innate and adaptive immune pathways distributed across the cellular components of the immune system and brain-resident immune cells, highlighting the importance of altered peripheral immune cells and glial cells responses in multiple sclerosis pathophysiology.[3]

The main environmental risk factors for multiple sclerosis include smoking,[4,5] obesity,[6] Epstein-Barr Virus infection,[7,8] sun exposure[9,10] and vitamin D (VitD) deficiency.[11] The latter has been recognized as a major risk factor for multiple sclerosis, with circulating levels modulated by specific genetic variants, skin colour and sun exposure, especially in early years of life.[2] Many reports have highlighted the clinical correlation of circulating VitD levels in multiple sclerosis with clinical disease activity[12,13] and of disability progression.[9,14] Moreover, VitD concentrations are significantly lower in females compared with males[15] and lack of sunlight and VitD deficiency seem to further increase the risk of multiple sclerosis in females than in males.[16]

There are different sources of VitD. Cholecalciferol ($VitD_3$) is a lipophilic cholesterol derivative (Fig. 1) synthesized in the skin from 7-dehydrocholesterol under the effect of UV radiation. It is also provided by animal food sources, especially oily fish. Cholecalciferol, which is biologically inactive, is stored in adipose tissue and transformed in the liver

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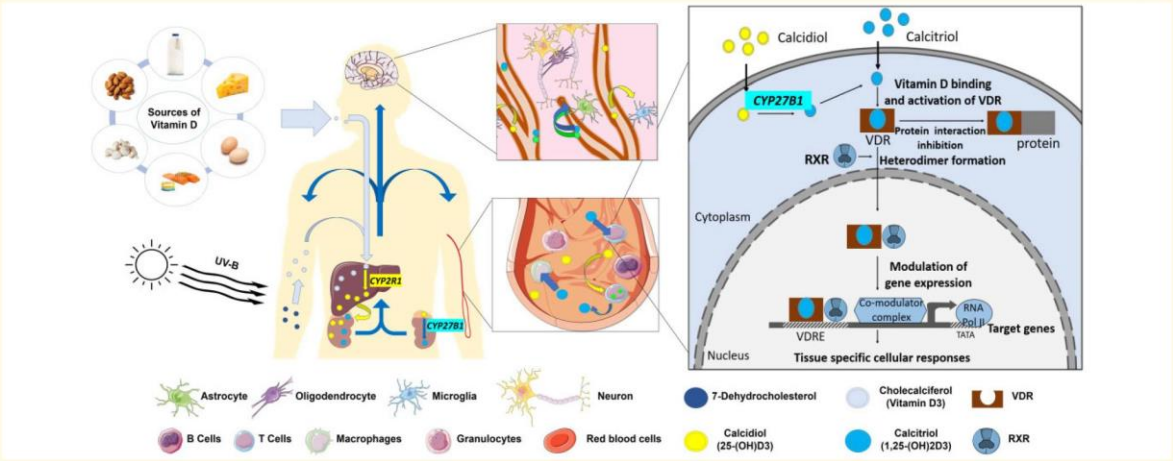

Figure 1 Sources, metabolism and cellular mode of action of VitD. VitD is synthesized in the skin under the effect of UV radiation and is also provided by food sources. Cholecalciferol (inactive form) is stored in adipose tissue and transformed in the liver into 25-hydroxyvitamin D_3 [$25(OH)D_3$, calcidiol—main circulating form]. Calcidiol is then hydroxylated in the kidney to form 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$, calcitriol—active metabolite]. VitD metabolites circulate ubiquitously, bound to its transporter: Vit DBP and is able to cross the blood–brain barrier to modulate CNS-resident cells (neurons, astrocytes, oligodendrocytes and microglia). Calcitriol acts intracellularly as a transcription factor by binding to the cytoplasmic VDR which heterodimerizes with RXR. Upon translocation of this heterodimer to the nucleus, VitD/VDR/RXR complex binds to the VDRE in the genomic DNA and modulates the expression of target genes. Drawings of the individual cell types were adapted from Servier Medical Art (http://smart.servier.com/), licenced under a Creative Common Attribution 3.0 Generic License.

into 25-hydroxyvitamin D_3 [$25(OH)D_3$, calcidiol], which is the main circulating form of VitD. Calcidiol is then hydroxylated in the kidney to form 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$, calcitriol], which is the active metabolite of VitD.[17] VitD metabolites circulate ubiquitously, bound to VitD binding protein (DBP), its transporter. Calcitriol acts intracellularly as a transcription factor by binding to the VitD receptor (VDR), which heterodimerizes with retinoid-X-receptor (RXR).[18,19] Upon translocation of this heterodimer to the nucleus, VitD recognizes VitD responsive elements (VDREs) in the genomic DNA and modulates the expression of over 200 genes. Most of these are associated with immune functions and VitD metabolism, highlighting the key role of VitD deficiency in numerous inflammatory diseases and cancer.[1]

The pleiotropic actions of VitD on the immune system globally result in immunomodulation by decreasing differentiation of effector T and B cells while promoting regulatory subsets.[20] VitD is also known to modulate cells of the innate immune system such as macrophages, monocytes and dendritic cells (DCs), that additionally contribute to multiple sclerosis pathophysiology.[20] Furthermore, VitD acts in the CNS by regulating microglial and astrocytic activation.[21] The immunomodulatory role of VitD in animal models of multiple sclerosis has suggested its potential therapeutic use in multiple sclerosis. In this context, a comprehensive analysis of data concerning the *in vitro* and *in vivo* effects of VitD in multiple sclerosis is mandatory to optimize its use in clinical practice. In this review, we focus on recent published data describing the biological effects of VitD in animal models of multiple sclerosis, on immune cells, blood–brain barrier (BBB) function, activation of glial cells and its potential neuroprotective effects. Based on the current knowledge, we discuss the optimization of therapeutic interventions with VitD in multiple sclerosis patients.

Immune cells and animal models

VitD ameliorates experimental autoimmune encephalomyelitis

Experimental autoimmune encephalitis (EAE), the main animal model of multiple sclerosis, is based on immunization of susceptible animals (especially rodents) with myelin antigens to trigger an autoinflammatory response in the CNS. Used in multiple strains or genotypes of rodents, it has been largely developed to recapitulate different aspects of the disease (clinical course, lesion localization or pathological patterns) and has significantly contributed to our understanding of the immunopathologic features of multiple sclerosis.[22]

EAE can be actively induced (active EAE) by the immunization of animals with peptides in emulsion of incomplete Freund's adjuvant containing heat killed Mycobacterium tuberculosis (H37Ra) in combination with pertussis toxin. Peptide injection triggers activation of myelin antigen-specific T cells and leads to CNS autoimmunity.[23] Alternatively, passive EAE is induced by the adoptive

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transfer of pre-activated myelin-specific T cells into naïve syngeneic recipients. The development of clinical EAE can be quantified using different clinical EAE scoring systems.[23,24]

Most used EAE models include immunization with peptides from myelin oligodendrocyte glycoprotein (MOG) (e.g. MOG_{35-55}) in C57BL/6 mice,[25–30] with myelin basic protein (MBP) in B10.PL mice or in Lewis rats[29,31–35] or with proteolipid protein (PLP) (e.g. $PLP_{139-151}$) in SJL mice.[27,28,36]

The efficacy of calcitriol in active EAE models has been analysed in a prophylactic or curative approach. Most studies show a clear protective effect of VitD in EAE. In the prophylactic method, a supplementation of calcitriol is added to the food before induction of EAE (generally, 50 ng/day for females and 200 ng/day for males).[32] In the curative method, calcitriol is administrated after the onset of clinical signs by intraperitoneal injection (50–300 ng), which may be combined with a supplementation in food (50 ng/day). Prophylactic administration of calcitriol inhibited EAE development, and therapeutic administration decreased the severity of active and passive EAE in B10.PL, C57BL/6 and SJL/J.[25–27,31,32,34,36,37] Moreover, cholecalciferol also reduced EAE severity in prevention.[29,33,35] In contrast, cholecalciferol alone failed to reverse the disease in the therapeutic setting in the MBP B10.PL mouse EAE model, while the combination of cholecalciferol and calcitriol did.[38]

An interesting study showed that the protective effect of VitD in EAE is transient as removal of VitD resulted in an increased severity of the disease in MOG-induced EAE in C57BL/6 mice.[26] This study also suggested that calcitriol does not inhibit the production of autoreactive $CD4^+$ T cells but that it may block their migration to the CNS. Indeed, there are fewer mononuclear cells infiltrating the CNS tissue after the induction of active EAE in mice and rats treated with cholecalciferol or calcitriol.[25,27,33,34,37] Calcitriol may decrease the migration of encephalitogenic T cells via the regulation of adhesion molecules involved in their trafficking into the CNS, as suggested by the lowered CXCR3 expression on $CD4^+$ T cells in calcitriol-treated mice (MOG-induced EAE in the C57BL/6 model).[26] *In vitro*, calcitriol reduced CCR6 expression and migration of Th17 cells.[25] Furthermore, reduced secretion of pro-inflammatory interleukin (IL)-17A and IL-22 and increased production of anti-inflammatory IL-10 by $Foxp3^+$ $CD4^+$ $CD25^{high}$ regulatory T cells (Tregs) was detected in the brain of $PLP_{139-151}$ EAE SJL/J mice treated with calcitriol.[27]

Several important studies have demonstrated the key role of the VDR in mediating the protective effect of VitD in EAE. VDR knockout (VDR KO) C57BL/6 mice with deleted exons coding for the first or second zinc finger of the DNA binding domain (VDR^{tm1Ska}-KO and VDR^{tm1Mbd}-KO, respectively) have been used.[39,40] In the absence of VDR, calcitriol does not confer a protection to active EAE in C57BL/6 VDR^{tm1Ska}-KO mice.[41] Furthermore, calcitriol failed to inhibit EAE in C57BL/6 mice lacking functional VDR specifically in $CD4^+$ T lymphocytes,[41] demonstrating that VDR expression in $CD4^+$ T cells is key for its protective effect.[42] Moreover, both *in vitro* and *in vivo*, an increase in autoreactive Th17 responses and a decrease in tolerogenic $CD103^+$ $CD11c^+$ DCs were observed in VDR KO mice.[43] Taken together, VDR seems indispensable to mediate the anti-inflammatory responses mediated by VitD.

Circulating lymphocytes

$CD4^+$ Th1 and Th17 cells

Most immunomodulatory effects of VitD have been well described in CD4+ T lymphocytes. Calcitriol exerts strong and direct immunomodulatory effects when added to $CD4^+$ T-cell cultures *in vitro*, by inhibiting effector Th1 and Th17 and promoting Th2 and Tregs calcitriol inhibits the differentiation of naïve $CD62L^+CD4^+$ T cells into Th1 and Th17 cells[25,44] and reduces the production of interferon gamma (IFN-γ) by Th1 cells.[45–47] In Jurkat T cells, the VDR/RXR complex was found to bind to a silencer region in the human IFN-γ promoter[46] (Fig. 2A). However, it is important to note that the decreased IFN-γ production could also result from an indirect action of calcitriol on IL-12 production by DCs and macrophages,[28,48] as further described below, as IL-12 promotes IFN-γ production and Th1 differentiation via the activation of STAT4[49] (Fig. 2E). *In vitro* treatment of activated T cells from SJL/J mice with calcitriol inhibits IL-12-induced tyrosine phosphorylation of STAT3, STAT4 and of the kinases JAK2 and TYK2[28] (Fig. 2A). Therefore, the modulation of the JAK-STAT signalling pathway by VitD impairs the development of Th1 cells.

Several studies have shown that calcitriol also inhibits IL-17A, IL-22 and IL-17F production by Th17 cells from EAE mice, both *in vivo* and *in vitro*.[27,50,51] Calcitriol reduces the level of mRNA that encodes IL-17A, IL-17F and IL-22, suggesting that inhibition of cytokines in Th17 cells occurs via transcriptional modulation[51] (Fig. 2B). The nuclear factor of activated T cells (NFAT)-AP-1 complex is important for the induction of IL-17 and IL-2 expression in $CD4^+$ T cells.[52] In one study using HUT102 cells and Jurkat T cells treated with calcitriol, there is a competition of calcitriol–VDR–RXR complex and NFAT to NFAT-binding sites in the gene coding IL-17A. However, in mice, the repression of IL-17A is independent of NFAT (Fig. 2B). Furthermore, in both human cell lines and in mice, binding of the calcitriol–VDR–RXR complex leads to the transcriptional repression of IL-17A expression through the recruitment of Histone Deacetylase 2.[27] In a similar fashion, IL-2 and GM-CSF are inhibited.[53,54] The VDR–RXR complex interferes with the formation of the NFATp–Jun–Fos transcriptional complex on the promoter of the IL-2 gene. RUNX1 has a key role in the differentiation of Th17 cells and the transcription of IL-17A. VDR binds to RUNX1 and prevents its binding to the IL-17a gene promoter. In addition, there is the formation of an inhibitory complex between FOXP3 and RUNX1, which blocks the activation of IL-17A (Fig. 2B). However, Chang *et al.* found no significance difference of the level of IL-17A mRNA upon addition of calcitriol in

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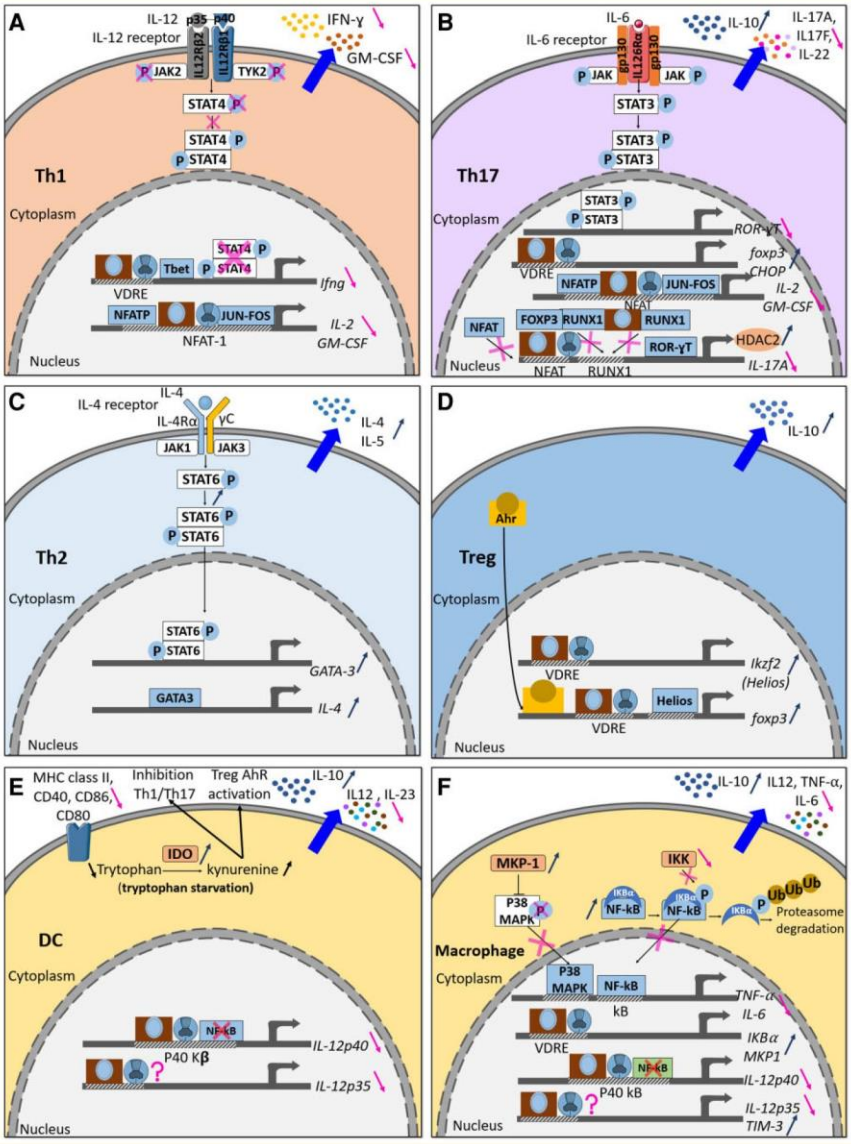

Figure 2 Immunomodulatory effects of VitD in mice. VitD has both a direct and indirect immunosuppressive effect on Th1 (**A**), Th17 (**B**), Th2 (**C**), Treg (**D**), DC (**E**) and macrophage (**F**).

MOG_{35-55} EAE in C57BL/6 mice. They proposed that the reduction of production of cytokines results from a translational regulation. *In vitro*, C/EBP homologous protein, endoplasmic reticulum stress sensor is induced by calcitriol in Th17 cells.[50]

$CD4^+$ Th2 cells

In contrast to Th1 and Th17, several studies have shown that calcitriol favours Th2 differentiation.[30,37,44] Following the addition of calcitriol to CD4+ T cells from BALB/C and C57BL/6 mice, a significant increase in *GATA-3*, a Th2-specific transcription factor, and c-maf was observed (Fig. 2C). Indeed, the Th2 signature cytokines IL-4 and IL-5 were upregulated by calcitriol while expression of IFN-γ and IL-17 was decreased. Moreover, STAT6 is important for calcitriol to confer a protection of MOG_{35-55} EAE in CD57BL/6 mice (Fig. 2C).

Regulatory T cells

Tregs play an important role in maintaining peripheral tolerance.[55] Impaired $Foxp3^+$ $CD4^+$ $CD25^{high}$ Treg have been identified as a key feature of multiple sclerosis.[56] Many

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studies have highlighted the role of VitD in promoting Tregs, and of the secretion of anti-inflammatory IL-10. Calcitriol and cholecalciferol increased the numbers of Tregs in the periphery and in the spinal cord of MOG_{35-55} induced EAE in C57BL/6 mice and MBP-induced EAE in B10.PL mice and Lewis rats.[27,33,37,38] Moreover, calcitriol increased their production of IL-10[57] (Fig. 2D). Indeed, key studies have shown that a functional IL-10 pathway is necessary for $1,25(OH)_2D_3$ to inhibit EAE.[51,58,59] Few studies have also reported an important role of VitD in Foxp3 regulation. The increase in Tregs is due to increased transcription of the *foxp3* gene as VDRE are present in the murine Foxp3 promoter.[27] Calcitriol also indirectly increased FOXP3 expression through the modulation of Helios, a positive regulator of FOXP3, and a marker of thymic-derived natural Tregs.[60] Injection of calcitriol (200 ng in oil) or the combination of calcitriol and cholecalciferol (gavage 5 µg in oil) increased $Helios^+Foxp3^+$ Treg in MOG_{35-55} EAE in C57BL/6 mice and MBP EAE in B10.PL mice.[38,61] VitD increases the expression of *Ikzf2* (encoding Helios) in $CD4^+$ T cells in a VDR-dependent mechanism,[61] and Helios was shown to bind to the *foxp3* promoter and increase its expression, like the aryl-hydrocarbon receptor (Ahr) does[62,63] (Fig. 2D).

In addition, there is evidence that VitD signalling plays a greater role in maintaining T-cell tolerance in females than in males. In this interesting study, a cooperative loop between VitD and oestrogen has been described as calcitriol promotes oestradiol (E2) that reduces transcription of *Cyp24a1*, the catabolic enzyme for active vitamin, and increases VDR in T cells, which in turn, promotes Treg development.[29,64] Therefore, lowered VitD and sex-specific VitD signalling may contribute to the breakdown in T-cell self-tolerance and increased multiple sclerosis incidence in females.

B cells

Much of the current literature has focused on $CD4^+$ T cells. However, understanding the role of VitD on B cells is essential, given the high efficacy of anti-CD20 treatments in multiple sclerosis.[65] VitD has been shown to modulate human B cells, by regulating their proliferation, cytokine secretion and differentiation into plasma cells.[20] However, as far as we know, no study analysed the direct role of VitD on B cells in EAE. One study reported the indirect effect of calcitriol through tolerogenic DCs in B cells.[66] The transfer of tolerogenic DCs induced by calcitriol in MOG_{35-55} EAE in C57BL/6 mice increased the proportion of $CD19^+$ $CD5^+$ $CD1d^+$ regulatory B cells (Breg) in the spleen. Breg play an important role in MOG_{G35-55} EAE C57BL/6 mice by increasing the production of IL-10.[67]

Innate immune cells

Dendritic cells

DCs are antigen presenting cells (APCs) that participate in the initiation and development of multiple sclerosis.[68] In the periphery, DCs activate naïve $CD4^+$ T cells into $CD4^+$ Th1 and Th17 cells, that in turn cross the BBB and are reactivated in the perivascular and subarachnoid CNS spaces by resident APCs allowing them to infiltrate the CNS parenchyma.[69,70]

Besides the established role of VitD in T cells, a clear role in DCs has also been clearly demonstrated. Addition of calcitriol generates tolerogenic DCs. Most studies have generated tolerogenic DCs *in vitro* and transferred them to MOG_{35-55} EAE in C57BL/6 or MBP_{73-86} EAE in Lewis rats.[66,71] Tolerogenic DCs are generated by adding GM-CSF (10 ng/ml) and/or IL-4 (10 ng/ml) and calcitriol (1–10 nM) or cholecalciferol (1 nM) to progenitor bone marrow cells from female CD57BL/6 mice or Lewis rats. They are characterized by a decreased expression of MHC Class II, CD40, CD86 and CD80 (co-stimulatory molecules), decreased IL-12, IL-23 and increased IL-10 production compared with untreated DCs[66,71,72] (Fig. 2E). DCs are generally pulsed with MOG_{35-55} peptide before transfer in EAE models at different time points: before immunization (preventive), after immunization (preclinical) and after the onset of the disease (therapeutic). This approach allows to determine the direct impact of VitD on DCs and its indirect effect on the adaptive immune response. The administration of tolerogenic DCs after immunization and after the onset of the disease led to a reduction of the severity of the disease in MOG_{35-55} EAE in C57BL/6 mice.[66,71] In the preventive approach, tolerogenic DCs abrogated EAE in MOG_{35-55} EAE in C57BL/6 mice and Lewis rat.[33,71] Furthermore, treatment of EAE by tolerogenic DCs led to an increase in Tregs and Bregs and secretion of IL-10,[33,66,71] with a reduced infiltration of autoreactive Th1 and Th17 cells into the spinal cord[66] and in a reduction of $CD11c^+$ DCs[66,73] (Fig. 2E).

Mechanistically, cholecalciferol and calcitriol increased the expression of indoleamine 2,3-dioxygenase (IDO), an enzyme that converts tryptophan to kynurenine (Fig. 2E). The inhibition of IDO on tolerogenic DCs abrogated the effect of cholecalciferol on MBP_{73-86} EAE course in Lewis rats.[33] IDO^+-tolerogenic DCs led to the generation of Treg through two mechanisms: tryptophan deficiency[74] and the activation of AhR on $CD4^+$ T cells by kynurenine, first metabolite of IDO.[63,75]

As mentioned earlier, it is also demonstrated that VitD reduces IL-12 production by myeloid cells. It inhibits IL-12p35 and p40 subunits at the transcriptional level in DCs and macrophages (Fig. 2E and F). Furthermore, calcitriol inhibits the mRNA expression of the IL-12p40 subunit in a VDR-dependent manner. Calcitriol may interfere with the binding of nuclear factor-κB (NF-kB) on the NF-kB binding site in the p40 promoter by competition with VDR–RXR heterodimers[48] or by downregulation of the activation of the NF-kB signalling pathway in DCs and macrophages.[76]

Monocytes/macrophages

Similarly to DCs, VitD modulates their function. In macrophages, calcitriol inhibits the production of tumour necrosis factor-α (TNFα) by reducing the activity of a major transcription factor (NF-kB), through the increase of inhibitor

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of kB-α (IkB-α) that sequesters NF-kB in the cytoplasm of these cells (Fig. 2F). The phosphorylation of IkB-α by IkB kinase (IKK) leads to its ubiquitination and degradation by the proteasome. It allows the release of NF-kB that can translocate to the nucleus where it binds to kB site on TNFα promoter and activates its expression. Calcitriol binds to the promoter of IkB-α and upregulates its mRNA expression, leading to an increase of IkB-α in the cytosol. In the presence of calcitriol, IkB-α mRNA is more stable. Calcitriol also decreases IKK phosphorylating activity on IkB-α, reducing IkB-α protein degradation and reducing production of TNFα.[76–78] In addition, calcitriol induces MAPK phosphatase-1 (MKP-1), which dephosphorylates p38 and reduces p38 MAPK activation in monocytes/macrophages (Fig. 2F) and the production of TNFα and IL-6,[79] a critical cytokine for the differentiation of Th17 cells. This leads to the phosphorylation of STAT3 and the activation of the transcription of retinoic acid-related orphan receptor. Furthermore, calcitriol promotes M2 macrophage polarization by increasing the expression of T-cell immunoglobulin-mucin3 (Tim-3), resulting in a decrease in TNFα and IL-6 and an increase in IL-10[80] (Fig. 2F). So, VitD indirectly inhibits Th17 cells and Th1 through the inhibition of production of IL-6 and TNFα by macrophages.

Altogether, these studies indicate that calcitriol inhibits EAE in a preventive approach and ameliorates EAE in a therapeutic approach. Clear evidence show that calcitriol reduces effector Th1 and Th17 while promoting Tregs, and decreases the infiltration of auto-aggressive T cells into the CNS. The reduced EAE depends on the expression of VDR in $CD4^+$ T cells. VitD has both a direct and indirect immunosuppressive effect on immune cells through the increase in tolerogenic immune responses (IDO^+-DCs, Treg and Breg) and in the anti-inflammatory cytokine IL-10. However, most studies focused on calcitriol, the active form of VitD, and relatively few animal model studies used cholecalciferol,[29,33,35] the main VitD metabolite administered to multiple sclerosis patients.

Immunomodulation in multiple sclerosis patients

While *in vivo* studies in EAE provided key molecular insights into the mode of action of VitD, several studies have analysed the effects of VitD on human lymphocytes from healthy donors or multiple sclerosis patients. Most studies determined the ability of VitD to modulate cell phenotype, proliferation and cytokine secretion *in vitro*. Several reports focused on the *in vivo* effects of VitD by directly assessing the levels of cytokines in serum or plasma of multiple sclerosis patients treated with high-dose VitD, or by analysing the proportions of key immune cell populations involved in multiple sclerosis pathophysiology (e.g. Treg, Th1, Th17).

An in-depth search of the recent literature (since 2010) identified 28 publications investigating the effects of VitD on peripheral immune cells and cytokines in multiple sclerosis patients. Fig. 3 summarizes the *in vivo* (Fig. 3A and B, Supplementary Fig. 1 and Table 1) and *in vitro* (Fig. 3C and D, Supplementary Fig. 1 and Table 2) studies analysing the changes observed in cell populations and expression of biomarkers in multiple sclerosis patients treated with high-dose VitD (range 7000–20 000 UI/day cholecalciferol) in therapeutic trials[81–94] (Supplementary Table 1) or by adding active VitD (10–100 nM calcitriol) *in vitro* to peripheral blood mononuclear cells (PBMCs) or purified T cells obtained from multiple sclerosis patients[83,95–106] (Supplementary Table 2).

In only one out of three *in vivo* studies looking at modifications of cell populations, VitD induced a significant decrease in circulating Th17 cells[82,84,85] (Fig. 3A). Different studies focusing on other immune cell populations showed a significant decrease of $CD4^+$ effector/memory T cells and a significant increase in circulating $CD4^+$ naïve and $CD4^+$ central memory T cells[85] (Fig. 3A). Concerning the modifications of circulating and secreted cytokine levels in multiple sclerosis patients treated with high-dose VitD, a significant increase of IL-10, transforming growth factor beta (TGFβ) and TNFα was reported in total PBMCs[47,83,86,87] and TGFβ in serum,[47,88,90] as well as a significant decrease in proliferation in PBMCs[86,94] (Fig. 3B). However, no significant effect of VitD was observed in cytokine secretion by T and B cells sorted by fluorescence activated cell sorting (FACS).

In contrast, *in vitro* studies showed a significant decrease in pro-inflammatory T-cell subsets, especially for those secreting IL-17 (Tc17 and Th17), and in $CD8^+$ T cells[97,100] (Fig. 1C, Supplementary Table 2). In four out of five studies, VitD induced a significant increase in circulating Treg.[95,97,98] These studies indicate a significant increase of IL-2RA in $CD4^+$ T cells, TGFβ and IL-5 in $CD8^+$ T cells and in total PBMCs, ILT3 and CCL2 in myeloid cells, and IL-4 in total PBMCs[83,95–98,100,103–106] and a significant decrease of pro-inflammatory cytokines such as IFN-γ, IL-17, TNFα, GM-CSF, IL-22, IL-6 in PBMCs, and in T-cell subtypes[95–98,100,101] (Fig. 3D). VitD also induces a significant decrease of IL-6 in B cells.[102] However, contrary to *in vivo* data, IL-10 is decreased in total PBMCs *in vitro*.[83]

Altogether, VitD decreases proliferation of $CD4^+$ T cells and B cells and reduces cell populations secreting pro-inflammatory cytokines (IFN-γ, IL-17, TNFα, IL-6, GM-CSF, IL-22) while increasing the proportion of cell populations secreting anti-inflammatory cytokines (such as IL-10 and TGFβ). *In vitro*, the decrease in IL-17 secretion in $CD4^+$ T cells is in accordance with the decrease in Th17 under VitD treatment (Fig. 3C and D). Similarly, the decrease of IFN-γ and TNFα secretion in $CD8^+$ T cells reflects the decrease in $CD8^+$ T cells, while the increase of IL-10 secretion and CD25 expression in $CD4^+$ T cells correlates with the increase in Treg. However, *in vivo* studies provide inconclusive data on the modulation of immune cell populations as compared with the cytokine secretion regulation. However, $CD8^+$ T cells, B cells and myeloid cells were not analysed in *in vivo* studies in multiple sclerosis patients and little is known on VitD effects on cytokine secretion profiles

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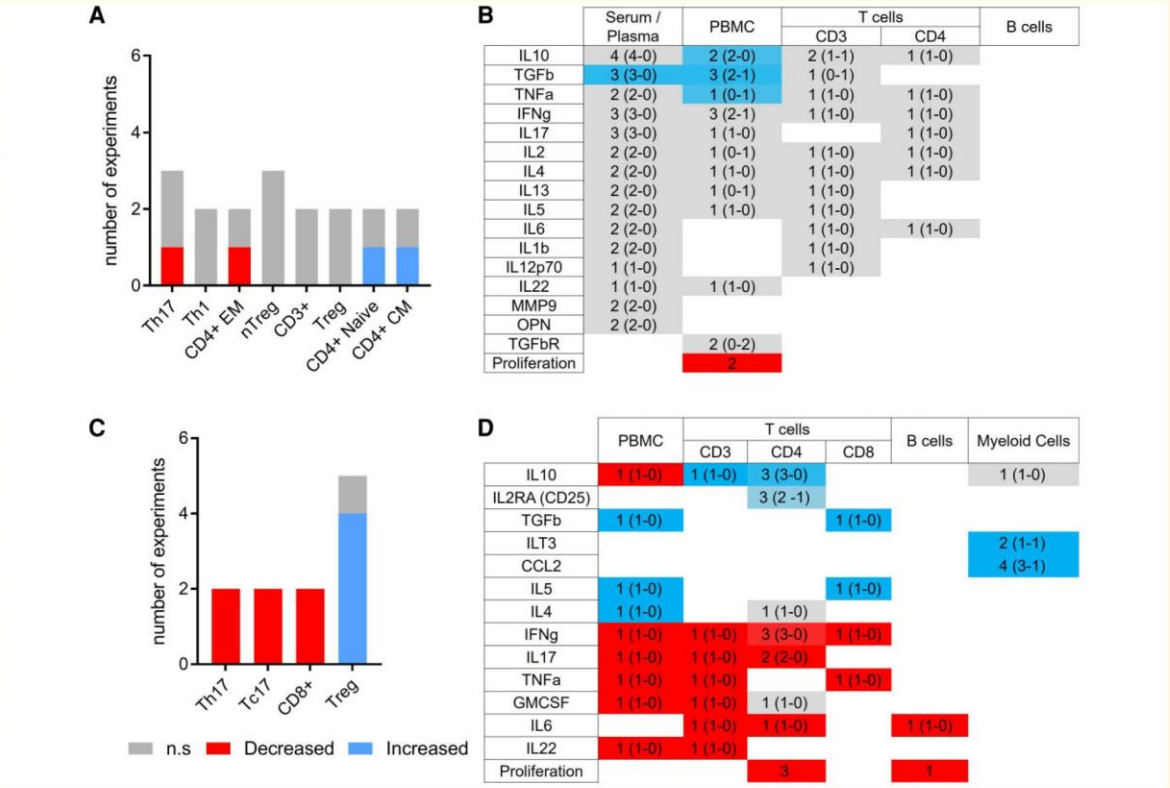

Figure 3 Main effects and modulations of VitD treatment on immune cells in multiple sclerosis. Effects and modulations of VitD investigated in more than one *in vivo* (**A** and **B**) and *in vitro* (**C** and **D**) studies. Graphs (**A** and **C**) represent the number of studies showing significantly increased (blue), decreased (red) or non-significantly modified (grey) cell populations after treatment by VitD. Heat-map (**B** and **D**) representing the modifications of circulating and secreted cytokines levels studied after VitD treatment/supplementation. Studies showing a significant increase ($n = a$), a significant decrease ($n = b$) or an absence of variation ($n = c$) were scored as follows: $[(a \times 2) - (b \times 2)]/[(a \times 2) + (b \times 2) + c]$. Red represents a global decrease, blue represents a global increase and grey non-significant or discrepant results. In each case, X (Y–Z) represents the total number of tests (X) and the number of tests realized using FACS/ELISA (Y) or PCR (Z).

in specific subpopulations like Th1, Th2, Th17, Treg in this disease.

VitD actions in the CNS

Immune regulations induced by VitD in the periphery may also protect the CNS from the inflammatory insult by local protection of the BBB and reduction of glial activation in the brain parenchyma. We particularly highlighted the effects of VitD on cellular mechanisms and cell types targeted by inflammation in multiple sclerosis (Fig. 4).

At the BBB level

Endothelial cells

The CNS is an immune-privileged site with unique anatomic, cellular and molecular elements that limit both physiologic and pathophysiologic inflammation and preserve CNS homeostasis.[107] The endothelial BBB of the CNS microvessels is composed of endothelial cells adjoined continuously by tight junctions (TJs), surrounded by pericytes and astrocyte end-feet that tightly ensheath the vessel, and possess the essential function of strictly controlling the cellular and molecular exchanges between the blood and the CNS.[70,108] It is well established that BBB breakdown is an early hallmark of multiple sclerosis pathogenesis which is detected by gadolinium enhanced (Gd) MRI in the CNS of multiple sclerosis patients.[109,110] Impaired BBB properties during multiple sclerosis are suggested by the observation of interrupted immunostaining for TJ and adherens junction (AJ) proteins, such as claudin-5 and VE-cadherin, respectively,[111–116] as well as leakage of fibrinogen and immunoglobulin G (IgG) outside of blood vessels in post-mortem CNS tissue of multiple sclerosis patients[117] (Fig. 4, Supplementary Table 3).

BBB endothelial cells express VDR, and VitD has been suggested to provide beneficial effects in multiple sclerosis by protecting BBB integrity as shown *in vitro* and *in vivo* (Table 1).

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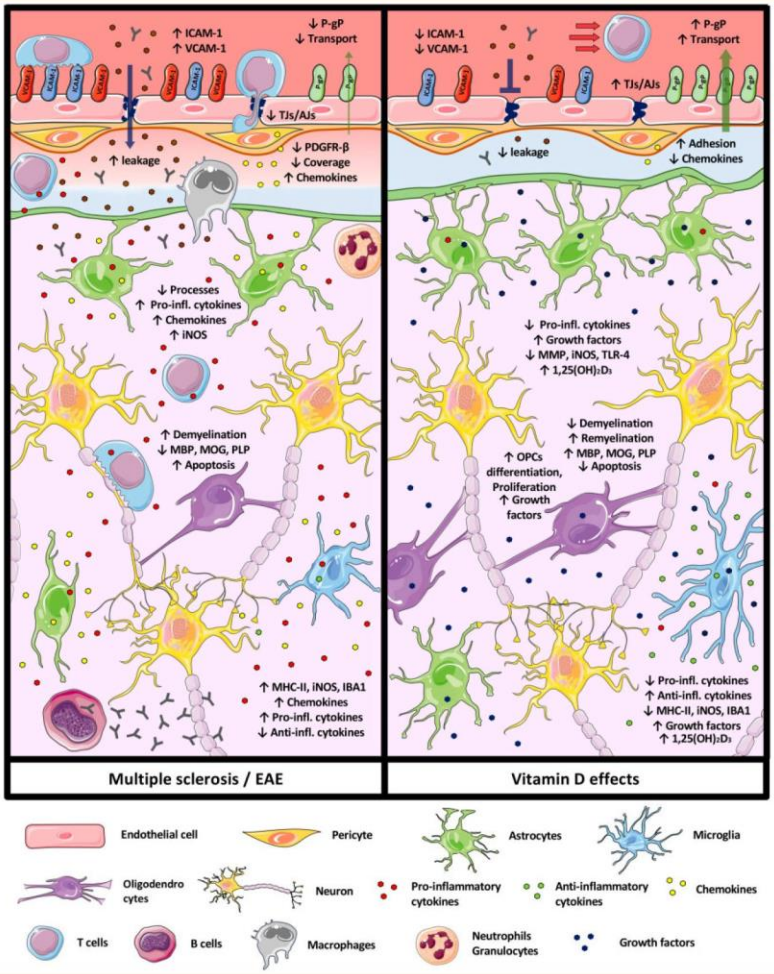

Figure 4 VitD modulates different cellular and molecular mechanisms of CNS-resident cells and of the blood–brain barrier involved in multiple sclerosis pathology. (Left) Schematic representation of the cellular and molecular mechanisms involved in multiple sclerosis/EAE pathology at the level of CNS-resident cells and the BBB. (Right) Schematic representation VitD impact on cellular and molecular mechanisms involved in multiple sclerosis/EAE pathology at the level of CNS-resident cells and the BBB. Changes in the cellular and molecular mechanisms are given as (↑) increased and (↓) decreased. Drawings of the individual cell types were adapted from Servier Medical Art (http://smart.servier.com/), licenced under a Creative Common Attribution 3.0 Generic License.

For instance, $1,25(OH)_2D_3$ prohibited the loss of TJs protein expression and decreased trans-endothelial electrical resistance (TEER) in brain microvascular endothelial cells (BMECs) incubated with TNFα or serum from RRMS patients *in vitro*.[143] Furthermore, $1,25(OH)_2D_3$ reduced endothelial cells apoptosis rate,[137,141,157] suggesting a potential beneficial role of VitD in sustaining BBB endothelial cells survival during multiple sclerosis. With respect to *in vivo* data, $1,25(OH)_2D_3$ treatment prohibited downregulation of zonula occludens-1 (ZO-) in spinal cord homogenates and reduced the extravasation of sodium fluorescein, Evans blue and IgG into the CNS of mice suffering from EAE.[26,149] Similarly, different randomized clinical trials (RCTs) on the effect of daily oral high dose of cholecalciferol in multiple sclerosis patients showed significantly reduced numbers of new Gd or new/enlarging T2 lesions compared with placebo group.[171–173]

Additional evidence of VitD impact on preventing BBB breakdown comes from the research fields of ischaemic stroke and obesity. For instance, $1,25(OH)_2D_3$ reduced the expression of matrix metalloproteinase-9 (MMP-9) and the permeability for fluorescein isothiocyanate (FITC)-dextran, as also increased TEER and TJs expression in a mouse brain endothelial cell line (bEnd3) cells under hypoxic conditions.[144] Furthermore, $1,25(OH)_2D_3$ treatment prior experimental stoke reduced Evans blue extravasation and infarct volumes in the CNS of different animal models for ischaemic stroke.[122,137,170] Similarly, oral administration of

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Table 1 Main cellular and molecular effects of VitD on CNS cells

	BBB (see Supplementary Table 3)	Pericytes (see Supplementary Table 4)	Astrocytes (see Supplementary Table 5)	Microglia (see Supplementary Table 6)	Oligodendrocytes (see Supplementary Table 7)
VitD metabolism					
VDR	↑ *in vitro*[118,119] n.s. *in vitro*[120,121] ↑ *in vivo*[122,123]	↑ *in vitro*[124,125]	↑ *in vitro*[126–128] n.s. *in vitro*[121]	↑ *in vitro*[121,129–131]	↑ *in vitro*[132–134]
Cyp24a1	↑ *in vitro*[118] n.s. *in vitro*[121] ↑ *in vivo*[135,136]		↑ *in vitro*[121,127]	↑ *in vitro*[121,127,129]	
Cyp24b1			n.s. *in vitro*[121,127]		
Growth factors					
BDNF	↑ *in vivo*[137,138]				
NGF			↑ *in vitro*[139,140]		
Signalling					
p-AKT	↑ *in vitro*[141,142]				
p-ERK	↑ *in vitro*[141,142]				
NF-kb	↓ *in vitro*[143,144]				
Activation					
Cell viability	↑ *in vitro*[141,144]			n.s. *in vitro*[145–147] ↑ *in vitro*[148]	
Leakage					
Claudin-5	↑ *in vitro*[143,144]				
ZO-1	↑ *in vitro*[143,144] ↑ *in vivo*[149,150]				
TEER	↑ *in vitro*[143,144]				
Brain efflux	↑ *in vivo*[135,151]				
Permeability	↓ *in vivo*[26,122,137,138,149,150]				
Trafficking					
Mrp1	n.s. *in vivo*[120,152]				
P-gP	↑ *in vivo*[120,135,136,151,152]				
BCRP	n.s. *in vivo*[135,136]				
Oxidative stress					
NO-production	↑ *in vitro*[141,142]			↓ *in vitro*[145,147,148,153] n.s. *in vitro*[154]	
ROS production	↓ *in vitro*[141,142]				
iNOS				↓ *in vitro*[129,131,148,155] n.s. *in vitro*[153]	
SOD	↑ *in vivo*[137,156]				
Arg1				↑ *in vitro*[131,155]	
Cytokines					
IL-10				↑ *in vitro*[129,130]	
TNFa				↓ *in vitro*[131,145,147,153,154] n.s. *in vitro*[148,155] ↓ *in vivo*[157,158]	
IL-1b				↓ *in vitro*[131,148,155]	
IL-6				↓ *in vitro*[129,131,147,148,153–155]	
Myelin					
Myelination					↑ *in vivo*[34,159]
MBP					↑ *in vivo*[159,160]
Demyelination					↓ *in vivo*[34,159–162]
CNS insult					
Osteopontin	↑ *in vivo*[122,123]				
GFAP			↓ *in vivo*[163–166] ↑ *in vivo*[161,167,168] n.s. *in vivo*[169]		
Radio clinical parameters					
Brain oedema	↓ *in vivo*[122,137,150]				
Neurological deficit	↓ *in vivo*[122,137,150] n.s. *in vivo*[170]				
New	↓ multiple sclerosis				

(continued)

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Table 1 (continued)

	BBB (see Supplementary Table 3)	Pericytes (see Supplementary Table 4)	Astrocytes (see Supplementary Table 5)	Microglia (see Supplementary Table 6)	Oligodendrocytes (see Supplementary Table 7)
Gd-enhancing lesions	Patients[171–173] n.s. multiple sclerosis patients[86]				

Molecules/mechanisms/endpoints showing concordant variation after VitD stimulation in more than one study for each cell types are represented in this table. See Supplementary Tables 3–7 for experimental details. Arg1, Arginase-1; BBB, blood–brain barrier; BCRP, breast cancer resistance protein; BDNF, brain-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; IL, interleukin; iNOS, inducible nitric oxide synthase; MBP, myelin basic protein; Mrp1, multidrug resistance-associated protein 1; NGF, neural growth factor; NO, nitric oxide; P-gP, permeability-glycoprotein; ROS, reactive oxygen species; SOD, superoxide dismutase; TEER, transepithelial/trans-endothelial electrical resistance; TNF-a, tumour necrosis factor alpha; VDR, vitamin D receptor.

cholecalciferol reduced BBB permeability for Evans blue in the hippocampus of obese rats,[138] which do also possess an altered BBB.[174–177] Furthermore, increased MMP-9 expression, IgG leakage and decreased immunostaining for TJs were observed in VitD-deficient rats after transient middle cerebral artery occlusion.[123]

Next to the presence of complex TJs, an additional property of the BBB is the presence of specific endothelial transporters, such as the efflux pump P-glycoprotein (P-gp), which actively removes unwanted compounds from the brain.[178] Interestingly, P-gp expression was reduced in BMECs in active lesions of multiple sclerosis patients[179] and P-gp transport function was significantly reduced during EAE in rats,[179] suggesting reduced P-gp function at the BBB in multiple sclerosis. To the best of our knowledge, there are no studies investigating the impact of VitD on BBB transporters in multiple sclerosis. However, in the context of Alzheimer's disease, different studies have demonstrated that VitD can modulate the expression of BBB transporters. In fact, $1,25(OH)_2D_3$ increased P-gp expression and transport activity in BMECs *in vitro* and *in vivo*.[118,135] Furthermore, $1,25(OH)_2D_3$ pre-treatment reduced amyloid-β accumulation in the parenchyma of two different Alzheimer's disease mouse models.[136] Although the role of BBB transporters in multiple sclerosis pathogenesis still needs further investigation, these observations provide some evidence on how VitD could potentially be beneficial for BBB function in multiple sclerosis.

Besides BBB breakdown and changes in expression of BBB transporters, post-mortem brain tissues of multiple sclerosis patients have also shown increased adhesion molecules expression such as intercellular adhesion molecule (ICAM)-1 and vascular cell-adhesion molecule (VCAM)-1, accompanied by an increased infiltration of leucocytes into the CNS parenchyma.[114,180] To this end, $1,25(OH)_2D_3$ treatment reduced ICAM-1 and VCAM-1 expression on BMECs incubated with TNFα or serum from acute phase RRMS patients *in vitro*,[143] highlighting the potential of VitD to modulate immune cell migration to the CNS by acting directly on the BBB endothelium. In line with this hypothesis, many studies have shown that VitD pre-treatment reduces immune cell infiltration into the CNS during EAE.[26,50,149,160,163,181,182] However, the exact mechanisms by which VitD might modulate the BBB to control immune cell migration into the CNS still have to be elucidated. Furthermore, the effect of VitD on the expression of other important BBB adhesion molecules, which contribute to the recruitment of immune cells into the CNS have not been investigated yet.[114,183–187]

Taken together, these observations suggest a beneficial effect of VitD supplementation on BBB integrity during different neuroinflammatory processes. Still, there is currently no evidence of a direct effect of VitD on BBB endothelial cells *in vivo* during EAE nor in multiple sclerosis patients. Further investigations are required to clarify if VitD can exert a direct or indirect effect on the BBB endothelium and how this may affect the course of multiple sclerosis.

Brain pericytes

Pericytes are mural cells associated with the walls of blood capillaries[188] and play a crucial role in sustaining BBB integrity.[189–195] Indeed, BBB permeability is inversely proportional to pericyte coverage,[196,197] and their loss leads to increased perivascular immune cells infiltration.[198,199]

The specific role of brain pericytes in EAE or multiple sclerosis has not been addressed in depth. Nevertheless, many studies have demonstrated the contribution of pericytes in mediating different neuroinflammatory processes.[199–203] Furthermore, atypical symptoms and sudden death were observed in pericyte-deficient mice during EAE, underscoring a protective role of pericytes in neuroinflammation.[204] During development, brain capillaries secrete platelet derived growth factor (PDGF)-β to recruit PDGF-receptor-beta (PDGFR-β) expressing pericytes to their wall.[190,205] Mice suffering from EAE were reported to have reduced numbers of PDGFR-β^+ pericytes, and lower expression of PDGFR-β expression in pericytes.[206,207] With respect to multiple sclerosis patients, CNS biopsies showed that active lesions harbour higher numbers of proliferative and quiescent perivascular cells (including pericytes) compared with inactive ones, but that these are lower in chronic lesions when compared with healthy tissue.[208] This indicates that the inflammatory status of the CNS plays an important role in determining the number of pericytes associated with microvessels.

Recent studies showed that brain pericytes lack the expression of the oxidative enzyme CYP27B1 and can

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therefore not synthesize $1,25(OH)_2D_3$,[197,209,210] suggesting that pericytes rely rather on paracrine or circulating $1,25(OH)_2D_3$. Accordingly, only a few studies have investigated the effects of VitD on brain pericytes (Fig. 4 and Table 1, Supplementary Table 4).

Brain pericytes express VDR and its expression is increased upon $1,25(OH)_2D_3$ exposure[124,125,143,210] (Table 1). In addition, $1,25(OH)_2D_3$ induced an anti-inflammatory phenotype in brain pericytes by downregulating the expression of chemokines like CCL2 *in* vitro.[210] In line with this, $1,25(OH)_2D_3$ increased the activity of the antioxidant enzyme γ-glutamyl transferase in brain pericytes *in vitro*.[211] Furthermore, primary brain pericytes isolated from $1,25(OH)_2D_3$ treated wild type (WT) mice showed reduced proliferation and migration *in vitro* but had increased adhesion to extracellular matrix proteins compared with cells isolated from VDR KO mice.[125] The latter might ultimately help strengthening BBB integrity.

The effect of VitD on pericyte coverage in multiple sclerosis has to the best of our knowledge not yet been investigated. Nevertheless, it has been shown that vessels of tumours transplanted into VDR KO mice have less pericytes coverage compared with those in WT mice, suggesting that pericyte attachment might be partially dependent on VDR.[212] In contrast, the endothelial cell/pericyte ratio was reduced in full retinal mounts of VDR KO mice compared with those of WT mice.[213]

Although the above-listed studies show variable effects of VitD on pericytes, further investigations are necessarily required to establish the therapeutic potential of VitD at this level.

Biological effects on glial cells

Astrocytes

Astrocytes are among the most crucial modulators of brain function. They maintain neurotransmitters homoeostasis, cerebral blood flow, and provide essential nutrients and growth factors to the entire CNS.[214] During development astrocytes play a major role in the maturation of the BBB by inducing for example the expression of TJs in CNS capillaries.[215] Furthermore, astrocytes contribute to making the CNS an immune-privileged site by forming a second barrier called the glia limitans, which ensheaths the entire CNS parenchyma and, along with the BBB, prevents unrestricted entrance of immune cells.[107,216]

It has been shown that astrocytes are early responders in nascent white matter lesions of multiple sclerosis patients[217,218] and in the CNS of mice after induction of EAE.[219–221] Indeed, they can react promptly to inflammatory stimuli such as TNFα and IL-1β by amplifying neuroinflammation in a NFkB-dependent manner,[222] and by recruiting leucocytes to the CNS via the expression of inflammatory chemokines, cell-adhesion molecules and nitric oxide synthase (iNOS).[223,224] In multiple sclerosis, astrocytes attain an hypertrophic morphology and a reduced number of cytoplasmic processes, leading to the retraction of the glia limitans and thereby increasing the chance of immune cell infiltration in the CNS.[217] On the other hand, activated astrocytes also exert beneficial effects by recruiting microglia and promoting clearance of myelin debris in the CNS of mice suffering from EAE.[225] This directs us toward the possibility of a dual role of astrocytes in multiple sclerosis, by not only sustaining but also preventing the development of neuroinflammation.[216]

Many studies have shown that astrocytes express CYP24A1, CYP27B1 and VDR.[121,126,127,132,226–228] These observations suggest that astrocytes can not only synthetize $1,25(OH)_2D_3$ but also respond to it in an autocrine or paracrine manner. To this end, different studies on effects of VitD on astrocytes and their contribution in multiple sclerosis/EAE and other neuroinflammatory diseases have been conducted (Fig. 4 and Table 1, Supplementary Table 5).

Regarding inflammation, a recent *in vitro* study showed that $25(OH)D_3$ leads to a significant decrease in the expression of pro-inflammatory cytokines such as TNFα and IL-1β, as also TLR4 in activated astrocytes.[226] Similarly, reduced TLR4 expression in the CNS of mice suffering from traumatic brain injury after combined $1,25(OH)_2D_3$ and progesterone treatment was observed.[164] Furthermore, a $VitD_2$ analogue could inhibit NF-kB signalling in astrocytes of mice suffering from EAE, leading also to reduced clinical score.[163] Taken together, these results point toward a beneficial effect of VitD treatment in multiple sclerosis by potentially reducing the pro-inflammatory response of astrocytes in the CNS.

With respect to CNS astrocytes density and GFAP expression, VitD treatment gave discrepant results in different neuroinflammatory animal models.[161,163–169] For example, the $VitD_2$ analogue reduced the populations of $GFAP^+$ astrocytes in the spinal cord of mice suffering from EAE,[163] while $1,25(OH)_2D_3$ increased the number of $GFAP^+$ astrocytes in the CNS of mice with cuprizone induced toxic demyelination.[161] $1,25(OH)_2D_3$ was also shown to rescue cell death by decreasing ROS production, iNOS and p53 activity in astrocytes *in vitro*.[229,230] Similarly, VitD treatment decreased immunostaining for $iNOS^+$ astrocytes in the CNS of EAE rats, suggesting VitD as a potential antioxidant treatment in multiple sclerosis, since ROS production is well established in the CNS of multiple sclerosis patients.[231]

Concerning cell survival, growth factors such as NGF and VEGF have been shown to be upregulated upon $1,25(OH)_2D_3$ treatment in activated astrocytes *in vitro*.[128,226(p3),139] However, no changes in the expression of their receptors were observed.[126] Furthermore, an analogue of $1,25(OH)_2D_3$ decreased the proliferation, migration and metalloprotease activity of astrocytes *in vitro*.[232]

Altogether, these observations suggest a beneficial effect of VitD supplementation in reducing astrocyte activation and increasing their growth factor production in different models of multiple sclerosis.

Microglial cells

Microglia are the tissue-resident macrophages of the CNS parenchyma[233,234] and play a key role in the brain and spinal

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cord by promoting synapse formation/refinement, oligodendrogenesis and myelin synthesis.[235] In the CNS, microglia remain selfsustained by proliferation and build the first line of defence in the CNS parenchyma.[236] In fact, microglia are very efficient in surveying the parenchymal environment for pathogens, cytokines, chemokines, neurotransmitters, neuromodulators and damaged cells.[237] When a potential danger is recognized, microglia get activated assuming a more amoeboid-like appearance, and start clearing the surrounding environment by phagocytosis, an inherent characteristic of these cells that permits the maintenance of CNS homeostasis.[238,239] Furthermore, they also secrete different growth factors such as TGF-β to promote cell survival.[240]

Microglia are recognized as a key player in many different inflammatory and neurodegenerative disorders. In the context of multiple sclerosis, microglia are abundant immune cells present in active lesions.[241] Within active lesions, microglia play a critical role in antigen presentation, recruitment of T cells, ROS production and release of pro-inflammatory cytokines, which further amplify neuroinflammation.[239,242] On the other hand, microglia are equally crucial for clearing myelin debris and enabling remyelination.[243] These observations suggest that microglia play both a detrimental but also a beneficial role in multiple sclerosis pathology.

With respect to VitD, it has been demonstrated that microglia can synthetize calcitriol *in vitro*,[244] since they express both CYP27A1 and CYP27B1.[121,127,129,145,245] Along with BBB endothelial cells, pericytes and astrocytes, microglia also express VDR and can respond in either an autocrine or paracrine manner to calcitriol.[121,127,129,130,145,153,245] Interestingly, a recent study showed that local synthesis of calcitriol by myeloid cells (microglia included) was essential for VitD-mediated EAE resistance.[246] Several *in vitro* and *in vivo* studies in the broader context of neuroinflammation have also investigated the impact of VitD treatment on microglia (Fig. 4 and Table 1, Supplementary Table 6). Calcidiol and calcitriol can reduce microglia activation and antigen presentation by downregulating the expression of Iba1, MHC-II, CD86 and TLR-4 *in vitro* and *in vivo* in different neuroinflammatory models, including EAE.[131,146,149,155,158,162,165,246,247] This was confirmed also by the observation of increased microglia activation in VitD-deficient mice.[146] On the other hand, different *in vitro* and *in vivo* studies showed increased CD163, CD206, CD204 expression on microglia after calcitriol treatment, suggesting a switch toward an anti-inflammatory phenotype.[131,165,170] Furthermore, calcidiol and calcitriol treatment reduced the production of pro-inflammatory cytokine such as IL-6, IL-1β, TNF-α, IFN-γ and MIP-1α, and on the other hand increased the expression of anti-inflammatory cytokines such as TGF-β1, IL-10, IL-4, IFN-α and IFN-β in microglia *in vitro* and *in vivo* in different neuroinflammatory models, including EAE.[129–131,147,148,153–155,158,165,248]

Along with a reduction in pro-inflammatory cytokine secretion, different *in vitro* studies have demonstrated that calcitriol can also reduce the production of ROS in microglia.[131,145,147,148,153] This might result from the downregulation of iNOS expression in microglia after cholecalciferol, calcidiol and calcitriol treatment, as shown *in vitro* and *in vivo*.[129,131,148,155,165,230,247,249] Furthermore, calcitriol increased microglia's TGF-β expression[153,165] pointing toward the possibility of a beneficial effect of VitD treatment in maintaining cell survival in the CNS of multiple sclerosis patients. Modulation of microglia phagocytosis by calcitriol has been also proposed by two different studies *in vitro*,[154,248] however, with discrepant results.

Altogether, the current evidence suggests that VitD may alleviate the pro-inflammatory phenotype of microglial cells promoting an anti-inflammatory milieu in the CNS by directly modulating microglia activity during multiple sclerosis.

Oligodendrocytes

Oligodendrocytes are a type of neuroglial cells of the CNS, which provide support and insulation to axons by ensheathing them with multiple lamellae of their membrane, the myelin.[250] They arise from oligodendrocyte precursor cells (OPCs)[251] and are the last cell type generated in the CNS during development.[252] Axon myelination is essential for fast and efficient signal transmission between neurons and proper neuronal function in the CNS.[253] Furthermore, oligodendrocytes provide essential metabolic support to neighbouring cells by releasing different growth factors such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1).[254]

Two different types of oligodendrocyte dysfunction, namely an immune-mediated oligodendrocyte dysfunction and a primary oligodendrogliopathy[255] with subsequent demyelination and neurodegeneration accompanied by oligodendrocyte apoptosis have been observed in multiple sclerosis.[256] In the first case, lymphocytes[257,258] and autoantibodies targeting myelin proteins (e.g. MBP, MOG and PLP)[259–261] induce direct cytotoxicity to myelin and oligodendrocytes. In the second case, oligodendrocyte apoptosis as a response to stress precedes both inflammation and the recruitment of immune cells to the lesion site.[262] Nevertheless, demyelinating plaques can sometimes undergo remyelination resulting in partial recovery of the neurological function in RRMS.[263] This process is mainly sustained by OPCs which after activation, proliferate and migrate to the damaged area, and differentiate into mature oligodendrocytes to replace harmed myelin sheaths.[264] On the other hand, in case of progressive multiple sclerosis, remyelination is impaired and neurological function is irreversibly lost.[265]

In contrast to microglia, oligodendrocytes lack the expression of CYP27B1 and are therefore unable to synthetize $1,25(OH)_2D_3$.[121] Nevertheless, oligodendrocytes and OPCs express VDR suggesting they can respond to calcitriol.[121,127,132–134,266] To this end, different *in vitro* and *in vivo* studies investigated the impact of VitD treatment of oligodendrocytes and myelin in the context of multiple sclerosis (Fig. 4 and Table 1, Supplementary Table 7).

In the last two decades, multiple studies have demonstrated that cholecalciferol and $1,25(OH)_2D_3$ treatment have a beneficial effect on myelination by reducing

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demyelination and increasing remyelination, as also restoring neurological function in the CNS in different animal models of multiple sclerosis including EAE.[34,159–162,266] Indeed, animals suffering from neurodegeneration showed increased expression of MBP, MOG and PLP upon cholecalciferol or calcitriol treatment compared with vehicle control.[159,160,266,267] Similarly, calcitriol treatment reduced OPC proliferation and lead to higher oligodendrocyte differentiation with consequent increase in MBP production *in vitro*.[133,266] In addition, rats suffering from ethidium–bromide induced demyelination showed decreased caspase-3 activation in the CNS when treated with calcitriol, suggesting also a beneficial effect of VitD on oligodendrocyte apoptosis in multiple sclerosis.[159] Along the beneficial effects of VitD on myelination, different *in vitro* studies showed that calcitriol could also increase the expression of growth factors (e.g. nerve growth factor in oligodendrocytes[132,134] and BDNF in neural stem cells[133] and therefore potentially sustain cell survival in the CNS).

Altogether, despite potential indirect effects of VitD on oligodendrocytes and the lack of evidence for a role of VitD on oligodendrocytes in multiple sclerosis brains, these results highlight VitD as a promising candidate to reducing demyelination (Table 1).

Globally, VitD protects the CNS from inflammation at the cellular level (BBB and glia) through the modulation of multiple mechanisms including cytokine and growth factors secretion, cell signalling, response to oxidative stress, BBB leakage and trafficking. The most robust effects of VitD in the CNS are summarized in Table 1.

VitD therapy in multiple sclerosis patients

Despite all previous data about the potential immunological regulation and neuroprotective effects of VitD in multiple sclerosis models and multiple sclerosis patients, controversial results have been obtained from clinical trials evaluating VitD supplementation in multiple sclerosis patients. Kimball *et al.* evaluated tolerance and safety of escalating doses of active VitD ranging from 28 000 to 280 000 IU/week in an open-label study including 12 RRMS patients over a total of 28 weeks.[172] Despite final mean serum concentrations of 25(OH)D of 386 ± 157 nmol/l, they did not observe any severe adverse events (SAEs) on this very small cohort. Since then, several SAEs (e.g. severe gastric symptoms, pseudotumour and seizures, tonic–clonic seizures, severe hypercalcemia) were observed at extremely high doses (100 000 IU/day for 1–36 months), suggesting that excessive supplementation with VitD can be dangerous despite a plateau concentration close to 400 nmol/l and that treatment duration might play a role in VitD toxicity.[268] Moreover, little is known about the immunological effects of very high levels of circulating VitD. In animal models, the higher VitD dose, the higher immunomodulatory effect is generally observed, although excessive VitD treatment achieving serum levels over 250 nmol/L resulted in pro-inflammatory effects through lymphocyte activation and demyelination and myeloid infiltration into the brain, in addition to perturbations of phosphocalcic homeostasis.[269]

Since the original study by Mahon *et al.* in 2003, we identified more than 20 published case–control studies and double-blind RCTs aiming at evaluating the potential clinical and radiological benefits of VitD supplementation in multiple sclerosis patients.[47,81–94,171,173,270–276] Some of these were recently reviewed and showed limited improvement in multiple sclerosis clinical and/or MRI features including fatigue, expanded disability status scale, relapses, T2-lesions, non-evidence of disease activity (NEDA-3) and in retinal nerve fibre layer thickness.[21,277] These trials also evaluated safety of VitD supplementation at more physiological dosages (<15 000 IU/day) and did not observe a significant increase of AEs or SAEs in VitD-treated groups compared with placebo groups (CHOLINE,[275] SOLAR[171]). Importantly, phosphocalcic homeostasis seemed respected at this level of supplementation, as no cases of calciuria and hypercalcemia were observed (SOLAR).

The safety range of VitD levels has been defined by an expert consensus in 2010 as of 30–100 ng/ml (equivalent to 75–250 nmol/l), considering that in healthy subjects who have spent prolonged periods in a sunny environment, measured 25(OH)D concentrations rarely exceed 100 ng/ml.[278] In populations with light skin frequently exposed to the sunlight, the upper limit of circulating VitD levels is located between 75 and 150 nmol/l, providing an optimal range to reach after VitD supplementation, as defined by experts of bone and calcium homeostasis in order to prevent osteoporosis and fractures.[279] However, so far, there are no recommendations for multiple sclerosis patients on potential VitD supplementation to achieve optimal levels for immune modulation and clinical effect.

In order to define VitD dosing to achieve optimal VitD therapeutic range after supplementation, we analysed 24 therapeutic trials accounting for 742 treated patients and providing their final VitD levels. When plotting the time of exposure with the daily dose of VitD supplementation of the treated group, we observed that protocols were very heterogeneous with treatment duration ranging from 2 to 24 months and dosages ranging from 1000 to 20 000 UI/day (Fig. 5A). However, we observed a strong correlation between the daily dose and the final circulating VitD levels of treated patients (Spearman $r = 0.803$, $P < 0.0001$, Fig. 5B), indicating that a daily dose of VitD between 3000 and 11 000 IU for 3–24 months achieves final blood VitD levels within the 75–150 nmol/l recommended range (red dots, Fig. 5A and B). Even at the higher doses used in the SOLAR study[171] where patients received 14 000 UI/day for 12 months, VitD levels above 200 nmol/l were obtained without occurrence of SAEs after 1 year. This potentially increases the safety of higher VitD supplementation reaching up to 250 nmol/l for short periods. However, the exact concentrations of VitD in multiple sclerosis needed to properly

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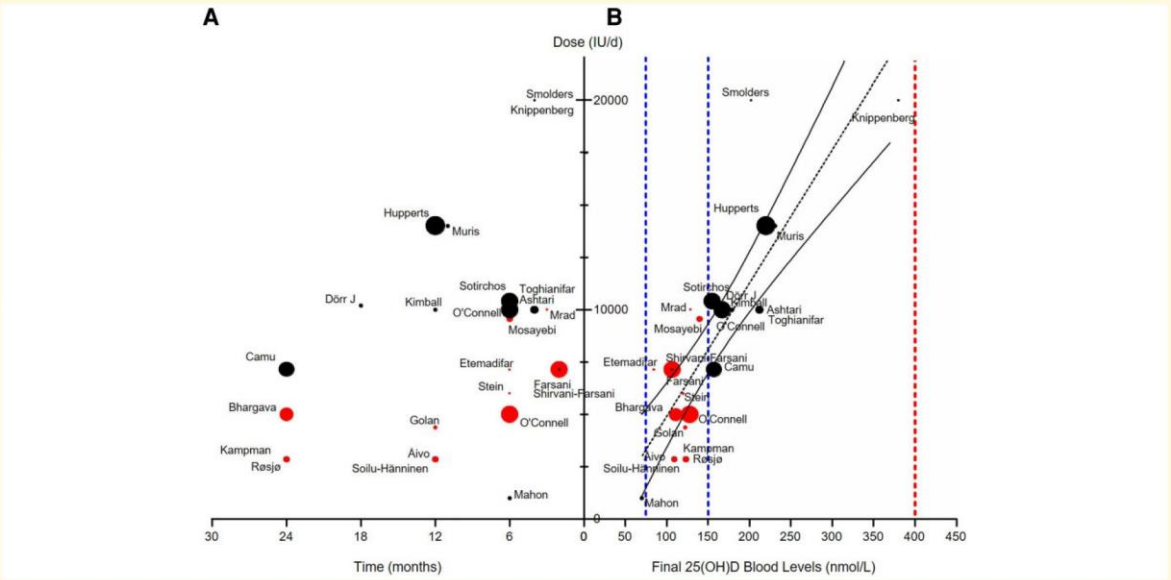

Figure 5 Correlation between daily dose of VitD and the final blood levels of 25(OH)D from different Phase 2 and Phase 3 RCTs in RRMS patients. (**A**) Representations of the different clinical studies on the effect of VitD in multiple sclerosis patients according to the dose administered (IU/day) as a function of time (months). (**B**) Representations of the different clinical studies in multiple sclerosis patients according to the final 25(OH)D blood levels (nmol/l) as a function of time (months). Dots are proportional to the number of patients in each study. Red dots represent studies with final blood VitD levels in the range 75–150 nmol/l as determined by Souberbielle *et al.*, 2019; other studies are represented in black. Linear regression shows a significant correlation between daily dose of VitD and the final blood levels of 25(OH)D [$r^2 = 0687$; 25(OH)D = daily dose/63.5 + 22.5].

restore immune homeostasis over the long term remains to be determined as well as the optimal risk/benefit balance for VitD supplementation. Given the weak clinical and radiological effects of VitD treatment in short-term RCTs involving multiple sclerosis patients, further analysis of the *in vivo* immunological efficacy of VitD are warranted to refine the objectives of chronic VitD supplementation in multiple sclerosis patients.

Discussion and perspectives

While it is indisputable that VitD exerts strong immunomodulatory effects, it is still debated whether VitD supplementation is efficacious in multiple sclerosis. Data from animal models and *in vitro* studies provide important information on VitD effects to optimize its clinical application. Despite the large number of studies published in the field of multiple sclerosis so far, sound review of the literature revealed that analysis of VitD modulation of the immune system only focused on subpopulations of PBMCs and cytokine secretion, principally by FACS, ELISA and RT-PCR. FACS relies on designed panels of antibodies coupled to serial wavelength fluorophores to target markers of cell surface (and also intracellular markers) and analyse ± sort cells of interest, providing an efficient tool to study immune cell populations. However, when dealing with complex immunological diseases like multiple sclerosis, limited numbers of lasers and channels does not provide the ability to study subpopulations of a given sample in a row. The recent development of high-parameter FACS equipped with 4–7 lasers allows the detection of up to 18–30 markers in each cell that is much more in line with the investigation of several subpopulations.

New and innovative techniques developed recently could also be of high interest to further discriminate lymphocyte subsets of importance in multiple sclerosis pathophysiology, regulation of which by treatments is mandatory to efficiently reduce inflammation and disease activity. Taking advantage of antibody coupling to non-radioactive isotopes of heavy metals that can be detected by mass spectrometry, cytometry by time of flight (CyTOF) enhances the study and analysis of very specific types of cells.[280] This technique allows the detection of a large number of cell types and the assessment of the immune cell activation state (around 60 markers) and overcomes the limitations of the use of fluorophores in flow cytometry. Using this technique, Couloume *et al.*[281] detected an increased abundance of a T-bet expressing B-cells subset and an increased abundance of a CD206$^+$ classical monocytes subset in the blood of multiple sclerosis patients with early multiple sclerosis. Johansson *et al.*[282] used CyTOF and identified an multiple sclerosis-associated

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B-cell population using an unsupervised clustering of all CSF cells characterized by the expression of CD49d, CD69, CD27, CXCR3 and also found that proteins involved in neural plasticity were reduced in multiple sclerosis. Comparing multiple sclerosis to other inflammatory and non-inflammatory conditions of the CNS, Galli *et al.*[283] identified an expanded T-helper cell subset characterized by the expression of GM-CSF and the C–X–C chemokine receptor Type 4 (CRCX4), revealing Th cells as the major contributor to GM-CSF production in the peripheral immune compartment of multiple sclerosis patients, with highest GM-CSF production in the effector memory fraction. Using intracellular staining after permeabilisation of cells, they combined surface and intracellular markers for mass cytometry and showed that the vast majority of GM-CSF producing Th cells co-expressed TNFα and IL-2.[283] Although intracellular markers can be used for additional analyses, one limitation of CyTOF is that cells are directly discarded at the end of the analysis and cannot be sorted for complementary functional tests *in vitro*. Another limitation of CyTOF is the important signal variation over time and among centres, precluding multicentre studies unless a reference sample is used to normalize the data and ensure reliable comparisons. Finally, CyTOF is limited by the targeted investigation of specific cell populations and markers using a panel of antibodies designed by the experimenter, precluding discovery of novel markers of new cell subtypes potentially playing an important role in multiple sclerosis. Despite these limitations, CyTOF emerges as a very innovative technique with a high potential to investigate immune regulation induced by VitD in multiple sclerosis patients.

Another approach to decipher changes in immune cell profiles and activation is RNA sequencing (RNAseq). This technique, relying on the identification and quantification of gene transcripts (coding RNA) in each sample is used to analyse qualitatively and quantitatively gene expression changes. It can also be used to investigate non-coding RNAs potentially playing the role of regulators of gene expression. Comparing the linear and circular transcriptomes of leucocytes from 50 multiple sclerosis patients and 20 healthy controls (HC), Iparraguirre *et al.*[284] found 689 upregulated and 683 downregulated transcripts in multiple sclerosis patients, most of them related to immune processes including neutrophil activation involved in immune response, neutrophil degranulation and granulocyte activation. Investigating circular RNA (circRNA) known as non-coding RNA regulating various immune responses, they observed a global upregulation of circRNAs in multiple sclerosis patients compared with HCs, with similar circRNA profiles between multiple sclerosis types. Long non-coding RNAs (lncRNAs) were also investigated in multiple sclerosis patients versus HCs by Han *et al.*,[285] who identified 1438 downregulated and 945 upregulated lncRNAs, some of which were found to influence the antigen processing/presentation and MAPK signalling pathway in multiple sclerosis. To provide more informative data regarding immune regulation, RNAseq can be used to investigate the modifications of specific lymphocyte populations sorted by FACS. Fernandes *et al.*[286] reported a detailed gene expression by RNAseq profiling analysis of FACS-sorted $CD4^+$ and $CD8^+$ T cells from HCs, RRMS and SPMS patients. Their analysis revealed 149 differentially expressed genes in both cell types, confirming previous findings (human-leucocyte antigen G) and discovering several novel genes and processes involved in multiple sclerosis, like IgG heavy chain and neurofilament-light chain. Looking at the effects of VitD in THP-1 cells (the human acute monocytic leukaemia cell line) by RNAseq, Neme *et al.*[287] identified 1284 out of 14 402 expressed genes (8.9% of the expressed transcriptome of THP-1 cells) regulated by $1,25(OH)2D_3$. In human PBMCs from HCs exposed to a bolus of VitD (80 000 UI), they also observed that 702 genes were significantly regulated, including genes involved in general protein translation, monocyte differentiation and cellular growth control, suggesting that this approach could be applied to multiple sclerosis patients.[288]

More recently, single cell RNAseq (scRNAseq) has emerged as a powerful method to analyse modifications of cell populations and activation state. Compared with CyTOF, scRNAseq data are much more highly dimensional. Compared with bulk RNAseq that provides data from a collection of mixed cell populations to which we cannot point to the individual contribution, ScRNAseq directly reveals all the transcripts expressed by a cell, thus ensuring a qualitative and quantitative analysis of gene changes of all cell subsets of the sample. In EAE, using a combination of scRNAseq and other techniques, Wheeler *et al.*[289] identified a reduced NRF2-driven gene expression and increased MAFG and MAT2α signalling in astrocytes, potentially promoting pro-inflammatory genomic programmes in astrocytes via GM-CSF, produced by inflammatory T cells recruited to the CNS in multiple sclerosis and EAE. Using scRNAseq to establish a detailed molecular description of microglia from HCs and multiple sclerosis patients, Masuda *et al.*[290] observed that the microglial profile from HCs was enriched for transcripts indicative of a homeostatic phenotype, while multiple sclerosis patients showed multiple activated subsets potentially indicating engagement with infiltrating autoreactive T cells, matrix remodelling or reduction of cytotoxic $CD8^+$ T-cell functions. Esaulova *et al.*[291] used scRNAseq to profile individual cells of CSF and blood from two subjects with RRMS and one with anti-MOG associated disorder and identified myeloid cell types expressing a microglial signature. Finally, using scRNAseq and FACS coupled to bulk RNAseq, Ramesh *et al.*[292] analysed paired CSF and blood B-cell subsets from multiple sclerosis patients, showing that B cells primarily clustered based on their subset type and the body compartment from which they were isolated. Independent analyses across technologies demonstrated that NF-κB and cholesterol biosynthesis pathways were activated, and specific cytokine and chemokine receptors were upregulated in CSF memory B cells.

Altogether, scRNAseq has a high potential to investigate the effects of VitD in immune cells in multiple sclerosis

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patients. However, scRNAseq has limitations, especially concerning the identification of cell subtypes according to the gene transcripts alone, especially concerning $CD4^+$ T cells that show very little expression of clusters of differentiation genes. To overcome this caveat, the use of antibodies generally used for characterization of cell subtypes by FACS has been barcoded (adjunction of a specific RNA sequence on the Fc fragment of the protein) to ensure faithful selection of the target population for genomic analysis. This method called Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq) allows accurate characterization of both phenotype and transcriptome in a scRNAseq experiment.[293–295]

All these data strongly support the accuracy of CYTOF/CITE-seq and scRNAseq to analyse the effects of VitD on the whole PBMC population to improve our understanding of the pleiotropic effects of VitD while keeping detailed analysis of key populations modified by VitD.

Conclusion

Despite conflicting results, the overall effect of VitD supplementation in multiple sclerosis patients and animal models of multiple sclerosis results in a global modulation of inflammation. Inconclusive results from the clinical studies analysing the effect of VitD supplementation as add-on therapy in small numbers of multiple sclerosis patients or during short periods do not exclude a potential protective effect *in vivo*. Despite an excellent clinical safety profile of VitD, excessive doses are associated with SAEs, limiting the therapeutic range of VitD much below the high doses generally used *in vitro*. Finally, well-powered multicentre randomized controlled trials of VitD monotherapy versus placebo are still needed to bring significant data highlighting the therapeutic effectiveness of VitD in multiple sclerosis, and to refine the optimal posology to reach clinical effects and allow generalization of VitD supplementation in multiple sclerosis. The ongoing RCT D-lay multiple sclerosis (NCT01817166) included 316 multiple sclerosis patients within 3 months from disease onset to show the ability of high-dose cholecalciferol versus placebo for 2 years to reduce disease activity. This study will also provide useful biological samples to decipher the complex modifications of immune cell hierarchy and functions by coupling high-throughput technologies such as high-parameter flow cytometry and CITE-seq.

Data sharing is not applicable to this article as no new data were created or analysed in this study. Statistical analysis is not applicable to this article.

Funding

This research has been supported by Agence Nationale de la Recherche (ANR) grant ANR-19-CE14-0043 to A.A., E.T., and Swiss National Science Foundation (SNSF) grant 310030E_189312 to B.E.

Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at Brain Communications online.

References

1. Ramagopalan SV, Heger A, Berlanga AJ, *et al.* A ChIP-seq defined genome-wide map of vitamin D receptor binding: Associations with disease and evolution. *Genome Res.* 2010;20(10):1352–1360. doi:10.1101/gr.107920.110.
2. Westerlind H, Ramanujam R, Uvehag D, *et al.* Modest familial risks for multiple sclerosis: A registry-based study of the population of Sweden. *Brain.* 2014;137(Pt 3):770–778. doi:10.1093/brain/awt356.
3. International Multiple Sclerosis Genetics Consortium. Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science.* 2019;365(6460):eaav7188. doi:10.1126/science.aav7188.
4. D'hooghe MB, Haentjens P, Nagels G, De Keyser J. Alcohol, coffee, fish, smoking and disease progression in multiple sclerosis. *Eur J Neurol.* 2012;19(4):616–624. doi:10.1111/j.1468-1331.2011.03596.x.
5. Hedström AK, Hillert J, Olsson T, Alfredsson L. Smoking and multiple sclerosis susceptibility. *Eur J Epidemiol.* 2013;28(11):867–874. doi:10.1007/s10654-013-9853-4.
6. Munger KL, Chitnis T, Ascherio A. Body size and risk of MS in two cohorts of US women. *Neurology.* 2009;73(19):1543–1550. doi:10.1212/WNL.0b013e3181c0d6e0.
7. Bjornevik K, Cortese M, Healy BC, *et al.* Longitudinal analysis reveals high prevalence of Epstein-barr virus associated with multiple sclerosis. *Science.* 2022;375(6578):296–301. doi:10.1126/science.abj8222.
8. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: The role of infection. *Ann Neurol.* 2007;61(4):288–299. doi:10.1002/ana.21117.
9. Ostkamp P, Salmen A, Pignolet B, *et al.* Sunlight exposure exerts immunomodulatory effects to reduce multiple sclerosis severity. *Proc Natl Acad Sci U S A.* 2021;118(1):e2018457118. doi:10.1073/pnas.2018457118.
10. Lucas RM, Byrne SN, Correale J, Ilschner S, Hart PH. Ultraviolet radiation, vitamin D and multiple sclerosis. *Neurodegener Dis Manag.* 2015;5(5):413–424. doi:10.2217/nmt.15.33.
11. Ascherio A, Munger KL, Simon KC. Vitamin D and multiple sclerosis. *Lancet Neurol.* 2010;9(6):599–612. doi:10.1016/S1474-4422(10)70086-7.
12. Simpson S, Taylor B, Blizzard L, *et al.* Higher 25-hydroxyvitamin D is associated with lower relapse risk in multiple sclerosis. *Ann Neurol.* 2010;68(2):193–203.
13. Mowry EM Jr, Waubant E, McCulloch CE, *et al.* Vitamin D status predicts new brain magnetic resonance imaging activity in multiple sclerosis. *Ann Neurol.* 2012;72(2):234–240. doi:10.1002/ana.23591.
14. Thouvenot E, Orsini M, Daures JP, Camu W. Vitamin D is associated with degree of disability in patients with fully ambulatory relapsing-remitting multiple sclerosis. *Eur J Neurol.* 2015;22(3):564–569. doi:10.1111/ene.12617.
15. Muscogiuri G, Barrea L, Somma CD, *et al.* Sex differences of vitamin D status across BMI classes: An observational prospective cohort study. *Nutrients.* 2019;11(12):3034. doi:10.3390/nu11123034.

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16. Orton SM, Wald L, Confavreux C, *et al.* Association of UV radiation with multiple sclerosis prevalence and sex ratio in France. *Neurology*. 2011;76(5):425–431. doi:10.1212/WNL.0b013e31820a0a9f.
17. VanAmerongen BM, Dijkstra CD, Lips P, Polman CH. Multiple sclerosis and vitamin D: An update. *Eur J Clin Nutr*. 2004;58(8):1095–1109. doi:10.1038/sj.ejcn.1601952.
18. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC. 1,25-Dihydroxyvitamin D_3 receptors in human leukocytes. *Science*. 1983;221(4616):1181–1183. doi:10.1126/science.6310748.
19. Veldman CM, Cantorna MT, DeLuca HF. Expression of 1,25-dihydroxyvitamin D_3 receptor in the immune system. *Arch Biochem Biophys*. 2000;374(2):334–338. doi:10.1006/abbi.1999.1605.
20. Peelen E, Knippenberg S, Muris AH, *et al.* Effects of vitamin D on the peripheral adaptive immune system: A review. *Autoimmun Rev*. 2011;10(12):733–743. doi:10.1016/j.autrev.2011.05.002.
21. Smolders J, Torkildsen Ø, Camu W, Holmøy T. An update on vitamin D and disease activity in multiple sclerosis. *CNS Drugs*. 2019;33(12):1187–1199. doi:10.1007/s40263-019-00674-8.
22. Simmons KM, Beaudin SG, Narvaez CJ, Welsh J. Gene signatures of 1,25-dihydroxyvitamin D_3 exposure in normal and transformed mammary cells: Genomic profiles of vitamin D treated mammary cells. *J Cell Biochem*. 2015;116(8):1693–1711. doi:10.1002/jcb.25129.
23. Burrows DJ, McGown A, Jain SA, *et al.* Animal models of multiple sclerosis: From rodents to zebrafish. *Mult Scler J*. 2019;25(3):306–324. doi:10.1177/1352458518805246.
24. Tietz SM, Zwahlen M, Haghayegh Jahromi N, *et al.* Refined clinical scoring in comparative EAE studies does not enhance the chance to observe statistically significant differences: Technical comment. *Eur J Immunol*. 2016;46(10):2481–2483. doi:10.1002/eji.201546272.
25. Chang JHH, Cha HRR, Lee DSS, Seo KY, Kweon MNN. 1,25-Dihydroxyvitamin D_3 inhibits the differentiation and migration of TH17 cells to protect against experimental autoimmune encephalomyelitis. *PLoS One*. 2010;5(9):e12925–e12925. doi:10.1371/journal.pone.0012925.
26. Grishkan IV, Fairchild AN, Calabresi PA, Gocke AR. 1,25-Dihydroxyvitamin D_3 selectively and reversibly impairs T helper-cell CNS localization. *Proc Natl Acad Sci U S A*. 2013;110-(52):21101–21106. doi:10.1073/pnas.1306072110.
27. Joshi S, Pantalena LC, Liu XK, *et al.* 1,25-Dihydroxyvitamin D_3 ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A. *Mol Cell Biol*. 2011;31(17):3653–3669. doi:10.1128/MCB.05020-11.
28. Muthian G, Raikwar HP, Rajasingh J, Bright JJ. 1,25 dihydroxyvitamin-D_3 modulates JAK–STAT pathway in IL-12/IFNγ axis leading to Th1 response in experimental allergic encephalomyelitis. *J Neurosci Res*. 2006;83(7):1299–1309. doi:10.1002/jnr.20826.
29. Nashold FE, Spach KM, Spanier JA, Hayes CE. Estrogen controls vitamin D_3-mediated resistance to experimental autoimmune encephalomyelitis by controlling vitamin D_3 metabolism and receptor expression. *J Immunol*. 2009;183(6):3672–3681. doi:10.4049/jimmunol.0901351.
30. Sloka S, Silva C, Wang J, Yong V. Predominance of Th2 polarization by vitamin D through a STAT6-dependent mechanism. *J Neuroinflammation*. 2011;8(1):56–56. doi:10.1186/1742-2094-8-56.
31. Cantorna MT, Hayes CE, DeLuca HF. 1,25-Dihydroxyvitamin D_3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. *Proc Natl Acad Sci U S A*. 1996;93-(15):7861–7864. doi:10.1073/pnas.93.15.7861.
32. Cantorna MT, Woodward WD, Hayes CE, DeLuca HF. 1,25-Dihydroxyvitamin D_3 is a positive regulator for the two anti-encephalitogenic cytokines TGF-β1 and IL-4. *J Immunol*. 1998;160(11):5314–5319.
33. Farias AS, Spagnol GS, Bordeaux-Rego P, *et al.* Vitamin D_3 induces IDO^+ tolerogenic DCs and enhances treg, reducing the severity of EAE. *CNS Neurosci Ther*. 2013;19(4):269–277. doi:10.1111/cns.12071.
34. Nashold FE, Miller DJ, Hayes CE. 1,25-dihydroxyvitamin D_3 treatment decreases macrophage accumulation in the CNS of mice with experimental autoimmune encephalomyelitis. *J Neuroimmunol*. 2000;103(2):171–179. doi:10.1016/S0165-5728(99)00247-7.
35. Spach KM, Hayes CE. Vitamin D_3 confers protection from autoimmune encephalomyelitis only in female mice. *J Immunol*. 2005;175(6):4119–4126. doi:10.4049/jimmunol.175.6.4119.
36. Lemire JM, Archer DC. 1,25-Dihydroxyvitamin D_3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. *J Clin Invest*. 1991;87(3):1103–1107. doi:10.1172/JCI115072.
37. Haghmorad D, Yazdanpanah E, Jadid Tavaf M, *et al.* Prevention and treatment of experimental autoimmune encephalomyelitis induced mice with 1, 25-dihydroxyvitamin D_3. *Neurol Res*. 2019;41(10):943–957. doi:10.1080/01616412.2019.1650218.
38. Nashold FE, Nelson CD, Brown LM, Hayes CE. One calcitriol dose transiently increases Helios+ FoxP3+ T cells and ameliorates autoimmune demyelinating disease. *J Neuroimmunol*. 2013;263(1–2):64–74. doi:10.1016/j.jneuroim.2013.07.016.
39. Yoshizawa T, Handa Y, Uematsu Y, *et al.* Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet*. 1997;16(4):391–396. doi:10.1038/ng0897-391.
40. Li YC, Pirro AE, Amling M, *et al.* Targeted ablation of the vitamin D receptor: An animal model of vitamin D-dependent rickets type II with alopecia. *Proc Natl Acad Sci U S A*. 1997;94(18):9831–9835. doi:10.1073/pnas.94.18.9831.
41. Mayne CG, Spanier JA, Relland LM, Williams CB, Hayes CE. 1,25-Dihydroxyvitamin D_3 acts directly on the T lymphocyte vitamin D receptor to inhibit experimental autoimmune encephalomyelitis. *Eur J Immunol*. 2011;41(3):822–832. doi:10.1002/eji.201040632.
42. Nashold FE, Hoag KA, Goverman J, Hayes CE. Rag-1-dependent cells are necessary for 1,25-dihydroxyvitamin D_3 prevention of experimental autoimmune encephalomyelitis. *J Neuroimmunol*. 2001;119(1):16–29. doi:10.1016/S0165-5728(01)00360-5.
43. Bruce D, Yu S, Ooi JH, Cantorna MT. Converging pathways lead to overproduction of IL-17 in the absence of vitamin D signaling. *Int Immunol*. 2011;23(8):519–528. doi:10.1093/intimm/dxr045.
44. Staeva-Vieira TP, Freedman LP. 1,25-Dihydroxyvitamin D_3 inhibits IFN-γ and IL-4 levels during in vitro polarization of primary murine $CD4^+$ T cells. *J Immunol*. 2002;168(3):1181–1189. doi:10.4049/jimmunol.168.3.1181.
45. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HFJ, O'Garra A. 1α,25-Dihydroxyvitamin D_3 has a direct effect on naive $CD4^+$ T cells to enhance the development of Th2 cells. *J Immunol*. 2001;167(9):4974–4980. doi:10.4049/jimmunol.167.9.4974.
46. Cippitelli M, Santoni A. Vitamin D_3: A transcriptional modulator of the interferon-gamma gene. *Eur J Immunol*. 1998;28(10):3017–3030. doi:10.1002/(SICI)1521-4141(199810)28:10<3017::AID-IMMU3017>3.0.CO;2-6.
47. Mahon BD, Gordon SA, Cruz J, Cosman F, Cantorna MT. Cytokine profile in patients with multiple sclerosis following vitamin D supplementation. *J Neuroimmunol*. 2003;134(1–2):128–132. doi:10.1016/S0165-5728(02)00396-X.
48. D'Ambrosio D, Cippitelli M, Cocciolo MG, *et al.* Inhibition of IL-12 production by 1,25-dihydroxyvitamin D_3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. *J Clin Invest*. 1998;101(1):252–262. doi:10.1172/JCI1050.
49. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol*. 2003;3(2):133–146. doi:10.1038/nri1001.

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50. Chang SH, Chung Y, Dong C. Vitamin D suppresses Th17 cytokine production by inducing C/EBP homologous protein (CHOP) expression. *J Biol Chem*. 2010;285(50):38751–38755. doi:10.1074/jbc.C110.185777.
51. Palmer MT, Lee YK, Maynard CL, *et al*. Lineage-specific effects of 1,25-dihydroxyvitamin D_3 on the development of effector CD4 T cells. *J Biol Chem*. 2011;286(2):997–1004. doi:10.1074/jbc.M110.163790.
52. Hermann-Kleiter N, Baier G. NFAT pulls the strings during CD4+ T helper cell effector functions. *Blood*. 2010;115(15):2989–2997. doi:10.1182/blood-2009-10-233585.
53. Alroy I, Towers TL, Freedman LP. Transcriptional repression of the interleukin-2 gene by vitamin D_3: Direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. *Mol Cell Biol*. 1995;15(10):5789–5799. doi:10.1128/MCB.15.10.5789.
54. Towers TL, Staeva TP, Freedman LP. A two-hit mechanism for vitamin D_3-mediated transcriptional repression of the granulocyte-macrophage colony-stimulating factor gene: Vitamin D receptor competes for DNA binding with NFAT1 and stabilizes c-jun. *Mol Cell Biol*. 1999;19(6):4191–4199. doi:10.1128/MCB.19.6.4191.
55. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol*. 2010;11(1):7–13. doi:10.1038/ni.1818.
56. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+ CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med*. 2004;199(7):971–979. doi:10.1084/jem.20031579.
57. Zhang X. IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+ CD4+ regulatory T cells. *Int Immunol*. 2004;16(2):249–256. doi:10.1093/intimm/dxh029.
58. Spach KM, Nashold FE, Dittel BN, Hayes CE. IL-10 Signaling is essential for 1,25-dihydroxyvitamin D_3-mediated inhibition of experimental autoimmune encephalomyelitis. *J Immunol*. 2006;177(9):6030–6037. doi:10.4049/jimmunol.177.9.6030.
59. Stohlman SA, Pei L, Cua DJ, Li Z, Hinton DR. Activation of regulatory cells suppresses experimental allergic encephalomyelitis via secretion of IL-10. *J Immunol*. 1999;163(11):6338–6344.
60. Thornton AM, Korty PE, Tran DQ, *et al*. Expression of Helios, an ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3$^+$ T regulatory cells. *J Immunol*. 2010;184(7):3433–3441. doi:10.4049/jimmunol.0904028.
61. Moore JR, Hubler SL, Nelson CD, Nashold FE, Spanier JA, Hayes CE. 1,25-Dihydroxyvitamin D_3 increases the methionine cycle, CD4+ T cell DNA methylation and Helios+ Foxp3+ T regulatory cells to reverse autoimmune neurodegenerative disease. *J Neuroimmunol*. 2018;324:100–114. doi:10.1016/j.jneuroim.2018.09.008.
62. Getnet D, Grosso JF, Goldberg MV, *et al*. A role for the transcription factor Helios in human CD4+ CD25+ regulatory T cells. *Mol Immunol*. 2010;47(7–8):1595–1600. doi:10.1016/j.molimm.2010.02.001.
63. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol*. 2010;185(6):3190–3198. doi:10.4049/jimmunol.0903670.
64. Spanier JA, Nashold FE, Mayne CG, Nelson CD, Hayes CE. Vitamin D and estrogen synergy in vdr-expressing CD4+ T cells is essential to induce Helios+ FoxP3+ T cells and prevent autoimmune demyelinating disease. *J Neuroimmunol*. 2015;286:48–58. doi:10.1016/j.jneuroim.2015.06.015.
65. Comi G, Bar-Or A, Lassmann H, *et al*. Role of B cells in multiple sclerosis and related disorders. *Ann Neurol*. 2021;89(1):13–23. doi:10.1002/ana.25927.
66. Xie Z, Chen J, Zheng C, *et al*. 1,25-dihydroxyvitamin D_3-induced dendritic cells suppress experimental autoimmune encephalomyelitis by increasing proportions of the regulatory lymphocytes and reducing T helper type 1 and type 17 cells. *Immunology*. 2017;152(3):414–424. doi:10.1111/imm.12776.
67. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol*. 2002;3(10):944–950. doi:10.1038/ni833.
68. Grigoriadis N, van Pesch V. A basic overview of multiple sclerosis immunopathology. *Eur J Neurol*. 2015;22:3–13. doi:10.1111/ene.12798.
69. Engelhardt B, Sorokin L. The blood–brain and the blood–cerebrospinal fluid barriers: Function and dysfunction. *Semin Immunopathol*. 2009;31(4):497–511. doi:10.1007/s00281-009-0177-0.
70. Marchetti L, Engelhardt B. Immune cell trafficking across the blood–brain barrier in the absence and presence of neuroinflammation. *Vasc Biol*. 2020;2(1):H1–H18. doi:10.1530/VB-19-0033.
71. Mansilla MJ, Sellès-Moreno C, Fàbregas-Puig S, *et al*. Beneficial effect of tolerogenic dendritic cells pulsed with MOG autoantigen in experimental autoimmune encephalomyelitis. *CNS Neurosci Ther*. 2015;21(3):222–230. doi:10.1111/cns.12342.
72. Adorini L, Penna G. Dendritic cell tolerogenicity: A key mechanism in immunomodulation by vitamin D receptor agonists. *Hum Immunol*. 2009;70(5):345–352. doi:10.1016/j.humimm.2009.01.016.
73. Besusso D, Saul L, Leech MD, *et al*. 1,25-Dihydroxyvitamin D_3-conditioned CD11c+ dendritic cells are effective initiators of CNS autoimmune disease. *Front Immunol*. 2015;6:575–575. doi:10.3389/fimmu.2015.00575.
74. Fallarino F, Grohmann U, You S, *et al*. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor ζ-chain and induce a regulatory phenotype in naive T cells. *J Immunol*. 2006;176(11):6752–6761. doi:10.4049/jimmunol.176.11.6752.
75. Quintana FJ, Basso AS, Iglesias AH, *et al*. Control of treg and TH17 cell differentiation by the aryl hydrocarbon receptor. *Nature*. 2008;453(7191):65–71. doi:10.1038/nature06880.
76. Chen Y, Zhang J, Ge X, Du J, Deb DK, Li YC. Vitamin D receptor inhibits nuclear factor κB activation by interacting with IκB kinase β protein. *J Biol Chem*. 2013;288(27):19450–19458. doi:10.1074/jbc.M113.467670.
77. Cohen-Lahav M, Shany S, Tobvin D, Chaimovitz C, Douvdevani A. Vitamin D decreases NFκB activity by increasing IκBα levels. *Nephrol Dial Transplant*. 2006;21(4):889–897. doi:10.1093/ndt/gfi254.
78. Wu S, Xia Y, Liu X, Sun J. Vitamin D receptor deletion leads to reduced level of IkappaBalpha protein through protein translation, protein–protein interaction, and post-translational modification. *Int J Biochem Cell Biol*. 2010;42(2):329–336. doi:10.1016/j.biocel.2009.11.012.
79. Zhang Y, Leung DYM, Richers BN, *et al*. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. *J Immunol*. 2012;188(5):2127–2135. doi:10.4049/jimmunol.1102412.
80. Liang S, Cai J, Li Y, Yang R. 1,25-Dihydroxy-Vitamin D_3 induces macrophage polarization to M2 by upregulating T-cell Ig-mucin-3 expression. *Mol Med Rep*. 2019;19(5):3707–3713.
81. Knippenberg S, Smolders J, Thewissen M, *et al*. Effect of vitamin D_3 supplementation on peripheral B cell differentiation and isotype switching in patients with multiple sclerosis. *Mult Scler*. 2011;17(12):1418–1423. doi:10.1177/1352458511412655.
82. Mrad MF, El Ayoubi NK, Esmerian MO, Kazan JM, Khoury SJ. Effect of vitamin D replacement on immunological biomarkers in patients with multiple sclerosis. *Clin Immunol*. 2017;181:9–15. doi:10.1016/j.clim.2017.05.017.
83. Muris AH, Smolders J, Rolf L, Thewissen M, Hupperts R, Damoiseaux J. Immune regulatory effects of high dose vitamin D_3 supplementation in a randomized controlled trial in relapsing remitting multiple sclerosis patients receiving IFNβ; the

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SOLARIUM study. *J Neuroimmunol*. 2016;300:47–56. doi:10.1016/j.jneuroim.2016.09.018.
84. Smolders J, Peelen E, Thewissen M, *et al*. Safety and T cell modulating effects of high dose vitamin D_3 supplementation in multiple sclerosis. *PLoS One*. 2010;5(12):e15235. doi:10.1371/journal.pone.0015235.
85. Sotirchos ES, Bhargava P, Eckstein C, *et al*. Safety and immunologic effects of high- vs low-dose cholecalciferol in multiple sclerosis. *Neurology*. 2016;86(4):382–390. doi:10.1212/WNL.0000000000002316.
86. Mosayebi G, Ghazavi A, Ghasami K, Jand Y, Kokhaei P. Therapeutic effect of vitamin D_3 in multiple sclerosis patients. *Immunol Invest*. 2011;40(6):627–639. doi:10.3109/08820139.2011.573041.
87. Shirvani-Farsani Z, Behmanesh M, Mohammadi SM, Naser Moghadasi A. Vitamin D levels in multiple sclerosis patients: Association with TGF-β2, TGF-βRI, and TGF-βRII expression. *Life Sci*. 2015;134:63–67. doi:10.1016/j.lfs.2015.05.017.
88. Åivo J, Hänninen A, Ilonen J, Soilu-Hänninen M. Vitamin D_3 administration to MS patients leads to increased serum levels of latency activated peptide (LAP) of TGF-beta. *J Neuroimmunol*. 2015;280:12–15. doi:10.1016/j.jneuroim.2015.01.005.
89. Ashtari F, Toghianifar N, Zarkesh-Esfahani SH, Mansourian M. Short-term effect of high-dose vitamin D on the level of interleukin 10 in patients with multiple sclerosis: A randomized, double-blind, placebo-controlled clinical trial. *Neuroimmunomodulation*. 2015;22(6):400–404. doi:10.1159/000439278.
90. Røsjø E, Steffensen LH, Jørgensen L, *et al*. Vitamin D supplementation and systemic inflammation in relapsing-remitting multiple sclerosis. *J Neurol*. 2015;262(12):2713–2721. doi:10.1007/s00415-015-7902-5.
91. Golan D, Halhal B, Glass-Marmor L, *et al*. Vitamin D supplementation for patients with multiple sclerosis treated with interferon-beta: A randomized controlled trial assessing the effect on flu-like symptoms and immunomodulatory properties. *BMC Neurol*. 2013;13(1):60. doi:10.1186/1471-2377-13-60.
92. Toghianifar N, Ashtari F, Zarkesh-Esfahani SH, Mansourian M. Effect of high dose vitamin D intake on interleukin-17 levels in multiple sclerosis: A randomized, double-blind, placebo-controlled clinical trial. *J Neuroimmunol*. 2015;285:125–128. doi:10.1016/j.jneuroim.2015.05.022.
93. Farsani ZS, Behmanesh M, Sahraian MA. Interleukin-10 but not transforming growth factor-β1 gene expression is up-regulated by vitamin D treatment in multiple sclerosis patients. *J Neurol Sci*. 2015;350(1–2):18–23. doi:10.1016/j.jns.2015.01.030.
94. Kimball S, Vieth R, Dosch HM, *et al*. Cholecalciferol plus calcium suppresses abnormal PBMC reactivity in patients with multiple sclerosis. *J Clin Endocrinol Metab*. 2011;96(9):2826–2834. doi:10.1210/jc.2011-0325.
95. Correale J, Ysrraelit MC, Gaitan MI. Immunomodulatory effects of vitamin D in multiple sclerosis. *Brain*. 2009;132(5):1146–1160. doi:10.1093/brain/awp033.
96. Correale J, Ysrraelit MC, Gaitán MI. Gender differences in 1,25 dihydroxyvitamin D_3 immunomodulatory effects in multiple sclerosis patients and healthy subjects. *J Immunol*. 2010;185(8):4948–4958. doi:10.4049/jimmunol.1000588.
97. da Costa DSMM, Hygino J, Ferreira TB, *et al*. Vitamin D modulates different IL-17-secreting T cell subsets in multiple sclerosis patients. *J Neuroimmunol*. 2016;299:8–18. doi:10.1016/j.jneuroim.2016.08.005.
98. Kickler K, Ni Choileain S, Williams A, Richards A, Astier AL. Calcitriol modulates the CD46 pathway in T cells. *PLoS One*. 2012;7(10):e48486. doi:10.1371/journal.pone.0048486.
99. Killick J, Hay J, Morandi E, *et al*. Vitamin D/CD46 crosstalk in human T cells in multiple sclerosis. *Front Immunol*. 2020;11:598727. doi:10.3389/fimmu.2020.598727.
100. Lysandropoulos AP, Jaquiéry E, Jilek S, Pantaleo G, Schluep M, Du Pasquier RA. Vitamin D has a direct immunomodulatory effect on CD8+ T cells of patients with early multiple sclerosis and healthy control subjects. *J Neuroimmunol*. 2011;233(1–2):240–244. doi:10.1016/j.jneuroim.2010.11.008.
101. Peelen E, Muris AH, Damoiseaux J, *et al*. GM-CSF production by CD4+ T cells in MS patients: Regulation by regulatory T cells and vitamin D. *J Neuroimmunol*. 2015;280:36–42. doi:10.1016/j.jneuroim.2015.02.007.
102. Haas J, Schwarz A, Korporal-Kuhnke M, Faller S, Jarius S, Wildemann B. Hypovitaminosis D upscales B-cell immunoreactivity in multiple sclerosis. *J Neuroimmunol*. 2016;294:18–26. doi:10.1016/j.jneuroim.2016.03.011.
103. Sanseverino I, Rinaldi AO, Purificato C, *et al*. CCL2 induction by 1,25(OH)$2D_3$ in dendritic cells from healthy donors and multiple sclerosis patients. *J Steroid Biochem Mol Biol*. 2014;144:102–105. doi:10.1016/j.jsbmb.2013.10.018.
104. Waschbisch A, Sanderson N, Krumbholz M, *et al*. Interferon beta and vitamin D synergize to induce immunoregulatory receptors on peripheral blood monocytes of multiple sclerosis patients. *PLoS One*. 2014;9(12):e115488. doi:10.1371/journal.pone.0115488.
105. Bartosik-Psujek H, Tabarkiewicz J, Pocinska K, Stelmasiak Z, Rolinski J. Immunomodulatory effects of vitamin D on monocyte-derived dendritic cells in multiple sclerosis. *Mult Scler*. 2010;16-(12):1513–1516. doi:10.1177/1352458510379611.
106. Berge T, Leikfoss IS, Brorson IS, *et al*. The multiple sclerosis susceptibility genes TAGAP and IL2RA are regulated by vitamin D in CD4+ T cells. *Genes Immun*. 2016;17(2):118–127. doi:10.1038/gene.2015.61.
107. Engelhardt B, Vajkoczy P, Weller RO. The movers and shapers in immune privilege of the CNS. *Nat Immunol*. 2017;18(2):123–131. doi:10.1038/ni.3666.
108. Mohseni S, Tabatabaei-Malazy O, Shadman Z, Khashayar P, Mohajeri-Tehrani M, Larijani B. Targeting dyslipidemia with antioxidative vitamins C, D, and E; a systematic review of meta-analysis studies: Dyslipidemia and antioxidative vitamins. *J Diabetes Metab Disord*. 2021;20(2):2037–2047. doi:10.1007/s40200-021-00919-8.
109. Kermode AG, Thompson AJ, Tofts P, *et al*. Breakdown of the blood–brain barrier precedes symptoms and other MRI signs of new lesions in multiple sclerosis. Pathogenetic and clinical implications. *Brain*. 1990;113(Pt 5):1477–1489. doi:10.1093/brain/113.5.1477.
110. Lassmann H. Pathogenic mechanisms associated with different clinical courses of multiple sclerosis. *Front Immunol*. 2019;9:3116. doi:10.3389/fimmu.2018.03116.
111. Plumb J, McQuaid S, Mirakhur M, Kirk J. Abnormal endothelial tight junctions in active lesions and normal-appearing white matter in multiple sclerosis. *Brain Pathol*. 2002;12(2):154–169. doi:10.1111/j.1750-3639.2002.tb00430.x.
112. Kirk J, Plumb J, Mirakhur M, McQuaid S. Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood–brain barrier leakage and active demyelination. *J Pathol*. 2003;201(2):319–327. doi:10.1002/path.1434.
113. Leech S, Kirk J, Plumb J, McQuaid S. Persistent endothelial abnormalities and blood–brain barrier leak in primary and secondary progressive multiple sclerosis. *Neuropathol Appl Neurobiol*. 2007;33(1):86–98. doi:10.1111/j.1365-2990.2006.00781.x.
114. Alvarez JI, Cayrol R, Prat A. Disruption of central nervous system barriers in multiple sclerosis. *Biochim Biophys Acta*. 2011;1812(2):252–264. doi:10.1016/j.bbadis.2010.06.017.
115. Alvarez JI, Saint-Laurent O, Godschalk A, *et al*. Focal disturbances in the blood–brain barrier are associated with formation of neuroinflammatory lesions. *Neurobiol Dis*. 2015;74:14–24. doi:10.1016/j.nbd.2014.09.016.
116. Padden M, Leech S, Craig B, Kirk J, Brankin B, McQuaid S. Differences in expression of junctional adhesion molecule-A and beta-catenin in multiple sclerosis brain tissue: Increasing evidence for the role of tight junction pathology. *Acta Neuropathol*. 2007;113(2):177–186. doi:10.1007/s00401-006-0145-x.

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117. Vos CM, Geurts JJ, Montagne L, *et al.* Blood–brain barrier alterations in both focal and diffuse abnormalities on postmortem MRI in multiple sclerosis. *Neurobiol Dis*. 2005;20(3):953–960. doi:10.1016/j.nbd.2005.06.012.
118. Durk MR, Chan GN, Campos CR, *et al.* 1α,25-Dihydroxyvitamin D_3-liganded vitamin D receptor increases expression and transport activity of P-glycoprotein in isolated rat brain capillaries and human and rat brain microvessel endothelial cells. *J Neurochem*. 2012;123(6):944–953. doi:10.1111/jnc.12041.
119. Guo YX, He LY, Zhang M, Wang F, Liu F, Peng WX. 1,25-Dihydroxyvitamin D_3 regulates expression of LRP1 and RAGE in vitro and in vivo, enhancing Aβ1-40 brain-to-blood efflux and peripheral uptake transport. *Neuroscience*. 2016;322:28–38. doi:10.1016/j.neuroscience.2016.01.041.
120. Alam C, Hoque MT, Finnell RH, Goldman ID, Bendayan R. Regulation of reduced folate carrier (RFC) by vitamin D receptor at the blood–brain barrier. *Mol Pharm*. 2017;14(11):3848–3858. doi:10.1021/acs.molpharmaceut.7b00572.
121. Landel V, Stephan D, Cui X, Eyles D, Feron F. Differential expression of vitamin D-associated enzymes and receptors in brain cell subtypes. *J Steroid Biochem Mol Biol*. 2018;177:129–134. doi:10.1016/j.jsbmb.2017.09.008.
122. Enkhjargal B, McBride DW, Manaenko A, *et al.* Intranasal administration of vitamin D attenuates blood–brain barrier disruption through endogenous upregulation of osteopontin and activation of CD44/P-gp glycosylation signaling after subarachnoid hemorrhage in rats. *J Cereb Blood Flow Metab*. 2017;37(7):2555–2566. doi:10.1177/0271678X16671147.
123. Sayeed I, Turan N, Stein DG, Wali B. Vitamin D deficiency increases blood–brain barrier dysfunction after ischemic stroke in male rats. *Exp Neurol*. 2019;312:63–71. doi:10.1016/j.expneurol.2018.11.005.
124. El-Atifi M, Dreyfus M, Berger F, Wion D. Expression of CYP2R1 and VDR in human brain pericytes: The neurovascular vitamin D autocrine/paracrine model. *Neuroreport*. 2015;26(5):245–248. doi:10.1097/WNR.0000000000000328.
125. Jamali N, Song YS, Sorenson CM, Sheibani N. 1,25$(OH)_2D_3$ regulates the proangiogenic activity of pericyte through VDR-mediated modulation of VEGF production and signaling of VEGF and PDGF receptors. *FASEB Bioadv*. 2019;1(7):415–434. doi:10.1096/fba.2018-00067.
126. Naveilhan P, Neveu I, Baudet C, *et al.* 1,25-Dihydroxyvitamin D_3 regulates the expression of the low-affinity neurotrophin receptor. *Brain Res Mol Brain Res*. 1996;41(1–2):259–268. doi:10.1016/0169-328X(96)00103-9.
127. Smolders J, Schuurman KG, van Strien ME, *et al.* Expression of vitamin D receptor and metabolizing enzymes in multiple sclerosis affected brain tissue. *J Neuropathol Exp Neurol*. 2013;72(2):91. doi:10.1097/NEN.0b013e31827f4fcc.
128. Neveu I, Naveilhan P, Baudet C, Brachet P, Metsis M. 1,25-dihydroxyvitamin D_3 regulates NT-3, NT-4 but not BDNF mRNA in astrocytes. *Neuroreport*. 1994;6(1):124–126. doi:10.1097/00001756-199412300-00032.
129. Boontanrart M, Hall SD, Spanier JA, Hayes CE, Olson JK. Vitamin D_3 alters microglia immune activation by an IL-10 dependent SOCS3 mechanism. *J Neuroimmunol*. 2016;292:126–136. doi:10.1016/j.jneuroim.2016.01.015.
130. He J, Guo X, Liu ZQ, Yang PC, Yang S. Vitamin D inhibits the staphylococcal enterotoxin B-induced expression of tumor necrosis factor in microglial cells. *Immunol Res*. 2017;65(4):913–919. doi:10.1007/s12026-017-8930-2.
131. Cui C, Xu P, Li G, *et al.* Vitamin D receptor activation regulates microglia polarization and oxidative stress in spontaneously hypertensive rats and angiotensin II-exposed microglial cells: Role of renin-angiotensin system. *Redox Biol*. 2019;26:101295. doi:10.1016/j.redox.2019.101295.
132. Baas D, Prufer K, Ittel ME, *et al.* Rat oligodendrocytes express the vitamin D_3 receptor and respond to 1,25-dihydroxyvitamin D_3. *Glia*. 2000;31(1):59–68. doi:10.1002/(SICI)1098-1136(200007)31:1<59::AID-GLIA60>3.0.CO;2-Y.
133. Shirazi HA, Rasouli J, Ciric B, Rostami A, Zhang GX. 1,25-Dihydroxyvitamin D_3 enhances neural stem cell proliferation and oligodendrocyte differentiation. *Exp Mol Pathol*. 2015;98(2):240–245. doi:10.1016/j.yexmp.2015.02.004.
134. Mengozzi M, Hesketh A, Bucca G, Ghezzi P, Smith CP. Vitamins D_3 and D_2 have marked but different global effects on gene expression in a rat oligodendrocyte precursor cell line. *Mol Med*. 2020;26(1):32. doi:10.1186/s10020-020-00153-7.
135. Durk MR, Fan J, Sun H, *et al.* Vitamin D receptor activation induces P-glycoprotein and increases brain efflux of quinidine: An intracerebral microdialysis study in conscious rats. *Pharm Res*. 2015;32(3):1128–1140. doi:10.1007/s11095-014-1524-y.
136. Durk MR, Han K, Chow EC, *et al.* 1α,25-Dihydroxyvitamin D3 reduces cerebral amyloid-β accumulation and improves cognition in mouse models of Alzheimer's disease. *J Neurosci*. 2014;34(21):7091–7101. doi:10.1523/JNEUROSCI.2711-13.2014.
137. Sadeghian N, Shadman J, Moradi A, Ghasem Golmohammadi M, Panahpour H. Calcitriol protects the blood–brain barrier integrity against ischemic stroke and reduces vasogenic brain edema via antioxidant and antiapoptotic actions in rats. *Brain Res Bull*. 2019;150:281–289. doi:10.1016/j.brainresbull.2019.06.010.
138. Hajiluian G, Nameni G, Shahabi P, Mesgari-Abbasi M, Sadigh-Eteghad S, Farhangi MA. Vitamin D administration, cognitive function, BBB permeability and neuroinflammatory factors in high-fat diet-induced obese rats. *Int J Obes (Lond)*. 2017;41(4):639–644. doi:10.1038/ijo.2017.10.
139. Hahn M, Lorez H, Fischer G. Effect of calcitriol in combination with corticosterone, interleukin-1beta, and transforming growth factor-beta1 on nerve growth factor secretion in an astroglial cell line. *J Neurochem*. 1997;69(1):102–109. doi:10.1046/j.1471-4159.1997.69010102.x.
140. Neveu I, Naveilhan P, Jehan F, *et al.* 1,25-Dihydroxyvitamin D_3 regulates the synthesis of nerve growth factor in primary cultures of glial cells. *Mol Brain Res*. 1994;24(1-4):70–76. doi:10.1016/0169-328X(94)90119-8.
141. Uberti F, Lattuada D, Morsanuto V, *et al.* Vitamin D protects human endothelial cells from oxidative stress through the autophagic and survival pathways. *J Clin Endocrinol Metab*. 2014;99(4):1367–1374. doi:10.1210/jc.2013-2103.
142. Molinari C, Uberti F, Grossini E, *et al.* 1α,25-Dihydroxycholecalciferol Induces nitric oxide production in cultured endothelial cells. *Cell Physiol Biochem*. 2011;27(6):661–668. doi:10.1159/000330075.
143. Takahashi S, Maeda T, Sano Y, *et al.* Active form of vitamin D directly protects the blood–brain barrier in multiple sclerosis. *Clin Exp Neuroimmunol*. 2017;8(3):244–254. doi:10.1111/cen3.12398.
144. Won S, Sayeed I, Peterson BL, Wali B, Kahn JS, Stein DG. Vitamin D prevents hypoxia/reoxygenation-induced blood–brain barrier disruption via vitamin D receptor-mediated NF-kB signaling pathways. *PLoS One*. 2015;10(3):e0122821.
145. Hur J, Lee P, Kim MJ, Cho YW. Regulatory effect of 25-hydroxyvitamin D_3 on nitric oxide production in activated microglia. *Korean J Physiol Pharmacol*. 2014;18(5):397–402. doi:10.4196/kjpp.2014.18.5.397.
146. Alessio N, Belardo C, Trotta MC, *et al.* Vitamin D deficiency induces chronic pain and microglial phenotypic changes in mice. *Int J Mol Sci*. 2021;22(7):3604. doi:10.3390/ijms22073604.
147. Kurtys E, Eisel ULM, Verkuyl JM, Broersen LM, Dierckx R, de Vries EFJ. The combination of vitamins and omega-3 fatty acids has an enhanced anti-inflammatory effect on microglia. *Neurochem Int*. 2016;99:206–214. doi:10.1016/j.neuint.2016.07.008.
148. Dulla YA, Kurauchi Y, Hisatsune A, Seki T, Shudo K, Katsuki H. Regulatory mechanisms of vitamin D_3 on production of nitric oxide and pro-inflammatory cytokines in microglial BV-2 cells. *Neurochem Res*. 2016;41(11):2848–2858. doi:10.1007/s11064-016-2000-3.

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149. de Oliveira LRC, Mimura LAN, Fraga-Silva TFC, *et al.* Calcitriol prevents neuroinflammation and reduces blood–brain barrier disruption and local macrophage/microglia activation. *Front Pharmacol*. 2020;11:161. doi:10.3389/fphar.2020.00161.
150. Yang J, Wang K, Hu T, Wang G, Wang W, Zhang J. Vitamin D_3 supplement attenuates blood–brain barrier disruption and cognitive impairments in a rat model of traumatic brain injury. *Neuromolecular Med*. 2021;23(4):491–499. doi:10.1007/s12017-021-08649-z.
151. Chow EC, Durk MR, Cummins CL, Pang KS. 1Alpha,25-dihydroxyvitamin D_3 up-regulates P-glycoprotein via the vitamin D receptor and not farnesoid X receptor in both *fxr*(−/−) and *fxr*(+/+) mice and increased renal and brain efflux of digoxin in mice in vivo. *J Pharmacol Exp Ther*. 2011;337(3):846–859. doi:10.1124/jpet.111.179101.
152. Peng HB, Noh K, Pan SR, *et al.* Human amyloid-β_{40} kinetics after intravenous and intracerebroventricular injections and calcitriol treatment in rats in vivo. *Drug Metab Dispos*. 2020;48(10):944–955. doi:10.1124/dmd.120.090886.
153. Lefebvre d'Hellencourt C, Montero-Menei CN, Bernard R, Couez D. Vitamin D_3 inhibits proinflammatory cytokines and nitric oxide production by the EOC13 microglial cell line. *J Neurosci Res*. 2003;71(4):575–582. doi:10.1002/jnr.10491.
154. Djukic M, Onken ML, Schutze S, *et al.* Vitamin d deficiency reduces the immune response, phagocytosis rate, and intracellular killing rate of microglial cells. *Infect Immun*. 2014;82(6):2585–2594. doi:10.1128/IAI.01814-14.
155. Lee PW, Selhorst A, Lampe SG, Liu Y, Yang Y, Lovett-Racke AE. Neuron-specific vitamin D signaling attenuates microglia activation and CNS autoimmunity. *Front Neurol*. 2020;11:19. doi:10.3389/fneur.2020.00019.
156. Ekici F, Ozyurt B, Erdogan H. The combination of vitamin D_3 and dehydroascorbic acid administration attenuates brain damage in focal ischemia. *Neurol Sci*. 2009;30(3):207–212. doi:10.1007/s10072-009-0038-6.
157. Dehghani L, Meamar R, Etemadifar M, *et al.* Can vitamin d suppress endothelial cells apoptosis in multiple sclerosis patients? *Int J Prev Med*. 2013;4(Suppl 2):S211–S215.
158. Kim JS, Ryu SY, Yun I, *et al.* 1alpha,25-dihydroxyvitamin D_3 protects dopaminergic neurons in rodent models of Parkinson's disease through inhibition of microglial activation. *J Clin Neurol*. 2006;2(4):252–257. doi:10.3988/jcn.2006.2.4.252.
159. Goudarzvand M, Javan M, Mirnajafi-Zadeh J, Mozafari S, Tiraihi T. Vitamins E and D_3 attenuate demyelination and potentiate remyelination processes of hippocampal formation of rats following local injection of ethidium bromide. *Cell Mol Neurobiol*. 2010;30(2):289–299. doi:10.1007/s10571-009-9451-x.
160. Shirazi HA, Rasouli J, Ciric B, Wei D, Rostami A, Zhang GX. 1,25-Dihydroxyvitamin D_3 suppressed experimental autoimmune encephalomyelitis through both immunomodulation and oligodendrocyte maturation. *Exp Mol Pathol*. 2017;102(3):515–521. doi:10.1016/j.yexmp.2017.05.015.
161. Nystad AE, Wergeland S, Aksnes L, Myhr KM, Bo L, Torkildsen O. Effect of high-dose 1.25 dihydroxyvitamin D_3 on remyelination in the cuprizone model. *APMIS*. 2014;122(12):1178–1186. doi:10.1111/apm.12281.
162. Wergeland S, Torkildsen O, Myhr KM, Aksnes L, Mork SJ, Bo L. Dietary vitamin D_3 supplements reduce demyelination in the cuprizone model. *PLoS One*. 2011;6(10):e26262. doi:10.1371/journal.pone.0026262.
163. Zhang D, Qiao L, Fu T. Paricalcitol improves experimental autoimmune encephalomyelitis (EAE) by suppressing inflammation via NF-κB signaling. *Biomed Pharmacother*. 2020;125:109528. doi:10.1016/j.biopha.2019.109528.
164. Tang H, Hua F, Wang J, *et al.* Progesterone and vitamin D combination therapy modulates inflammatory response after traumatic brain injury. *Brain Inj*. 2015;29(10):1165–1174. doi:10.3109/02699052.2015.1035330.
165. Calvello R, Cianciulli A, Nicolardi G, *et al.* Vitamin D treatment attenuates neuroinflammation and dopaminergic neurodegeneration in an animal model of Parkinson's disease, shifting M1 to M2 microglia responses. *J Neuroimmune Pharmacol*. 2017;12(2):327–339. doi:10.1007/s11481-016-9720-7.
166. Spach KM, Pedersen LB, Nashold FE, *et al.* Gene expression analysis suggests that 1,25-dihydroxyvitamin D_3 reverses experimental autoimmune encephalomyelitis by stimulating inflammatory cell apoptosis. *Physiol Genomics*. 2004;18(2):141–151. doi:10.1152/physiolgenomics.00003.2004.
167. Gomez-Pinedo U, Cuevas JA, Benito-Martín MS, *et al.* Vitamin D increases remyelination by promoting oligodendrocyte lineage differentiation. *Brain Behav*. 2020;10(1):e01498. doi:10.1002/brb3.1498.
168. Malinic M, Dozudic JG. Effects of vitamin D on the expression of markers of principal neurons, interneurons and astrocytes in cerebral cortex and hippocampus in gerbils exposed to transient global cerebral ischemia: PS231. *Porto Biomed J*. 2017;2(5):209. doi:10.1016/j.pbj.2017.07.082.
169. Hua F, Reiss JI, Tang H, *et al.* Progesterone and low-dose vitamin D hormone treatment enhances sparing of memory following traumatic brain injury. *Horm Behav*. 2012;61(4):642–651. doi:10.1016/j.yhbeh.2012.02.017.
170. Evans MA, Kim HA, Ling YH, *et al.* Vitamin D_3 supplementation reduces subsequent brain injury and inflammation associated with ischemic stroke. *Neuromolecular Med*. 2018;20(1):147–159. doi:10.1007/s12017-018-8484-z.
171. Hupperts R, Smolders J, Vieth R, *et al.* Randomized trial of daily high-dose vitamin D_3 in patients with RRMS receiving subcutaneous interferon β-1a. *Neurology*. 2019;93(20):e1906–e1916.
172. Kimball SM, Ursell MR, O'Connor P, Vieth R. Safety of vitamin D_3 in adults with multiple sclerosis. *Am J Clin Nutr*. 2007;86(3):645–651. doi:10.1093/ajcn/86.3.645.
173. Soilu-Hänninen M, Åivo J, Lindström BM, *et al.* A randomised, double blind, placebo controlled trial with vitamin D_3 as an add on treatment to interferon β-1b in patients with multiple sclerosis. *J Neurol Neurosurg Psychiatry*. 2012;83(5):565–571. doi:10.1136/jnnp-2011-301876.
174. Chang HC, Tai YT, Cherng YG, *et al.* Resveratrol attenuates high-fat diet-induced disruption of the blood–brain barrier and protects brain neurons from apoptotic insults. *J Agric Food Chem*. 2014;62(15):3466–3475. doi:10.1021/jf403286w.
175. Davidson TL, Monnot A, Neal AU, Martin AA, Horton JJ, Zheng W. The effects of a high-energy diet on hippocampal-dependent discrimination performance and blood–brain barrier integrity differ for diet-induced obese and diet-resistant rats. *Physiol Behav*. 2012;107(1):26–33. doi:10.1016/j.physbeh.2012.05.015.
176. Elahy M, Lam V, Pallebage-Gamarallage MM, Giles C, Mamo JC, Takechi R. Nicotine attenuates disruption of blood–brain barrier induced by saturated-fat feeding in wild-type mice. *Nicotine Tob Res*. 2015;17(12):1436–1441. doi:10.1093/ntr/ntv044.
177. Pallebage-Gamarallage M, Lam V, Takechi R, Galloway S, Clark K, Mamo J. Restoration of dietary-fat induced blood–brain barrier dysfunction by anti-inflammatory lipid-modulating agents. *Lipids Health Dis*. 2012;11:117. doi:10.1186/1476-511X-11-117.
178. Löscher W, Potschka H. Blood–brain barrier active efflux transporters: ATP-binding cassette gene family. *NeuroRx*. 2005;2(1):86–98. doi:10.1602/neurorx.2.1.86.
179. Kooij G, van Horssen J, de Lange EC, *et al.* T lymphocytes impair P-glycoprotein function during neuroinflammation. *J Autoimmun*. 2010;34(4):416–425. doi:10.1016/j.jaut.2009.10.006.
180. Sospedra M, Martin R. Immunology of multiple sclerosis. *Annu Rev Immunol*. 2005;23:683–747. doi:10.1146/annurev.immunol.23.021704.115707.
181. Chiuso-Minicucci F, Ishikawa LLW, Mimura LAN, *et al.* Treatment with vitamin D/MOG association suppresses experimental autoimmune encephalomyelitis. *PLoS One*. 2015;10(5):e0125836. doi:10.1371/journal.pone.0125836.

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182. Hochmeister S, Aeinehband S, Dorris C, *et al.* Effect of vitamin D on experimental autoimmune neuroinflammation is dependent on haplotypes comprising naturally occurring allelic variants of CIITA (Mhc2ta). *Front Neurol*. 2020;11:600401. doi:10.3389/fneur.2020.600401.
183. Cayrol R, Wosik K, Berard JL, *et al.* Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. *Nat Immunol*. 2008;9(2):137–145. doi:10.1038/ni1551.
184. Lyck R, Lécuyer MA, Abadier M, *et al.* ALCAM (CD166) is involved in extravasation of monocytes rather than T cells across the blood–brain barrier. *J Cereb Blood Flow Metab*. 2017;37(8):2894–2909. doi:10.1177/0271678X16678639.
185. Larochelle C, Cayrol R, Kebir H, *et al.* Melanoma cell adhesion molecule identifies encephalitogenic T lymphocytes and promotes their recruitment to the central nervous system. *Brain*. 2012;135(Pt 10):2906–2924. doi:10.1093/brain/aws212.
186. Ifergan I, Kebir H, Terouz S, *et al.* Role of ninjurin-1 in the migration of myeloid cells to central nervous system inflammatory lesions. *Ann Neurol*. 2011;70(5):751–763. doi:10.1002/ana.22519.
187. Odoardi F, Sie C, Streyl K, *et al.* T cells become licensed in the lung to enter the central nervous system. *Nature*. 2012;488(7413):675–679. doi:10.1038/nature11337.
188. Brown LS, Foster CG, Courtney JM, King NE, Howells DW, Sutherland BA. Pericytes and neurovascular function in the healthy and diseased brain. *Front Cell Neurosci*. 2019;13:282. doi:10.3389/fncel.2019.00282.
189. Stratman AN, Davis GE. Endothelial cell–pericyte interactions stimulate basement membrane matrix assembly: Influence on vascular tube remodeling, maturation, and stabilization. *Microsc Microanal*. 2012;18(1):68–80. doi:10.1017/S1431927611012402.
190. Armulik A, Genove G, Betsholtz C. Pericytes: Developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell*. 2011;21(2):193–215. doi:10.1016/j.devcel.2011.07.001.
191. Stratman AN, Malotte KM, Mahan RD, Davis MJ, Davis GE. Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation. *Blood*. 2009;114(24):5091–5101. doi:10.1182/blood-2009-05-222364.
192. Daneman R, Zhou L, Kebede AA, Barres BA. Pericytes are required for blood–brain barrier integrity during embryogenesis. *Nature*. 2010;468(7323):562–566. doi:10.1038/nature09513.
193. Armulik A, Genove G, Mae M, *et al.* Pericytes regulate the blood–brain barrier. *Nature*. 2010;468(7323):557–561. doi:10.1038/nature09522.
194. Lindahl P, Johansson BR, Leveen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science*. 1997;277(5323):242–245. doi:10.1126/science.277.5323.242.
195. Quaegebeur A, Segura I, Carmeliet P. Pericytes: Blood–brain barrier safeguards against neurodegeneration? *Neuron*. 2010;68(3):321–323. doi:10.1016/j.neuron.2010.10.024.
196. Shepro D, Morel NM. Pericyte physiology. *FASEB J*. 1993;7(11):1031–1038. doi:10.1096/fasebj.7.11.8370472.
197. He L, Vanlandewijck M, Mae MA, *et al.* Single-cell RNA sequencing of mouse brain and lung vascular and vessel-associated cell types. *Sci Data*. 2018;5:180160. doi:10.1038/sdata.2018.160.
198. Ogura S, Kurata K, Hattori Y, *et al.* Sustained inflammation after pericyte depletion induces irreversible blood-retina barrier breakdown. *JCI Insight*. 2017;2(3):e90905. doi:10.1172/jci.insight.90905.
199. Rustenhoven J, Jansson D, Smyth LC, Dragunow M. Brain pericytes as mediators of neuroinflammation. *Trends Pharmacol Sci*. 2017;38(3):291–304. doi:10.1016/j.tips.2016.12.001.
200. Thomas H, Cowin AJ, Mills SJ. The importance of pericytes in healing: Wounds and other pathologies. *Int J Mol Sci*. 2017;18(6):1129. doi:10.3390/ijms18061129.
201. Duan L, Zhang XD, Miao WY, *et al.* PDGFRbeta cells rapidly relay inflammatory signal from the circulatory system to neurons via chemokine CCL2. *Neuron*. 2018;100(1):183–200.e8. doi:10.1016/j.neuron.2018.08.030.
202. Herland A, van der Meer AD, FitzGerald EA, Park TE, Sleeboom JJ, Ingber DE. Distinct contributions of astrocytes and pericytes to neuroinflammation identified in a 3D human blood–brain barrier on a chip. *PLoS One*. 2016;11(3):e0150360. doi:10.1371/journal.pone.0150360.
203. Rustenhoven J, Aalderink M, Scotter EL, *et al.* TGF-beta1 regulates human brain pericyte inflammatory processes involved in neurovasculature function. *J Neuroinflammation*. 2016;13:37. doi:10.1186/s12974-016-0503-0.
204. Torok O, Schreiner B, Schaffenrath J, *et al.* Pericytes regulate vascular immune homeostasis in the CNS. *Proc Natl Acad Sci U S A*. 2021;118(10):e2016587118. doi:10.1073/pnas.2016587118.
205. Girolamo F, de Trizio I, Errede M, Longo G, d'Amati A, Virgintino D. Neural crest cell-derived pericytes act as pro-angiogenic cells in human neocortex development and gliomas. *Fluids Barriers CNS*. 2021;18(1):14. doi:10.1186/s12987-021-00242-7.
206. Grygorowicz T, Dabrowska-Bouta B, Struzynska L. Administration of an antagonist of P2X7 receptor to EAE rats prevents a decrease of expression of claudin-5 in cerebral capillaries. *Purinergic Signal*. 2018;14(4):385–393. doi:10.1007/s11302-018-9620-9.
207. Tuzun E, Şekerdağ E, Atak D, *et al.* The Role of Pericytes in the Pathophysiology of Multiple Sclerosis (P2.2-075). *Neurology* [Internet]. 2019;92(15 Supplement). Available from: https://n.neurology.org/content/92/15_Supplement/P2.2-075.
208. Iacobaeus E, Sugars RV, Tornqvist Andren A, *et al.* Dynamic changes in brain mesenchymal perivascular cells associate with multiple sclerosis disease duration, active inflammation, and demyelination. *Stem Cells Transl Med*. 2017;6(10):1840–1851. doi:10.1002/sctm.17-0028.
209. Vanlandewijck M, He L, Mae MA, *et al.* A molecular atlas of cell types and zonation in the brain vasculature. *Nature*. 2018;554(7693):475–480. doi:10.1038/nature25739.
210. Nissou MF, Guttin A, Zenga C, Berger F, Issartel JP, Wion D. Additional clues for a protective role of vitamin D in neurodegenerative diseases: 1,25-dihydroxyvitamin D_3 triggers an anti-inflammatory response in brain pericytes. *J Alzheimers Dis*. 2014;42(3):789–799. doi:10.3233/JAD-140411.
211. Nataf S, Garcion E, Darcy F, Chabannes D, Muller JY, Brachet P. 1,25 Dihydroxyvitamin D_3 exerts regional effects in the central nervous system during experimental allergic encephalomyelitis. *J Neuropathol Exp Neurol*. 1996;55(8):904–914. doi:10.1097/00005072-199608000-00006.
212. Chung I, Han G, Seshadri M, *et al.* Role of vitamin D receptor in the antiproliferative effects of calcitriol in tumor-derived endothelial cells and tumor angiogenesis *in vivo*. *Cancer Res*. 2009;69(3):967–975. doi:10.1158/0008-5472.CAN-08-2307.
213. Jamali N, Wang S, Darjatmoko SR, Sorenson CM, Sheibani N. Vitamin D receptor expression is essential during retinal vascular development and attenuation of neovascularization by 1,25(OH)2D3. *PLoS One*. 2017;12(12):e0190131. doi:10.1371/journal.pone.0190131.
214. Sofroniew MV, Vinters HV. Astrocytes: Biology and pathology. *Acta Neuropathol*. 2010;119(1):7–35. doi:10.1007/s00401-009-0619-8.
215. Liebner S, Dijkhuizen RM, Reiss Y, Plate KH, Agalliu D, Constantin G. Functional morphology of the blood–brain barrier in health and disease. *Acta Neuropathol*. 2018;135(3):311–336. doi:10.1007/s00401-018-1815-1.
216. Agrawal S, Anderson P, Durbeej M, *et al.* Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. *J Exp Med*. 2006;203(4):1007–1019. doi:10.1084/jem.20051342.
217. Brosnan CF, Raine CS. The astrocyte in multiple sclerosis revisited. *Glia*. 2013;61(4):453–465. doi:10.1002/glia.22443.
218. Ponath G, Ramanan S, Mubarak M, *et al.* Myelin phagocytosis by astrocytes after myelin damage promotes lesion pathology. *Brain*. 2017;140(2):399–413. doi:10.1093/brain/aww298.

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219. Wang D, Ayers MM, Catmull DV, Hazelwood LJ, Bernard CCA, Orian JM. Astrocyte-associated axonal damage in pre-onset stages of experimental autoimmune encephalomyelitis. *Glia*. 2005;51(3):235–240. doi:10.1002/glia.20199.
220. Pham H, Ramp AA, Klonis N, *et al.* The astrocytic response in early experimental autoimmune encephalomyelitis occurs across both the grey and white matter compartments. *J Neuroimmunol*. 2009;208(1–2):30–39. doi:10.1016/j.jneuroim.2008.12.010.
221. D'Amelio FE, Smith ME, Eng LF. Sequence of tissue responses in the early stages of experimental allergic encephalomyelitis (EAE): Immunohistochemical, light microscopic, and ultrastructural observations in the spinal cord. *Glia*. 1990;3(4):229–240. doi:10.1002/glia.440030402.
222. Shih RH, Wang CY, Yang CM. NF-kappaB Signaling pathways in neurological inflammation: A mini review. *Front Mol Neurosci*. 2015;8:77.
223. Sheng WS, Hu S, Feng A, Rock RB. Reactive oxygen species from human astrocytes induced functional impairment and oxidative damage. *Neurochem Res*. 2013;38(10):2148–2159. doi:10.1007/s11064-013-1123-z.
224. Lee SJ, Benveniste EN. Adhesion molecule expression and regulation on cells of the central nervous system. *J Neuroimmunol*. 1999;98(2):77–88. doi:10.1016/S0165-5728(99)00084-3.
225. Colombo E, Cordiglieri C, Melli G, *et al.* Stimulation of the neurotrophin receptor TrkB on astrocytes drives nitric oxide production and neurodegeneration. *J Exp Med*. 2012;209(3):521–535. doi:10.1084/jem.20110698.
226. Jiao KP, Li SM, Lv WY, Jv ML, He HY. Vitamin D_3 repressed astrocyte activation following lipopolysaccharide stimulation in vitro and in neonatal rats. *Neuroreport*. 2017;28(9):492–497. doi:10.1097/WNR.0000000000000782.
227. Eyles DW, Liu PY, Josh P, Cui X. Intracellular distribution of the vitamin D receptor in the brain: Comparison with classic target tissues and redistribution with development. *Neuroscience*. 2014;268:1–9. doi:10.1016/j.neuroscience.2014.02.042.
228. de la Fuente AG, Errea O, van Wijngaarden P, *et al.* Vitamin D receptor–retinoid X receptor heterodimer signaling regulates oligodendrocyte progenitor cell differentiation. *J Cell Biol*. 2015;211(5):975–985. doi:10.1083/jcb.201505119.
229. Molinari C, Morsanuto V, Ghirlanda S, *et al.* Role of combined lipoic acid and vitamin D_3 on astrocytes as a way to prevent brain ageing by induced oxidative stress and iron accumulation. *Oxid Med Cell Longev*. 2019;2019:2843121. doi:10.1155/2019/2843121.
230. Garcion E, Nataf S, Berod A, Darcy F, Brachet P. 1,25-Dihydroxyvitamin D_3 inhibits the expression of inducible nitric oxide synthase in rat central nervous system during experimental allergic encephalomyelitis. *Brain Res Mol Brain Res*. 1997;45(2):255–267. doi:10.1016/S0169-328X(96)00260-4.
231. Ohl K, Tenbrock K, Kipp M. Oxidative stress in multiple sclerosis: Central and peripheral mode of action. *Exp Neurol*. 2016;277:58–67. doi:10.1016/j.expneurol.2015.11.010.
232. Ferronato MJ, Alonso EN, Salomon DG, *et al.* Antitumoral effects of the alkynylphosphonate analogue of calcitriol EM1 on glioblastoma multiforme cells. *J Steroid Biochem Mol Biol*. 2018;178:22–35. doi:10.1016/j.jsbmb.2017.10.019.
233. Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci*. 2007;10(12):1538–1543. doi:10.1038/nn2014.
234. Mildner A, Schmidt H, Nitsche M, *et al.* Microglia in the adult brain arise from ly-6ChiCCR2+ monocytes only under defined host conditions. *Nat Neurosci*. 2007;10(12):1544–1553. doi:10.1038/nn2015.
235. Hagemeyer N, Hanft KM, Akriditou MA, *et al.* Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood. *Acta Neuropathol*. 2017;134(3):441–458. doi:10.1007/s00401-017-1747-1.
236. Ginhoux F, Greter M, Leboeuf M, *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science*. 2010;330(6005):841–845. doi:10.1126/science.1194637.
237. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. *Physiol Rev*. 2011;91(2):461–553. doi:10.1152/physrev.00011.2010.
238. Sierra A, Encinas JM, Deudero JJ, *et al.* Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell*. 2010;7(4):483–495. doi:10.1016/j.stem.2010.08.014.
239. Olson JK. Immune response by microglia in the spinal cord. *Ann N Y Acad Sci*. 2010;1198:271–278. doi:10.1111/j.1749-6632.2010.05536.x.
240. Jurga AM, Paleczna M, Kuter KZ. Overview of general and discriminating markers of differential microglia phenotypes. *Front Cell Neurosci*. 2020;14:198. doi:10.3389/fncel.2020.00198.
241. Bogie JFJ, Mailleux J, Wouters E, *et al.* Scavenger receptor collectin placenta 1 is a novel receptor involved in the uptake of myelin by phagocytes. *Sci Rep*. 2017;7(1):44794. doi:10.1038/srep44794.
242. Lassmann H, van Horssen J, Mahad D. Progressive multiple sclerosis: Pathology and pathogenesis. *Nat Rev Neurol*. 2012;8(11):647–656. doi:10.1038/nrneurol.2012.168.
243. Voß EV, Škuljec J, Gudi V, *et al.* Characterisation of microglia during de- and remyelination: Can they create a repair promoting environment? *Neurobiol Dis*. 2012;45(1):519–528. doi:10.1016/j.nbd.2011.09.008.
244. Neveu I, Naveilhan P, Menaa C, Wion D, Brachet P, Garabédian M. Synthesis of 1,25-dihydroxyvitamin D_3 by rat brain macrophages in vitro. *J Neurosci Res*. 1994;38(2):214–220. doi:10.1002/jnr.490380212.
245. Cui LY, Chu SF, Chen NH. The role of chemokines and chemokine receptors in multiple sclerosis. *Int Immunopharmacol*. 2020;83:106314. doi:10.1016/j.intimp.2020.106314.
246. Spanier JA, Nashold FE, Nelson CD, Praska CE, Hayes CE. Vitamin D_3-mediated resistance to a multiple sclerosis model disease depends on myeloid cell 1,25-dihydroxyvitamin D_3 synthesis and correlates with increased CD4(+) T cell CTLA-4 expression. *J Neuroimmunol*. 2020;338:577105. doi:10.1016/j.jneuroim.2019.577105.
247. Garcion E, Sindji L, Nataf S, Brachet P, Darcy F, Montero-Menei CN. Treatment of experimental autoimmune encephalomyelitis in rat by 1,25-dihydroxyvitamin D_3 leads to early effects within the central nervous system. *Acta Neuropathol*. 2003;105(5):438–448. doi:10.1007/s00401-002-0663-0.
248. Clarke J, Yaqubi M, Futhey NC, *et al.* Vitamin D regulates MerTK-dependent phagocytosis in human myeloid cells. *J Immunol*. 2020;205(2):398–406. doi:10.4049/jimmunol.2000129.
249. Garcion E, Sindji L, Montero-Menei C, Andre C, Brachet P, Darcy F. Expression of inducible nitric oxide synthase during rat brain inflammation: Regulation by 1,25-dihydroxyvitamin D_3. *Glia*. 1998;22(3):282–294. doi:10.1002/(SICI)1098-1136(199803)22:3<282::AID-GLIA7>3.0.CO;2-7.
250. Bean BP. The action potential in mammalian central neurons. *Nat Rev Neurosci*. 2007;8(6):451–465. doi:10.1038/nrn2148.
251. Cameron-Curry P, Le Douarin NM. Oligodendrocyte precursors originate from both the dorsal and the ventral parts of the spinal cord. *Neuron*. 1995;15(6):1299–1310. doi:10.1016/0896-6273(95)90009-8.
252. Thomas JL, Spassky N, Perez Villegas EM, *et al.* Spatiotemporal development of oligodendrocytes in the embryonic brain. *J Neurosci Res*. 2000;59(4):471–476. doi:10.1002/(SICI)1097-4547(20000215)59:4<471::AID-JNR1>3.0.CO;2-3.
253. Stadelmann C, Timmler S, Barrantes-Freer A, Simons M. Myelin in the central nervous system: Structure, function, and pathology. *Physiol Rev*. 2019;99(3):1381–1431. doi:10.1152/physrev.00031.2018.
254. Du Y, Dreyfus CF. Oligodendrocytes as providers of growth factors. *J Neurosci Res*. 2002;68(6):647–654. doi:10.1002/jnr.10245.

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255. Nakahara J, Aiso S, Suzuki N. Autoimmune versus oligodendrogliopathy: The pathogenesis of multiple sclerosis. *Arch Immunol Ther Exp (Warsz)*. 2010;58(5):325–333. doi:10.1007/s00005-010-0094-x.
256. Lubetzki C, Stankoff B. Demyelination in multiple sclerosis. *Handb Clin Neurol*. 2014;122:89–99. doi:10.1016/B978-0-444-52001-2.00004-2.
257. Na SY, Cao Y, Toben C, *et al.* Naive CD8 T-cells initiate spontaneous autoimmunity to a sequestered model antigen of the central nervous system. *Brain*. 2008;131(Pt 9):2353–2365.
258. Lassmann H. Multiple sclerosis: Lessons from molecular neuropathology. *Exp Neurol*. 2014;262(Pt A):2–7. doi:10.1016/j.expneurol.2013.12.003.
259. Ramanathan S, Dale RC, Brilot F. Anti-MOG antibody: The history, clinical phenotype, and pathogenicity of a serum biomarker for demyelination. *Autoimmun Rev*. 2016;15(4):307–324. doi:10.1016/j.autrev.2015.12.004.
260. Hecker M, Fitzner B, Wendt M, *et al.* High-density peptide microarray analysis of IgG autoantibody reactivities in serum and cerebrospinal fluid of multiple sclerosis patients. *Mol Cell Proteomics*. 2016;15(4):1360–1380. doi:10.1074/mcp.M115.051664.
261. Linington C, Bradl M, Lassmann H, Brunner C, Vass K. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. *Am J Pathol*. 1988;130(3):443–454.
262. Cui QL, Rone M, Khan D, *et al.* Oligodendrogliopathy in multiple sclerosis: Relation to low glycolytic metabolic rate of oligodendrocytes (I10.004). *Neurology*. 2016;86(Suppl. 16):I10.004.
263. Patani R, Balaratnam M, Vora A, Reynolds R. Remyelination can be extensive in multiple sclerosis despite a long disease course. *Neuropathol Appl Neurobiol*. 2007;33(3):277–287. doi:10.1111/j.1365-2990.2007.00805.x.
264. Lytle JM, Chittajallu R, Wrathall JR, Gallo V. NG2 cell response in the CNP-EGFP mouse after contusive spinal cord injury. *Glia*. 2009;57(3):270–285. doi:10.1002/glia.20755.
265. Dulamea AO. Role of oligodendrocyte dysfunction in demyelination, remyelination and neurodegeneration in multiple sclerosis. *Adv Exp Med Biol*. 2017;958:91–127. doi:10.1007/978-3-319-47861-6_7.
266. De La Fuente AG, Lange S, Silva ME, *et al.* Pericytes stimulate oligodendrocyte progenitor cell differentiation during CNS remyelination. *Cell Rep*. 2017;20(8):1755–1764. doi:10.1016/j.celrep.2017.08.007.
267. Mashayekhi F, Salehi Z. Administration of vitamin D_3 induces CNPase and myelin oligodendrocyte glycoprotein expression in the cerebral cortex of the murine model of cuprizone-induced demyelination. *Folia Neuropathol*. 2016;54(3):259–264. doi:10.5114/fn.2016.62535.
268. Fragoso YD, Adoni T, Damasceno A, *et al.* Unfavorable outcomes during treatment of multiple sclerosis with high doses of vitamin D. *J Neurol Sci*. 2014;346(1–2):341–342. doi:10.1016/j.jns.2014.08.019.
269. Häusler D, Torke S, Peelen E, *et al.* High dose vitamin D exacerbates central nervous system autoimmunity by raising T-cell excitatory calcium. *Brain*. 2019;142(9):2737–2755. doi:10.1093/brain/awz190.
270. Etemadifar M, Janghorbani M. Efficacy of high-dose vitamin D_3 supplementation in vitamin D deficient pregnant women with multiple sclerosis: Preliminary findings of a randomized-controlled trial. *Iran J Neurol*. 2015;14(2):67–73.
271. Stein MS, Liu Y, Gray OM, *et al.* A randomized trial of high-dose vitamin D2 in relapsing-remitting multiple sclerosis. *Neurology*. 2011;77(17):1611–1618. doi:10.1212/WNL.0b013e3182343274.
272. Bhargava P, Steele SU, Waubant E, *et al.* Multiple sclerosis patients have a diminished serologic response to vitamin D supplementation compared to healthy controls. *Mult Scler*. 2016;22(6):753–760. doi:10.1177/1352458515600248.
273. O'Connell K, Sulaimani J, Basdeo SA, *et al.* Effects of vitamin D_3 in clinically isolated syndrome and healthy control participants: A double-blind randomised controlled trial. *Mult Scler J Exp Transl Clin*. 2017;3(3):205521731772729.
274. Kampman MT, Steffensen LH, Mellgren SI, Jørgensen L. Effect of vitamin D_3 supplementation on relapses, disease progression, and measures of function in persons with multiple sclerosis: Exploratory outcomes from a double-blind randomised controlled trial. *Mult Scler*. 2012;18(8):1144–1151. doi:10.1177/1352458511434607.
275. Camu W, Lehert P, Pierrot-Deseilligny C, *et al.* Cholecalciferol in relapsing-remitting MS: A randomized clinical trial (CHOLINE). *Neurol Neuroimmunol Neuroinflamm*. 2019;6(5):e597. doi:10.1212/NXI.0000000000000597.
276. Dörr J, Bäcker-Koduah P, Wernecke KD, *et al.* High-dose vitamin D supplementation in multiple sclerosis – results from the randomized EVIDIMS (efficacy of vitamin D supplementation in multiple sclerosis) trial. *Mult Scler J Exp Transl Clin*. 2020;6(1):205521732090347.
277. Feige J, Moser T, Bieler L, Schwenker K, Hauer L, Sellner J. Vitamin D supplementation in multiple sclerosis: A critical analysis of potentials and threats. *Nutrients*. 2020;12(3):783. doi:10.3390/nu12030783.
278. Souberbielle JC, Body JJ, Lappe JM, *et al.* Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: Recommendations for clinical practice. *Autoimmun Rev*. 2010;9(11):709–715. doi:10.1016/j.autrev.2010.06.009.
279. Souberbielle JC, Cormier C, Cavalier E, *et al.* La supplémentation en vitamine D en France chez les patients ostéoporotiques ou à risque d'ostéoporose : Données récentes et nouvelles pratiques. *Revue du Rhumatisme*. 2019;86(5):448–452. doi:10.1016/j.rhum.2019.02.014.
280. Fernández-Zapata C, Leman JKH, Priller J, Böttcher C. The use and limitations of single-cell mass cytometry for studying human microglia function. *Brain Pathol*. 2020;30(6):1178–1191. doi:10.1111/bpa.12909.
281. Couloume L, Ferrant J, Le Gallou S, *et al.* Mass cytometry identifies expansion of T-bet+ B cells and CD206+ monocytes in early multiple sclerosis. *Front Immunol*. 2021;12:653577. doi:10.3389/fimmu.2021.653577.
282. Johansson D, Rauld C, Roux J, *et al.* Mass cytometry of CSF identifies an MS-associated B-cell population. *Neurol Neuroimmunol Neuroinflamm*. 2021;8(2):e943. doi:10.1212/NXI.0000000000000943.
283. Galli E, Hartmann FJ, Schreiner B, *et al.* GM-CSF and CXCR4 define a T helper cell signature in multiple sclerosis. *Nat Med*. 2019;25(8):1290–1300. doi:10.1038/s41591-019-0521-4.
284. Iparraguirre L, Alberro A, Sepúlveda L, *et al.* RNA-Seq profiling of leukocytes reveals a sex-dependent global circular RNA upregulation in multiple sclerosis and 6 candidate biomarkers. *Hum Mol Genet*. 2020;29(20):3361–3372. doi:10.1093/hmg/ddaa219.
285. Han Z, Xue W, Tao L, Lou Y, Qiu Y, Zhu F. Genome-wide identification and analysis of the eQTL lncRNAs in multiple sclerosis based on RNA-seq data. *Brief Bioinform*. 2020;21(3):1023–1037. doi:10.1093/bib/bbz036.
286. Fernandes SJ, Morikawa H, Ewing E, *et al.* Non-parametric combination analysis of multiple data types enables detection of novel regulatory mechanisms in T cells of multiple sclerosis patients. *Sci Rep*. 2019;9(1):11996. doi:10.1038/s41598-019-48493-7.
287. Neme A, Nurminen V, Seuter S, Carlberg C. The vitamin D-dependent transcriptome of human monocytes. *J Steroid Biochem Mol Biol*. 2016;164:180–187. doi:10.1016/j.jsbmb.2015.10.018.
288. Neme A, Seuter S, Malinen M, *et al.* In vivo transcriptome changes of human white blood cells in response to vitamin D. *J Steroid Biochem Mol Biol*. 2019;188:71–76. doi:10.1016/j.jsbmb.2018.11.019.
289. Wheeler MA, Clark IC, Tjon EC, *et al.* MAFG-driven astrocytes promote CNS inflammation. *Nature*. 2020;578(7796):593–599. doi:10.1038/s41586-020-1999-0.

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290. Masuda T, Sankowski R, Staszewski O, *et al.* Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. *Nature*. 2019;566(7744):388–392. doi:10.1038/s41586-019-0924-x.
291. Esaulova E, Cantoni C, Shchukina I, *et al.* Single-cell RNA-seq analysis of human CSF microglia and myeloid cells in neuroinflammation. *Neurol Neuroimmunol Neuroinflamm*. 2020;7(4):e732. doi:10.1212/NXI.0000000000000732.
292. Ramesh A, Schubert RD, Greenfield AL, *et al.* A pathogenic and clonally expanded B cell transcriptome in active multiple sclerosis. *Proc Natl Acad Sci U S A*. 2020;117(37):22932–22943. doi:10.1073/pnas.2008523117.
293. Stoeckius M, Hafemeister C, Stephenson W, *et al.* Large-scale simultaneous measurement of epitopes and transcriptomes in single cells. *Nat Methods*. 2017;14(9):865–868. doi:10.1038/nmeth.4380.
294. Xu G, Liu Y, Li H, Liu L, Zhang S, Zhang Z. Dissecting the human immune system with single cell RNA sequencing technology. *J Leukoc Biol*. 2020;107(4):613–623. doi:10.1002/JLB.5MR1019-179R.
295. Peterson VM, Zhang KX, Kumar N, *et al.* Multiplexed quantification of proteins and transcripts in single cells. *Nat Biotechnol*. 2017;35(10):936–939. doi:10.1038/nbt.3973.

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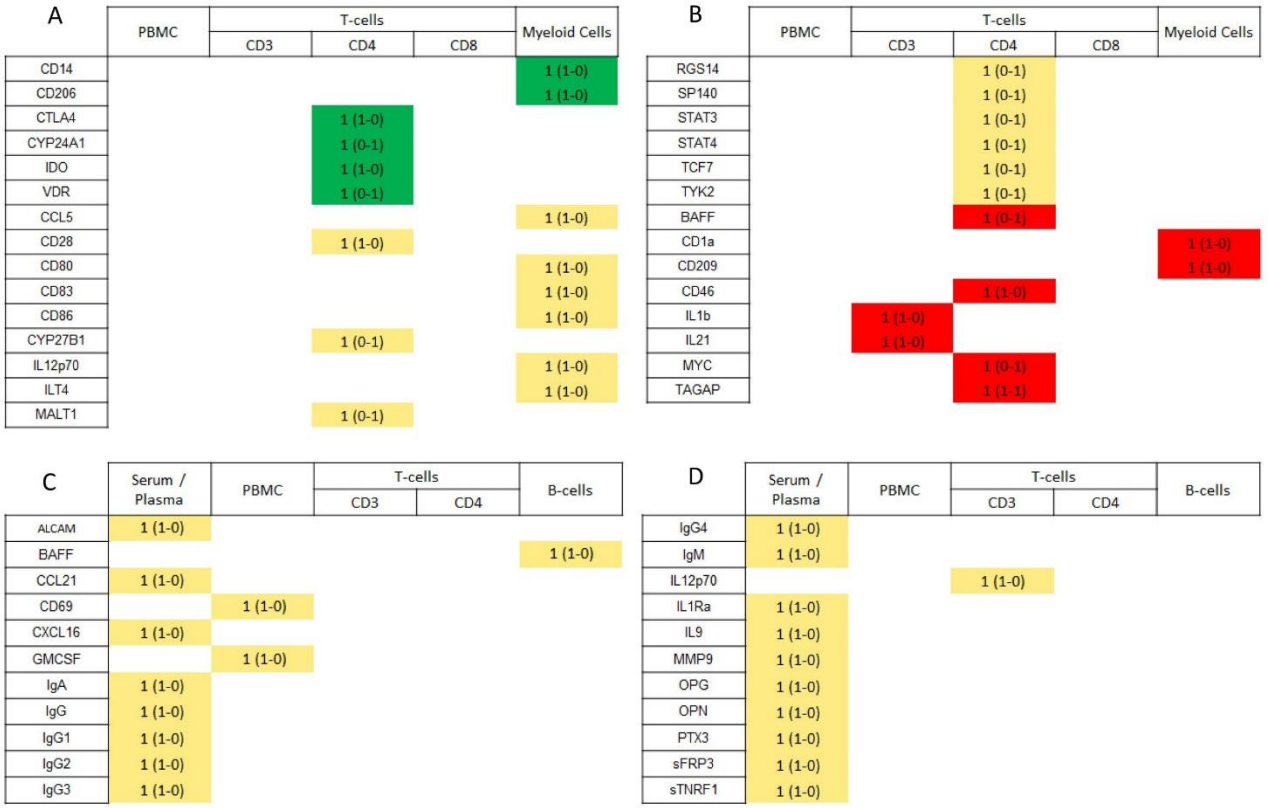


Supplementary Figure 1. Heat-Map representing the modifications of expression of biomarkers studied in *in vitro* (A,B) and *in vivo* (C,D) studies. Studies showing a significant increase (n=a), a significant decrease (n=b) or an absence of variation (n=c) were scored as follows: ((a*2)-(b*2))/((a*2)+(b*2)+c). Red represents a global decrease, green represents a global increase and yellow non-significant or discrepant results. In each case, X (Y-Z) represents the total number of tests (X) and the number of tests realized using FACS/ELISA (Y) or PCR (Z).

	Population characteristics			Vit D (cholecalciferol) supplementation						Immune effects of Vitamin D			
	MS (F/M)	DMD	Relapse free (months)	Dose UI/ week	Duration (months)	Control	Vit D isoform	Method	Mean Vitamin D levels (baseline – end)	Cell population	Methods	Cytokines/Chemokines	Immune cells
Äivo et al., 2015	30 (18/12)	IFN-beta-1b	1	20,000	12	Placebo	25(OH)D2 + 25(OH)D3	RIA	54 - 109	Serum	ELISA	↑ LAP (TGF-β), IFN-γ, IL-17A, IL-2, IL-10, IL-9, IL-22, IL-6, IL-13, IL-4, IL-5, IL-1β, TNF-α	
Ashtari et al., 2015	44 (35/9)	IFN-beta	1	70,000	3	Placebo	25(OH)D3	ECL	28 - 85	Serum	ELISA	IL 10	
Farsani et al., 2015	32 (27/5)	No	MD	50,000	2	Before/ after	25(OH)D2 + 25(OH)D3	ELISA	20 - 60	CD3	qRT- PCR	↑ IL 10 mRNA *, TGF B1 mRNA,	
Golan et al., 2013	20	IFN-beta	MD	30,590	3	800 IU/day	25(OH)D2 + 25(OH)D3	CLIA	48 - 122	Serum	ELISA	IL 17, IFN-γ, IL 10	
Kimball et al., 2011	25	IFN-beta, GA, none	2	70,000	12	maximum 4000 IU/D open label	1,25(OH)2D3 / 25(OH)D2 + 25(OH)D3	RIA	155 – 184 / 78 - 179	CD3	ELISA, [3H] thymidine incorporation	IL-1 béta, IL-4, IL-5, IL-6, IL-10, IL-12p70, and IL-13, IFN-γ, and TNF-α, MMP-9, tissue inhibitor of MMP-1, kallikrein-6, CRP, OPN	↓ proliferative response of lymphocyte for neuron antigens (Ex-2 and MBP)
Knippenberg et al., 2011	15	IFN-beta 1a or 1b	1,5	140,000	3	Before/ after	25(OH)D2 + 25(OH)D3	RIA	50 - 380	Plasma, B cells	Nephelometry, ELISA, FACS	IgG1, IgG2, IgG3 or IgG4, IgM and IgA, BAFF levels,	Total B cells (CD19+), proportions of circulating naive B cells (CD19+CD276+), memory B cells (CD19+CD27+), unswitched memory B cells (CD19+CD27+IgD+), switched memory B cells (CD19+CD27+IgD-)
Mahon et al., 2003	17	MD	MD	7,000	6	Placebo	25(OH)D2 + 25(OH)D3	RIA	17 - 28	PBMCs and serum	ELISA, Semi-quantitative PCR	↑ TGF-β1 IL-2 mRNA, IL13 mRNA, TNF-α mRNA, IFN-γ mRNA	
Mosayebi et al., 2011	26 (17/9)	IFN-beta-1a	During study	70,000	6	Placebo IM	25(OH)D2 + 25(OH)D3	Enzyme immunoassay	25 - 140	PBMCs	ELISA, MTT assay method	↑ TGF-β1, IL 10, IFN γ	↓ lymphoproliferation of PBMC
Mrad et al., 2017	21	IFN-beta-1a or 1b	1	10,000	3	Before after	25(OH)D2 + 25(OH)D3	Roche Diagnostics total assay	16 - 51	CD4+	ELISA, FACS	IFNγ, IL-17, TNFα, IL-10, IL-6, IL-4, IL-2	Naïve T-CD4 (CD4+RO−CCR7+), central memory T-CD4(CD45CD4+CR7+), effector memory T-CD4 (CD45RO+-CD4+7−), Th17 (CCR6+-IL23R+CD161+IL-17+GMCSF+), Th1, Treg, CD3+ CD4-
Muris et al., 2016	23	IFN-beta-1a	1	49,000 for 4 weeks, followed by 98,000	11	Placebo	25(OH)D2 + 25(OH)D3	RIA	60 - 231	PBMCs and serum	ELISA, FACS	↑ TNF α, IL- 10, IL-4, IL-5, IFN γ, IL-17, IL-22, GMCSF, LAP, IFNγ/IL5	Breg (CD24hiCD38hiCD19+IL10+), CD39+ nTreg (CD25+CD127-CD39+), IFNγ+ Th cells, IL17+ Th cells, IL22+ Th cells, IL4+ Th cells, iTreg (CD4+IL10), nTregs : CD25+CD127- ; CD25+CD127-FoxP3+ ; CD25+FoxP3+ memory nTreg (CD4+CD25+CD127-CD45RA-), Th 1 ou 17 GMCSF+, TNFα+ Th cells, Th1/Th2 ratio
Røsjø et al., 2015	36 (25/11)	IFN-beta, GA, NTZ	MD	20,000	24	Placebo	25(OH)D2 + 25(OH)D3	MSpec	56 - 123	serum	ELISA	ALCAM, CCL21, CXCL16, IL-1 Ra, MMP9, OPN, OPG, PTX3, sFRP3, sTNF R1, TGF β 1	
Shirvani-Farsani et al., 2015	31 (26/5)	MD	2	50,000	2	Before after	25(OH)D2 + 25(OH)D3	Enzyme immunoassay	12 – 43 (12 – 44 / 12 – 33)	PBMCS	qRT- PCR	↑ TGF-β2 mRNA, TGF-βRI mRNA,TGF-βRII mRNA	
Smolders et al., 2010	15 (8/7)	IFN-beta-1a or 1b	1,5	140,000	3	Before after	1,25(OH)2D3 / 25(OH)D2 + 25(OH)D3	RIA	146 – 202 / 50 - 380	CD4+	FACS	CD69	↑ Treg ou Tr1 ou Th2 : CD4+IL10+ ↓ ratio IFN-γ+CD4+ and IL-4 +CD4+/ (Th2 >Th1) T cells (CD3) in circulation, T activated (CD25+FoxP3+), Th1 (CD4+IFN gamma+), Th17 (CD4+IL17+), Th2 (CD4+IL4+), Treg (CD25+CD127-CD4+), Treg memory (CD25+CD127-CD45RA-), Treg Naive (CD25+CD127-CD45RA+), Tresp cell proliferation, Treg suppressive function
Sotirchos et al., 2016	19 (14/5)	IFN-beta, GA, NTZ	1	72,800	6	800 IU/D	25(OH)D2 + 25(OH)D3	CLIA	27 - 60	PBMCs	FACS		↑ naive CD4+ (D45RA+ 4+D4+ls +), CM CD4+, ↓ EM CD4+, Th17 (CD4+IL-17+), Th17 ou MAIT (CD161+CD4+), CD85j+ proportion of CD8+ cells, IFN-g+CD4+ T cells, IFN-g+IL-17 + CD4+ T cells,
Toghianifar et al., 2015	44 (35/9)	IFN-beta	1	70,000	3	Placebo	25(OH)D3	ECL	28 - 85	serum	ELISA	IL 17	

Supplementary Table 1: Effects of VitD on MS patients immune cells *in vivo*.
The effects of Vitamin D (cholecalciferol) are given as ↑ increased; ↓ decreased; not affected when not specified. * A significant increase of IL 10 mRNA occurred only when vitamin D plasmatic levels after treatment raised more than 20 g/ml.
ALCAM : Activated leukocyte cell adhesion molecule, BAFF : B cell activating factor, CCL : Chemokine (C–C motif) ligand, CLIA : Chemiluminescent Immuno-assay, CXCL : Chemokine (C–X–C motif) ligand, DMD : disease modifying drug, ECL : Electrochemiluminescence, ELISA : enzyme-linked immunosorbent assay, FACS : fluorescence-activated cell sorting analysis with cytometry, GA : glatiramer acetate, GM-CSF : Granulocyte-macrophage colony-stimulating factor, IL: interleukin, INF :interferon, Ig : Immunoglobulin, LAP : latency activated protein of TGF β, MSpec : Mass Spectroscopy, MD : missing data, MMP :Matrix metallopeptidase, NTZ : natalizumab, OPG : Osteoprotegerin, OPN : osteopontin, PBMC : peripheral blood mononuclear cells, PTX3 : Pentraxin 3, qRT-PCR : quantitative real-time polymerase chain reaction, RIA : radio Immuno-assay, sFRP3 : Secreted frizzled-related protein 3, sTNF R1 : Soluble tumor necrosis factor receptor 1, TGF :transforming growth factor, TNF : tumor necrosis factors,

	Population (female/male)	Culture conditions						Immune effects of Vitamin D		
		Cells type used	Vit D metabolite	Dose	Duration (days)	Control	Stimulation	Detection methods	Cytokines/Chemokines	Immune cells
Berge et al., 2016	RRMS (8)	T CD4+	1,25(OH)2D3 (calcitriol)	10 nM	1	vehicle control (ethanol)	αCD3/ CD28 coated beads	qRT-PCR, FACS	↑ CD25, IL2RA mRNA, CYP24A1 mRNA (24 hydroxylase), ↓ TAGAPprot, TAGAP mRNA	
Bartosik et al., 2009	RRMS (26) F18/M8	Mo-DCs	1,25(OH)2D3 (calcitriol)	1nM	2	without	TNF α	ELISA, FACS	↑ CD14, CD206, CCL2 ↓ CD1a, CD209, IL-23/IL-12p40, IL-23/IL-12p40, IL10, IL12p70 and CCL5, CD83, CD80, CD86,	
Correale et al., 2009	RRMS (20)	T CD4+	1,25(OH)2D3 (calcitriol)	10 nM	3	24,25 (OH)2 Vitamin D and 25,26 (OH)2 Vitamin D or without	1 mg/ml of plate-bound anti-CD3 mAb	[3H] thymidine incorporation, qRT-PCR ELISA	↑ VDR mRNA, IDO mRNA, IL-10 ↓ IL-6 and L-17, CYP27B1 mRNA (1-hydroxylase), IFN γ, IL4	↑ fraction T reg (CD4+CD25+FoxP3+) ↓ proliferation of CD4+ T cells
Correale et al., 2010 †	RRMS (32) 17F/15M	T CD4+	1,25(OH)2D3 (calcitriol)	10 nM	3	without	9 mg/ml PHA or myelin peptide-specific TCLs + immobilized anti-CD3 mAb	[3H] thymidine incorporation, ELISA, FACS, qRT-PCR	↑ IL-10, ↓ *IFN γ*, IL-17, CYP24A1 transcript induction (24 hydroxylase mRNA), CYP27B1 mRNA (1-hydroxylase), VDR mRNA	↑ Fraction T reg (CD4+CD25+FoxP3+)
Da Costa et al., 2016	RRMS (20) 15F/5M	T CD3+	1,25(OH)2D3 (calcitriol)	10 nM	3	without	PMA (20 ng/mL) + ionomycin (600 ng/mL) with brefeldin A (10 μg/mL)	ELISA, FACS	↑ IL-10, ↓ IL-1β, IL-6, TNF α, IL 21, IFN γ, IL-17, IL-22 and GM-CSF	Fraction of : ↑ CD4 IL10+IL17+, Tr1 (Treg CD25+CD127-FoxP3-IL10+) ; Treg : CD4 IL10+IL17- ; CD25+CD127-FoxP3+ ; CD25+CD127-FoxP3+IL10+ ; CD8 IL10+IL17+, CD8 Treg (CD8 IL10+IL17-) ↓ Th1 (CD4 IL17-IFN γ +), Th17 (CD4 IL10-IL17+), Th17 (CD4 IL17+IFN γ -), Th17.1 (CD4 IL17+IFN γ +), CD8 Tc1 (CD8 IL17-IFN γ +), CD8 Tc1* (CD8 IL17+IFN γ +), CD8 Tc17 (CD8 IL10-IL17+), CD8 Tc17 (CD8 IL17+IFN γ -)
Haas et al., 2016	RRMS (10)	B CD19+	1α,25(OH)2D3 (Calcitriol)	10-100ng/ml	2	without	2 μg/ml CpG-ODN2006 or human CD40 mAb (5 μg/ml) plus IgM (12.5 μg/ml)	[3H] thymidine incorporation, ELISA	↓ IL-6	↑ total B cells B cells switched memory (CD20+CD27+IgD-) naive B cells (CD20+CD27-IgD+)
Kickler et al., 2012	RRMS (11) 9F/2M	T CD4+	1,25(OH)2D3 (calcitriol)	100 nM	5	vehicle control (ethanol)	immobilized anti-CD3 or anti-CD3/CD46 + IL-2 (10 U/ml)	FACS, ELISA	↑ CTLA4, ↓ CD25, INF γ, CD46 if not associated with CD28+, CD 28, IL10	↓ proliferation of CD4+ T cells Treg (FoxP3+)
Lysandropoulos et al., 2011	CIS (4) /RRMS (6)	T CD8+	1,25(OH)2D3 (calcitriol)	100 nM	7	without	1 μg/ml of a pool of immunodominant 8–15 mer peptide EBV epitopes	FACS, ELISA	↑ IL-5, TGF β, ↓ IFN γ, TNF α, IL-2, IL-4 and IL-10	↓ faction of IFN γ-secreting CD8+ T cells, faction of TNFα-secreting CD8+ T cells
Muris et al., 2016	30 RRMS 21F/9M	PBMC	1,25(OH)2D3 (calcitriol)	10 nM	3	without	Anti-CD3 stimulated PBMC	ELISA	↑ IL-4, IL-5, LAP *, ↓ IFN γ, IL-17, IL-22, GM-CSF, TNF α, IL-10	
Peelen et al., 2015	8 RRMS 5F/3M	T CD4+	1,25(OH)2D3 (calcitriol)	10 nM	5	without	anti-CD3 antibody + Brefeldin A	FACS	↓ percentage inhibition of GM-CSF secretion GM-CSF protein in cultur	percentage inhibition of GM-CSF+ cells Fraction GMCSF cells
Sanseverino., et al 2014	RRMS (10)	Mo-DCs	1,25(OH)2D3 (calcitriol), 25(OH)D3 (calcidiol)	10 nM, 1uM,	6	without	TLR4 agonist lipopolysaccharide (LPS) 10ng/ml	ELISA, qRT-PCR	↑ CCL2, ↑ CCL2 mRNA	
Waschbisch., et al 2014	RRMS (9)	Mo CD14	1,25(OH)2D3 (calcitriol)	100 nM	2	Without	10% of heat inactivated human AB serum, glutamine and antibiotics	FACS, qRT-PCR	↑ ILT3, ILT3mRNA, ↓ ILT4	

Supplementary Table 2: Effects of VitD on MS patients immune cells *in vitro*.
The effects of Vitamin D are given as ↑ increased; ↓ decreased; not affected when not specified. † significant difference have been proved in female population against male population.
CCL : Chemokine (C–C motif) ligand, CLTA : cytotoxic T-lymphocyte-associated protein, ELISA : enzyme-linked immunosorbent assay, FACS : fluorescence-activated cell sorting analysis with cytometry, GM-CSF : Granulocyte-macrophage colony-stimulating factor; IDO : indoleamine 2 3-dioxygenase, IL: interleukin, ILT: Immunoglobulin-like transcript, INF :interferon, MMP :Matrix metallopeptidase, MoCD14 : Monocyte CD14, Mo-DCs : monocyte-derived dendritic cells, PBMC : peripheral blood mononuclear cells, qRT-PCR : quantitative real-time polymerase chain reaction, RRMS : relapsing remitting Multiples sclerosis, TGF :transforming growth factor, TNF : tumor necrosis factors, VDR : Vitamin D receptor

Reference	Study Model/Type	Vitamin D supplementation		Effects of Vitamin D	
		Isoform - Dose - Duration - Administration	Control	Method/Type of experiment	Observation
In Vitro					
Garcion et al. 1996	Primary brain micro vessels	1,25(OH)2D3 - 10 ng/100g - 3-4 hours - i.p., only once	Vehicle (not mentioned)	γ-GT kit	= γ-GT activity (but 50-fold higher that in brain homogenate)
	BBEC bovine cell line	1,25(OH)2D3 - 1nM - 4, 18 hours - Addition to medium	Vehicle (not mentioned)	γ-GT kit	↑ γ-GT activity (only when co-cultured with primary rat astrocytes)
Ito et al. 2011	TM-BBB4 mouse cell line	N/A	N/A	Western Blot	VDR+
Molinari et al. 2011	HUVECs	1,25(OH)2D3 - 0.01, 0.1, 1, 10 nM - 1 to 5 minutes - Addition to medium	EtOH	Griess Assay, DAF-FM	↑ NO production (after 1 min treatment, completely reduced to 0 after 5 min) (VDR and NO synthase dependent)
				Western Blot	↑ p-eNOS, ↑ p-p38, ↑ p-Akt, ↑ p-ERK (VDR and NO synthase dependent)
Durk et al. 2012	Sprague-Dawley rat brain capillaries	1,25(OH)2D3 - 1, 10, 100 nM - 4 or 24 hours - Addition to medium	0.1% EtOH	NBD-CSA assay	↑ P-gP trasport activity
				Western Blot	↑ P-gP
	RBE4 rat cell line	1,25(OH)2D3 - 5, 10, 100 nM - 1, 3 days - Addition to medium	0.1% EtOH	RT-qPCR	↑ *Vdr*, ↑ *Cyp24a1*, = *Mdr1a*, ↑ *Mdr1b*
				Western Blot	↑ P-gP
				Rhodamine 6G accumulation	↓ Rhodamine 6G accumulation
				HiLyte Fluor 488 human Ameloid-β42 accumulation	↓ HiLyte Fluor 488 human Amyloid-β1-42 accumulation
	hCMEC/D3 human cell line	1,25(OH)2D3 - 10, 100 nM - 1, 3 days - Addition to medium	0.1% EtOH	RT-qPCR	↑ *Vdr*, ↑ *Cyp24*, ↑ *Mdr1*
				Western Blot	↑ P-gP, = RAGE, = LRP-1
				Rhodamine 6G accumulation	↓ Rhodamine 6G accumulation
				HiLyte Fluor 488 human Ameloid-β42 accumulation	↓ HiLyte Fluor 488 human Amyloid-β1-42 accumulation
Uberti et al. 2013	HUVECs (H_2O_2 stimulation)	1,25(OH)2D3 - 1 nM - 15 minutes - Addition to medium, prior H_2O_2 stimulation	EtOH	Griess Assay	↑ NO production
				MTT assay	↑ Cell viability
				Cytochrome C reduction	↓ ROS production
				Western Blot, Immunocytochemistry	↑ p-Akt, ↑ p-ERK, ↑ Bectin-1 ↓ Bax, ↓ Caspase-3, ↓ Caspase-8, ↓ Caspase-9, ↓ Cytochrome C
				Immunocytochemistry, TUNEL assay	↓ Cell death
				Measurement of MPTP, Membrane potential assay	Prevention of mitochondrial membrane potential loss and MPTP opening
Won et al., 2015	bEnd.3 mouse cell line (Hypoxic condition)	1,25(OH)2D3 - 0, 5, 20, 200 nM - 24 hours - Addition to medium, prior hypoxic condition	Untreated cells	FITC-Dextran fluorescein permeability test	↓ Permeability
				Millicell ERS-2 volt/ohm meter	↑ TEER
				Western Blot, Immunocytochemistry	↑ ZO-1, ↑ Claudin-5, ↑ Occludin
				MTT assay	↑ Cell viability
				MitoSOX Red dye	↓ ROS production
				Western Blot	↓ MMP-9
				Immunocytochemistry	↓ NF-kB activation (lower pNF-kB nuclear staining)
Guo et al. 2016	bEnd.3 mouse cell line	1,25(OH)2D3 - 1, 10, 100 nM - 60 hours - Addition to medium during hypoxic condition	0.01% DMSO	Ameloid-β1–40 vectorial transport	↑ Amyloid-β efflux
				RT-qPCR, Western Blot	↑ LRP-1, ↓ RAGE, ↑ VDR
Alam et al. 2017	hCMEC/D3 human cell line	1,25(OH)2D3 - 50, 100, 500 nM - 6, 24 hours - Addition to medium	EtOH	RT-qPCR	↑ *RFC*, = *VDR*, ↑ *PCFT*
				Western Blot, siRNA	↑ RFC (VDR dipendent), ↑ PCFT
	C57BL/129/LM/Bc mouse brain capillaries	1,25(OH)2D3 - 100 nM - 4 hours - Addition to medium	EtOH	RT-qPCR	↑ *Rfc*, = *Vdr*
				Western Blot	↑ RFC
Takahashi et al., 2017	TY10 human cell line	1,25(OH)2D3 - 1, 10, 100 nM - 24 hours - Addition to medium	Untreated cells	RT-qPCR, Western Blot, Immunocytochemistry	VDR+ (nuclear staining with VitD treatment; cytoplasmic without VitD treatment)
				RT-qPCR	*Cyp24a1*+ (nuclear staining with VitD treatment; cytoplasmic without VitD treatment)
				Western Blot	↓ ICAM-1, ↑ Claudin-5, ↑ ZO-1
				Millicell electrical resistance apparatus	↑ TEER
		1,25(OH)2D3 - 1, 10, 100 nM - 24 hours - Addition to medium, prior TNFa stimulation	Untreated cells	RT-qPCR, Western Blot, Immunocytochemistry	↓ ICAM-1, ↑ Claudin-5, ↑ ZO-1
				Millicell electrical resistance apparatus	↑ TEER
				Immunocytochemistry	↓ NFkB activation (lower pNF-kB nuclear staining)
Landel et al. 2018	Primary brain endothelial cell culture from Wistar rats	N/A	N/A	RT-qPCR	*Cyp27a1*+, *Cyp27b1*+, *Cyp24a1*-, *Pdia3*+, *Vdr*+ (low)
		1,25(OH)2D3 - 20 nM - 12 hours - Addition to medium	EtOH	RT-qPCR	= *Cyp27a1*, = *Cyp27b1*, = *Vdr*, = *Cyp24a1*, ↓ *Pdia3*
Molinari et al. 2019	HUVECs co-cultured with primary C57BL/6J mouse astrocytes	25(OH)D3 - 100 nM - 15 to 1440 minutes - Addition to medium	Untreated cells	ELISA	Time-dependent ↑ in 25(OH)D3 permeability (stronger permeability in combination with lipoic acid)

In Vivo					
Garcion et al. 1996	Sprague-Dawley rats	1,25(OH)2D3 - 10 ng/100g - 2-3-4-8-24 hours - i.p., only once	Vehicle (not mentioned)	γ-GT kit	↑ γ-GT cerebral activity
Ekici et al. 2009	Wistar albino rats (tMCAO)	1,25(OH)2D3 - 1 µg/kg - 8 days - i.p., every day, starting 8 days prior tMCAO	Sham-operated rats	Lipid peroxidation measurement	↓ MDA levels (↓ Lipid peroxidation) (only in combination with DHA treatment)
				GSH measurement	↑ GSH activity (only in combination with DHA treatment)
				SOD measurement	↑ SOD activity (only in combination with DHA treatment)
Chow et al. 2011	C57BL/6 mice fxr (-/-)	1,25(OH)2D3 - 2.5 µg/kg - 8 days - i.p., every day	Sterile corn oil	RT-qPCR, Western Blot	↑ P-gP, = VDR (FXR indipendent)
				[3H] Digoxin analyses	↑ Brain efflux
Ito et al. 2011	C57BL/6J mice	1,25(OH)2D3 - 1 µg/100µl/animal - 24 hours - i.p., once	5% EtOH	Brain Efflux Index, ELISA	↑ [125I] human and ↑ endogenous Amyloid-β1-40 elimination
				Western Blot	VDR+ brain capillaries
Grishkan et al., 2013	C57BL/6J mice (MOG_{35-55} EAE)	1,25(OH)2D3 - 1.5 µg/kg - 15 days - Oral gavage, every day	1% EtOH	Evans Blue dye extravasation	↓ Permeability
				Immunohistochemistry	↓ IgG permeability
Durk et al. 2014	C57BL/6 mice	1,25(OH)2D3 - 2.5 µg/kg - 8 days - i.p., every second day	Sterile corn oil	Immunocytochemistry	P-gP+, VDR+ brain capillaries
				Western Blot	↑ Pg-P in the hippocampus
				RT-qPCR	↑ *Mdr1a*, ↑ *Cyp24a1*
	C57BL/6 mice (Vitamin D-deficiet diet)	N/A	N/A	Enzyme immunoassay	↓ Plasma 1,25(OH)2D3
				RT-qPCR, Western Blot	↓ P-gP
		1,25(OH)2D3 - 2.5 µg/kg - 8 days - i.p., every second day	Sterile corn oil	Enzyme immunoassay	Rescued plasma 1,25(OH)2D3 levels
				RT-qPCR, Western Blot	Rescued P-gP expression
	human Ameloid-β transgenic mouse model (Tg2576)	1,25(OH)2D3 - 2.5 µg/kg - 8 days - i.p., every second day	Sterile corn oil	RT-qPCR, Western Blot	↑ P-gP, = BCRP, = LRP-1, = RAGE
				ELISA	↓ Human Amyloid-β1-40 and 1-42 accumulation
	human Ameloid-β transgenic mouse model (TgCRND8)	1,25(OH)2D3 - 2.5 µg/kg - 8 weeks - i.p., every third day	Sterile corn oil	RT-qPCR, Western Blot	↑ P-gP, = LRP-1, = RAGE
				ELISA	↓ Human soluble and insoluble Amyloid-β1-40 and 1-42 accumulation
				Immunocytochemistry	↓ Cortical and hyppocampal plaque density, ↑ Cerebrovascular plaque density
				Fear conditioning	↑ Conditioned fear memory
Durk et al. 2015	Sprague Dawley rats	1,25(OH)2D3 - 4.8, 6.4 nmol/kg - 8 days - i.p., every 2 days	Sterile corn oil	Immunohistochemistry	P-gP+, VDR+ brain capillaries
				RT-qPCR	↑ *Cyp24a1*, ↑ *Mdr1a*, = *Mdr1*, = *Mdr4*, = *Bcrp*
				Western Blot	↑ P-gP, = MRP-1, = MRP-4, = BCRP
				Equilibrium dialysis (Quinidine)	↑ Brain efflux
Guo et al. 2016	Kun Ming mice	1,25(OH)2D3 - 500 nM - 0-60 minutes - Addition to medium	EtOH	[3H]-methotrexate uptake	↑ Folate transport
Enkhjargal et al. 2017	Sprague-Dawley rats	1,25(OH)2D3 - 30, 60, 120 ng/kg - 24, 48, 72 hours - i.n., once	Not mentioned	Western Blot, Immunocytochemistry	↑ Osteopontin (in astrocytes and endothelial cells)
	Sprague-Dawley rats (SAH model)	1,25(OH)2D3 - 30 ng/kg - 24, 72 hours - i.n., 24 hours before or 1 hour after SAH	Not mentioned	Neurobehavioral tests	↓ Neurological deficit
				Brain water content analysis	↓ Brain edema
				Evans Blue dye extravasation	↓ Permeability
				Western Blot, Immunocytochemistry	↑ VDR, ↑ Osteopontin (in astrocytes)
				RT-qPCR, siRNA	↑ *Opn-a*, ↑ *Opn-c* (VDR dependent)
				Western Blot, siRNA	Regulation of CD44 splice variants (VRD and OPN dependent)
				Western Blot	↑ P-gP glycosilation
Hajiluian et al. 2017	Wistar rats (High fat diet)	Not mentioned - 500 IU/kg - 5 weeks - Oral gavage, every day	Migliol	Weighting	↓ Body weight
				ELISA	↑ plasma 1,25(OH)2D3 levels
				Morris water maze test	↑ Cognitive function
				Evans Blue dye extravasation	↓ Permeability
				Western Blot	↓ NF-kB, ↑ BDNF
Evans et al. 2018	C57BL/6 mice (tMCAO)	1,25(OH)2D3 - 100 ng/kg - 5 days - i.p., every day, starting 5 days prior tMCAO	Sterile water, propylene glycol, EtOH (5:4:1 ratio)	Thionin staining	↓ Brain infarct volume
				Neurobehavioral tests	= Neurological deficit
Alam et al. 2019	C57BL/129/LM/Bc mouse (WT or Folr1 KO)	1,25(OH)2D3 - 2.5 µg/kg - 8 to 16 days - i.p., every day	Sterile corn oil	Body weighting	↓ Body weight
					↑ Folate uptake (↑ Transport activity of RFC) (in Folr1 KO mice only)
	C57BL/129/LM/Bc mouse isolated brain capillaries (WT or Folr1 KO)	1,25(OH)2D3 - 2.5 µg/kg - 8 days - i.p., every day	Sterile corn oil	RT-qPCR	↑ *Rfc*, ↑ *P-gP*, ↑ *Pcft*, = *Oat3*, = *Mrp1*, = *Mrp2*
Sadeghian et al. 2019	Sprague Dawley rats (tMCAO)	1,25(OH)2D3 - 12 µg/kg - 24 hours - i.p., once, 4 hours after tMCAO	EtOH	TCC staining	↓ Infarct size
				Brain water content analysis	↓ Brain edema
				Evans Blue dye extravasation	↓ Permeability
				Assay kits	↓ MDA concentration, ↑ SOD, ↑ CAT, ↑ GPX activities
	Sprague Dawley rats (tMCAO)	1,25(OH)2D3 - 2 µg/kg - 5-7 days - i.p., every day	EtOH in saline solution	Neurological deficit score (NDS)	↓ Neurological deficit
				H&E staining	↓ Pathological changes
				Tunel staining	↓ Apoptosis
				Immunocytochemistry	↑ BDNF

Sayeed et al. 2019	Wistar rats (tMCAO, Vitamin D-deficiet diet)	N/A	N/A	ELISA	↓ Serum 1,25(OH)2D3 levels
				Nissl staining	↑ Brain infarct volume
				Immunohistochemistry, Western Blot	↑ IgG permeability, ↑ MMP-9, ↓ Claudin-5, ↓ Occludin, ↓ β-Catenin, ↑ VDR, ↑ GLUT-1, ↑ Osteopontin
de Oliveira et al., 2020	C57BL/6J mice (MOG_{35-55} EAE)	1,25(OH)2D3 - 5 µg/kg - 8 days - i.p., every day, starting 24 hours after immunization	15% EtOH	NaFlu permeability test	↓ Permeability
				RT-qPCR	↑ *ZO-1*
Peng et al. 2020	Sprague Dawley rats (Human Amyloid-β-40 injected)	1,25(OH)2D3 - 1 µg/kg - 4 days - i.p., every day, starting 4 days prior Amyloid-β-40 injection	Sterile corn oil	Western Blot	↑ P-gP, = Neprilysin, = Lrp1, = Mrp1
				ELISA	↑ Human Amyloid-β-40 efflux
Yang et al. 2021	Sprague–Dawley rats (TBI model)	Cholecalciferol - 1, 2, 5 µg/kg - 7 days - i.p., every day, starting 7 days prior TBI	Almond oil	Enzyme-linked immunosorbent assay	↑ Serum 25(OH)D3
				Weighing wet and dry weight	↓ Brain edema
				Motor, sensory, reflex, and balance tests	↓ Neurological deficit
				MWM test	↓ Cognitive impairment
				Evans Blue dye extravasation	↓ Permeability
				Western Blot	↑ ZO-1, ↑ Occludin
				RT-qPCR	↓ *TNF-α*, ↓ *IL-6*, ↓ *IL-1β*
MS patients/ derived samples					
Kimball et al. 2007	RRMS or SPMS patients	Cholecalciferol - From 28'000 to 280'000 - 28 weeks - Oral administration, increasing dosage through the 28 weeks of treatment	Placebo	MRI	↓ Numbers of new Gd-enhancing lesions
Mosayebi et al. 2011	MS patients	Cholecalciferol - 300'000 IU - 6 month - i.m., every month	Placebo	MRI	= Numbers of new Gd-enhancing lesions
Soilu-Hänninen et al. 2012	RRMS patients with at least 1-month IFNβ-1b treatment)	Cholecalciferol - 20'000 IU - 12 month - i.m., every week	Placebo	MRI	↓ Numbers of new Gd-enhancing lesions
Dehghani et al., 2013	HUVECs (MS serum treatment)	1,25(OH)2D3 - 100 nM - 24 hours - Addition to medium, prior MS patient's serum addition	Untreated cells	MTS assay	↑ Cell viability
				ELISA	↓ Apoptosis
Takahashi et al., 2017	TY10 human cell line (MS serum treatment)	1,25(OH)2D3 - 1, 10, 100 nM - 24 hours - Addition to medium, prior MS patient's serum addition	Untreated cells	Western Blot	↓ VCAM-1, ↓ NFkB, ↑ Claudin-5, ↑ ZO-1
Hupperts et al., 2019	RRMS patients with INFβ-1a treatment	Cholecalciferol - 14'007 IU - 48 weeks - Oral administration, every day	Placebo	MRI	↓ Numbers of new Gd-enhancing lesions, ↓ New/enlarging T2 lesions

Supplementary Table 3. The effects of vitamin D on the BBB.
The effects of Vitamin D are given as ↑ increased; ↓ decreased; = not affected. Changes in protein and mRNA expression levels are indicated respectively with normal and *italic* text. Combined data on protein and mRNA are written with normal text.

Reference	Study Model/Type	Vitamin D supplementation		Effects of Vitamin D	
		Isoform - Dose - Duration - Administration	Control	Method/Type of experiment	Observation
In Vitro					
Nissou et al. 2014	Human brain pericytes	N/A	N/A	Western Blot	VDR+
		1,25(OH)2D3 - 10 nM - 24 hours - Addition to medium	Ethanol	RT-qPCR	↑ *Cyp24a1*
				Microarray, RT-qPCR	*Cyp27b1*-, ↑ *Cyp24a1*, ↑ *Vcam-1*, ↑ *Eftud1*, ↑ *Thbd*, ↑ *Osr2*, ↑ *Klf4*, ↑ *Serinc2*, ↑ *Tnfaip6*, ↑ *Gata6*, ↑ *Kcjn2*, ↑ *Cables1*, ↑ *Col16a1*, ↑ *Elfn1*, ↑ *Chrdl1*, ↑ *Cd248*, ↑ *Serpine2*, ↑ *Igfbp5*, ↑ *Rarres1*, ↑ *Lrrn3*, ↑ *Epas1*, ↓ *Ccl2*, ↓ *Fabp4*, ↓ *Tnfs4/Ox40l*, ↓ *Ak5*, ↓ *Adamtsl1*
El-Atifi et al. 2015	Human brain pericytes TNFα and IFNγ treatment	N/A	N/A	RT-qPCR	↑ *Cyp1*, ↑ *Vdr*
Takahashi et al. 2017	Human brain pericyte cell line (HBPCT)	N/A	N/A	RT-qPCR, Western Blot	VDR+
Jamali et al. 2019	Primary retinal pericytes from WT C57BL/6J mice and (VDR)-deficient mice (B6.129S4-Vdr^{tm1Mbd}/J)	N/A	N/A	FACS	↑ NG2 and ↑ Smooth Muscle Actin (in VDR KO cells)
		1,25(OH)2D3 - 10 µM - 48 hours - Addition to medium	Ethanol	Counting, Click-It-EdU Alexa Flour 488 assay, TUNEL assay, VEGF trap (sFLT-1)	↓ Cell count (VDR dependent), ↓ DNA synthesis (VDR dependent), no change in apoptosis rate, ↑ enhanced G1 arrest (VEGF dependent)
				Scratch wound assay	↓ Wound closure
				Transwell migration assay, VEGF trap (sFLT-1)	↓ Migration (VDR independent, VEGF dependent)
				Cell adhesion assay	↑ Adhesion to ECM (in both WT and Vdr-deficient cells)
				FACS	↑ α4 integrin, ↑ α5β1 integrin
				ELISA	↑ VEGF production
				Migration assay	↑ Migration of endothelial cells (upon pericytes conditioned treatment)
				Amnis imaging flow cytometry analysis	↑ VEGFR2-PDGFβR heterodimer
		1,25(OH)2D3 - 10 µM - 24 hours - Addition to medium	Ethanol	Western Blot, RT-qPCR, VEGF trap (sFLT-1)	↑ p38 activation, ↑ ERK activation, ↓ AKT activation, ↑ VDR (VEGF dependent)
In Vivo					
Garcion et al. 1996	Primary brain pericytes from Sprague-Dawley rats	1,25(OH)2D3 - 1nM - 18 hours - Addition to medium	Vehicle (not mentioned)	γ-GT kit	↑ γ-GT activity
Chung et al. 2009	C57BL/6j mice VDR (-/-) TRAMP tumours implantation	N/A	N/A	Immunohistochemistry	↓ Pericytes coverage on TRAMP-2 tumor's vessels
				ELISA	VEGF secretion ↑
				Western Blot	PDGF-Rβ ↓
Jamali et al. 2017	C57BL/6j mice (Vdr)-deficient (B6.129S4-Vdr^{tm1Mbd}/J)	N/A	N/A	Retinal mount, Immunocytochemistry	No change in angiogenic sprouting and retina vascular coverage (P5) No change in number of arteries, branches and point branches (P7, P21) ↓ Endothelial/pericyte cell ratio (P42)
				Body weighting	No change in body weight (P17)
	C57BL/6j mice (Vdr)-deficient (B6.129S4-Vdr^{tm1Mbd}/J) Oxygen induced ischemic retinopathy model (OIR)	N/A	N/A	Retinal mount, Immunocytochemistry	No change in neovascularization and area of vessel obliteration (P17)
		1,25(OH)2D3 - 2.5 µg/kg - 5 days - i.p. every day	Saline solution	Retinal mount, Immunocytochemistry	↓ Neovascularization (VDR dependent) (P17), no change in area of vessel obliteration (P17)
				Body weighting	No change in body weight (VDR dependent) (P17)

Supplementary Table 4. The effects of vitamin D on pericytes.
The effects of Vitamin D are given as ↑ increased; ↓ decreased; = not affected. Changes in protein and mRNA expression levels are indicated respectively with normal and *italic* text. Combined data on protein and mRNA are written with normal text.

Reference	Study Model/Type	Vitamin D supplementation		Effects of Vitamin D	
		Isoform - Dose - Duration - Administration	Control	Method/Type of experiment	Observation
In Vitro					
Neveu, et al. 1994	Primary astrocyte culture, C6 glioma cells	1,25(OH)2D3 - 10 nM - 24 hours - Addition to medium	Ethanol	RNAse protection assay	↑ *NGF* (astrocytes), ↓ *NT-4* (C6 glioma)
Neveu, et al. 1994	Primary rat brain astrocytes, C6 glioma cells	1,25(OH)2D3 - 10 nM - 6 , 48 hours - Addition to medium	Untreated cells (N/A)	Northern blotting	↑ *Ngf*, ↑ *App* (astrocytes and glioma cells)
				ELISA	↑ NGF, ↑ VDR (astrocytes and glioma cells)
Naveilhan et al., 1996	Primary astrocyte culture, C6 glioma cells	N/A	N/A	Northern blotting	*trkA-*, *trkB+ & trkC+* (astrocytes and glioma cells)
		1,25(OH)2D3 - 0.1 to 10 nM - 12-48 hours - Addition to medium	10% ethanol in propylene glycol	Northern blotting	↑ $P75^{NTR}$, ↑ *Vdr*
Garcion et al. 1996	Primary brain astrocytes from Sprague-Dawley rats	1,25(OH)2D3 - 1nM - 4, 18 hours - Addition to medium	Vehicle (not mentioned)	γ-GT kit	↑ γ-GT activity
Hahn et al., 1997	RC7 immortalized cerebral astrocytes and/or with corticosterone	1,25(OH)2D3 - 1 µM - 38 hours - Addition to medium	Vehicle (not mentioned)	Two-sided enzyme assay	↑ NGF
Baas et al. 2000	Rat CG-4 cell line	1,25(OH)2D3 - 100 nM - 24 hours - Addition to medium	Vehicle (not mentioned)	Northern blot	*Vdr*+ type-2 astrocytes
Smolders et al. 2013	Astroglioma cell lines (U343, U373)	1,25(OH)2D3 - 100, 1000 nM - 48 hours - Addition to medium	EtOH	RT-qPCR	= *Cyp27b1*, ↑ *Cyp24a1*, ↑ *Vdr*
Shirazi et al. 2015	Neural stem cells (NSCs) from C57BL/6J mice	1,25(OH)2D3 - from 10 pM to 10nM - 14 days - Addition to medium	None	Immunocytochemistry	↑ GFAP+ cell differentiation (low) (astrocytes)
Jiao et al. 2017					
Landel et al., 2018	Primary astrocytes culture from Wistar rats	N/A	N/A	RT-qPCR	*Cyp27a1+*, *Cyp27b1-*, *Cyp24a1-*, *Pdia3+*, *VDR+* (high)
		1,25(OH)2D3 - 20 nM - 12 hours - Addition to medium	EtOH	RT-qPCR	= *Cyp27a1*, = *Cyp27b1*, = *Vdr*, ↑ *Cyp24a1*, = *Pdia3*
Ferronato et al. 2018	Glioblastoma cells (U251, T98G); Human primary astrocytes	1,25(OH)2D3 or EM-1 (analogue of 1,25(OH)2D3) - 100 nM - 17 hours - Addition to medium	Isopropanol	Cell Proliferation assay	↑ % cells in G0/G1 phase
				Western blot	↑ p21, ↑ p27, ↑ p57, ↓ cyclin
				Cell viability assay	↓ Primary astrocytes Viability % (1,25(OH)2D3), ↑ Viability % (EM-1)
				Wound healing assay	↓ Migration (T98G and U251)
				Transwell invasion assays	↓ Cell invasion (EM-1), ↑ 1,25(OH)2D3 (relative to EM-1) (T98G)
				Gelatin Zymography assay	↓ MMP9 activity (T98G)
Molinari et al. 2019	Primary astrocytes from C57BL/6J mice (H_2O_2 stimulation)	1,25(OH)2D3 - 100 nM - 15 to 1440 min - Addition in medium in combination or not with LA	Untreated cells	Cell viability assay	↑ Viability (alone and combined with LA)
				ROS Production assay & Membrane potential assessment	↓ ROS production (alone and combined with LA), ↑ Mitochondrial activity (alone and combined with LA)
				ELISA kit -p53 transcription factor assay kit	↓ p53 activity (alone and combined with LA)
				APP Quantification	↓ APP
				ERK and Akt Activation Assay	↑ p-EKR activity, ↑ p-Akt activity (alone and combined with LA)
				Iron Quantification Assay	↓ Iron uptake
				Western blot	↓ SOD3, ↓ iNOS, ↑ p-Akt, ↑ Cytochrome C
Clarke et al. 2020	Primary human microglia from patients undergoing surgery for intractable epilepsy	1,25(OH)2D3 - 100 nM - 6 days - Addition to medium	Vehicle (not mentioned)	RT-qPCR, FACS	= MerTK
In Vivo					
Garcion et al. 1997	Lewis rats (GPSC EAE)	1,25(OH)2D3 - 5 and 1 µg/kg - 23 days - i.p., starting with 5 µg/kg on d11 and d13 post immunisation and continuing with 1 µg/kg on d19, d21, d23	Propylene glycol and disodium phosphate	Immunohistochemistry, In-situ hybridisation	↓ GFAP+iNOS+ astrocytes in the cerebellum, brainstem and spinal cord
Spach et al. 2004	B10.PL(73NS)/Sn mice (MBP EAE)	1,25(OH)2D3 - 2 µg/ml - 6 hours - i.p.	Soybean oil	RT-qPCR	↓ *GFAP*; ↑ *Calcium/calmodulin-dependent protein kinase IIδ*
Hua et.al, 2012	Male Sprague–Dawley rat (TBI model)	1,25(OH)2D3 - 1, 2.5 or 5 µg/kg – 1h post-injury – i.p, only once, given with 16 mg/kg Progesterone (PROG)	Lesion (CCl)-PROG	Immunohistochemistry	= % GFAP+ cells, ↑ Size of processes and cell body (significant for 1 µg/kg)
			Sham + Vehicle (22.5% 2-hydroxyropyl-β-cyclodextran (HBC)+ Sterile water)		= % GFAP + cells, ↑ Size of processes and cell body
Nystad et al. 2014	C57BL/6J mice (Cuprizone Model)	1,25(OH)2D3 - 0.2 ug/mouse - 4 weeks - i.p., twice weekly, starting 5 weeks after cuprizone treatment	Vehicle (not mentioned)	Immunohistochemistry	↑ GFAP+ astrocytes (astrocytosis)
	Sprague-Dawley rats (TBI model)	N/A	N/A	Immunocytochemistry	GFAP+ cells arranged around lesion site

Tang et al. 2015		1,25(OH)2D3 - 1 mg/kg - 1, 6 hours post-injury - i.p., once	PBS	Western Blot	↓ GFAP, ↓ TLR4
Malinic & Dozudic 2017	Mongolian Gerbils (Transient global cerebral ischemia)	Not mentioned - Not mentioned - 7 days - Prior to ischemia (method not mentioned)	Untreated mice	Western Blot	↑ GFAP
Calvello et al. 2017	C57BL/6N mice (MPTP model of Parkinson's disease)	1,25(OH)2D3 - 1 µg/kg - 10 days - Intragastric gavage, starting 3 days before MPTP injection	Saline solution	Immunohistochemistry	↑ GFAP+ cells
				Western Blot	↓ GFAP
Gomez-Pinedo et al. 2019	Wistar rats (Surgical lesions to the corpus callosum and lysolecithin injection)	Cholecalciferol - 5'000 IU/kg - 30 days - not mentioned, every day, 15 days prior and after to lysolecithin injection	None	Immunohistochemistry	Subventricular zone: = GFAP+ cells Corpus callosum (lesion site): ↑ GFAP+ cells
Zhang et al. 2020	C57BL/6J (MOG_{35-55} EAE)	Paricalcitriol (Vitamin D2 analogue) - 0.25, 0.5 µg/kg - i.p., every 2 days from d2 to d28	Untreated mice	Western Blot, Immunohistochemistry	↓ GFAP+ astrocytes, ↓ Iba1+ microglia
				Immunohistochemistry	↓ F4/80, ↓ iNOS, ↓ COX2
MS patients / derived samples					
Smolders et al. 2013	Post-mortem brain tissue of MS and control patients (NAWM)	N/A	N/A	Immunohistochemistry	GFAP+VDR+ cells (astrocytes) (nuclear, but also cytoplasmic, unlike controls) GFAP+CYP24A1+ cells (astrocytes)
	Primary human astrocyte culture from post-mortem MS-brain tissue	1,25(OH)2D3 - 100-1000 nM - 48 hours - Addition to medium	EtOH	Immunocytochemistry	↑ CYP24A1
de la Fuente et al. 2015	Post-mortem brain tissue of MS and control patients (NAWM, active and chronic lesions, PPWM)	N/A	N/A	Immunohistochemistry	GFAP+VDR+ cells (astrocytes) ↑ VDR+ cell in active lesions, ↑ nuclear VDR staining in active lesions, = cytoplasmic VDR staining in all lesions

Supplementary Table 5. The effects of vitamin D on astrocytes.
The effects of Vitamin D are given as ↑ increased; ↓ decreased; = not affected. Changes in protein and mRNA expression levels are indicated respectively with normal and *italic* text. Combined data on protein and mRNA are written with normal text.

Reference	Study Model/Type	Vitamin D supplementation		Effects of Vitamin D	
		Isoform - Dose - Duration - Administration	Control	Method/Type of experiment	Observation
In Vitro					
Baas et al. 2000	Secondary Oligodentrocytes cultures from Sprague-Dawley rat	1,25(OH)2D3 - 100 nM - 24 hours - addition to medium	Vehicle (not mentioned)	In situ hybridization	*Vdr*+ (cytoplasmic localization)
				Immunocytochemistry	↑ VDR+ cells (nuclear and cytoplasmic localization)
				Northern blot	↑ *Vdr*, ↑ *Ngf*, ↑ $p75^{NTR}$, = *Mbp*, = *Plp*
	Rat CG-4 cell line	1,25(OH)2D3 - 100 nM - 24 hours - addition to medium	Vehicle (not mentioned)	Northern blot	*VDR*+ O-2A progenitors, *VDR*+ Oligodentrocytes
Chabas et al. 2013	Schwann cells and mixed culture of neurons and schwann cells from dorsal root ganglia of Sprague Dawley rats	1,25(OH)2D3 - 500 nM - 24 hours - Addition to medium	Vehicle (not mentioned)	Microarray, qPCR Analysis for significant gene ontology clusters using DAVID Bioinformatics Resources	↑ *89 and* ↓ *42 genes expression, Regulation of the expression of genes involved in axogenesis and myelination (Acsl4, Arhgef19, Il33, Pank1, Relt and Spp1 in both cultures),* Altered functions in DRG/Schwann cell cultures: *mRNA processing, ribonucleotide binding, neurological system process, regulation of apoptosis, muscle development* Altered functions in Schwann cell only cultures: *anion binding, steroid metabolic process, response to hormone stimulus, inflammatory response, cell cycle* Commonly activated pathways: *regulation of axogenesis, regulation of cytoskeleton, regulation of blood pressure, regulation of cell migration, regulation of transcription (some genes in myelination)*
Shirazi et al. 2015	Neural stem cells (NSCs) from C57BL/6J mice	1,25(OH)2D3 - from 10 pM to 10 nM - 5 days - addition to medium	None	RT-qPCR	*Vdr*+, ↑ *Vdr* (concentration dependent manner), ↑ *Nt-3*, ↑ *Bdnf*, ↑ *Cntf*, ↑ *Gdnf*
				Hemocytometer counting, BrdU assay	↑ Number of cells, ↑ Cell size, ↑ Neurosphere formation
		1,25(OH)2D3 - from 10 pM to 10 nM - 14 days - addition to medium	None	Widefield microscopy	↑ Mature/differentiated morphology
				Immunocytochemistry	↑ GalC+ cell differentiation (oligodendrocytes), = Nestin+ undifferentiation (undifferentiated NSCs)
de la Fuente et al. 2015	Oligodendrocytes and oligodendrocytes progenitor cells (OPCs) from Sprague-Dawley rats	N/A	N/A	Immunocytochemistry, Western Blot, Co-IP, Proximity ligand assay	VDR+, RXRγ+, VDR+RXRγ+, (= nuclear and cytoplasmic amount) (both OLGs and OPCs) VDR antagonists or VDR siRNA: ↓ VDR, ↑ OPC Proliferation (Ki67+ cells), ↓ Differentiation (MBP+ cells), ↓ MBP production, = Cell death VDR antagonist + RXR agonist: ↓ MBP+ cells
		1,25(OH)2D3 - 10-50-100 nM - 24 hours - addition to medium	DMSO	Immunocytochemistry, Western blot	= OPCs Proliferation, = Differentiation, = Survival (normal FBS in medium) ↓ OPCs Proliferation (Ki67+ cells), ↑ Differentiation (MBP+ cells), ↑ MBP production, = Survival (charcoal-stripped FBS in medium) (VDR dependent)
Landel et al. 2018	Wistar rats derived Oligodentrocytes	N/A	N/A	RT-qPCR	*Cyp27a1*+, *Cyp27b1*-, *Cyp24a1*-, *Pdia3*+, *VDR*+ (low)
Mengozzi et al. 2020	Rat CG-4 cell line	1,25(OH)2D2 or 1,25(OH)2D3 - 100 nM - 24 or 72 hours hours - addition to medium	96% EtOH	RT-qPCR, Microarray	↑ *Vdr* (for both 24- and 72-hours incubation) Vitamin D3 influenced 1272 genes and vitamin D2 574 at 24 hours (D3 > D2) ↓ *Ontology category gene expression,* ↓ *Transcription factor genes (Sox4, Tfeb),* ↓ *Notch pathway genes (Jag1, Dll3, Psen1),* ↑ *Ras pathway genes (RRas2, Ngf, Tgfb2)*
In Vivo					
Baas et al. 2000	Wistar rats	N/A	N/A	Immunohistochemistry	VDR+ (30-50% in the striatum, 10% in the corpus callosum or spinal cord)
Nashold et al. 2000	B10.PL(73NS)/Sn mice MBP EAE	1,25(OH)2D3 - 100 ng (injection) + 50 ng (diet) - 72 hours - i.p., every day, starting at moderate EAE severity + Additional food supply every day	Soybean oil (injection), 1,25(OH)2D3 devoid diet	Immunohistochemistry	↓ Demyelination, ↑ Myelination
Goudarzvand et al. 2010	Sprague Dawley rats Ethidium bromide model	1,25(OH)2D3 - 5 µg/kg - 2-7-28 days - i.p., every day, starting post lesion	Soybean oil	Immunohistochemistry	= Demyelination, = Myelination (2- and 7-days post lesion) ↓ Demyelination, ↑ Myelination (28 days post lesion)
				Western Blot	= Caspase-3 activation (2 days), ↓ Caspase-3 activation (7- and 28-days) = MBP (7 days), ↑ MBP (2- and 28-days)
Wergeland et al. 2011	C57BL/6J mice Cuprizone Model	Not mentioned (Vitamin D3) - 50, 500, 6200, 12'500 IU/kg - 10 weeks - 2 weeks before cuprizone addition, diet supplement	Cuprizone untreated mice	LC/MS-detector, Body weighting	↑ Serum 25(OH)D3 (dose dependent), ↑ Serum calcium (dose independent), = Body weight
				Immunohistochemistry, LFB/PLP scores	↓ Demyelination, = Number of NOGO-A+ oligodendrocytes, = Remyelination (high vs low Vitamin D diet)
Chabas et al. 2013	Sprague-Dawley rats with peroneal nerve injury	Cholecalciferol (Vitamin D3) or ergocalciferol (Vitamin D2) - 100 or 500 IU/kg/day - 3 months - Oral gavage, every week	Triglicerides	Peroneal functional index (PFI),	↑ Locomotor recovery (after 3 months in 500 IU/kg/day treated animals)
				Maximum relaxation rate (MRR), Tetanus threshold	↑ Muscle mechanical properties (after 3 months in 500 IU/kg/day treated animals)
				Ventilation response, Metabosensitive afferent activity	↑ Ventilation and metabosensitive afferent responses after active muscle electrical stimulation or intramuscular capsaicin injection
				Tibialis anterior muscle weighting	↓ Muscular atrophy

				Immunohistochemistry (G-ratio)	↑ Axon numbers (in the proximal area), ↑ axon diameter, ↑ Myelination (in the proximal and distal area)
				ELISA	= Calcium levels, ↑ Serum 25(OH)D3
Nystad et al. 2014	C57BL/6J mice Cuprizone Model	1,25(OH)2D3 - 0.2 ug/mouse - 4 weeks - i.p., twice weekly, starting 5 weeks after cuprizone treatment	Vehicle (not mentioned)	Immunohistochemistry, LFB scores	↓ Demyelination, ↑ Remyelination, ↑ Number of NOGO-A+ oligodendrocytes
				LC/MS-detector	= Serum calcium, ↓ Serum 25(OH)D3, ↓ Serum 24,25(OH)2D3
				Body weighting, RotaRod test	= Body weight, = Motor performance
de la Fuente et al. 2015	Sprague-Dawley rats Ethidium bromide model	N/A	N/A	Immunohistochemistry	APC+VDR+ cells, Olig2+VDR+ cells (nuclear staining) (21 day post lesion (dpl)) ↓ Olig2+VDR+ cells (5 dpl), ↓ Olig2+VDR+ cells (14 and 21 dpl), ↑ Olig2+APC-VDR+ cells (OPCs and immature OLGs) (5 dpl), ↑ APC+VDR+ cells (14 and 21 dpl)
	Cerebellar slices culture from Sprague-Dawley rats Lysolecithin-induced demyelination	VDR antagonist (ZK159222)	DMSO	Immunohistochemistry	↓ Myelination (Area MBP-NHF/NHF) (wo Lysolecithin) ↓ Remyelination (ArearMBP-NHF/NHF) (with Lysolecithin)
Montava et al. 2015	New Zealand white rabbits with surgical lesion to the facial nerve	Cholecalciferol - 200 IU/kg - 12 week - Oral administration, every day, starting 1 post-surgery	Triglycerides	Facial functional index (FFI)	↓ Facial paralysis (from week 6 on)
				Immunohistochemistry	= Inner diameter of myelin sheath, ↑ Myelination (G ratio)
Mashayekhi et al. 2016	Balb/c mice Cuprizone Model	Not mentioned (Vitamin D3) - 5 µg/kg - 6 weeks - i.p., every day, starting 5 weeks after cuprizone treatment	Olive oil	Bio-Rad protein assay, Western Blot	= Total brain protein amount, ↑ CNPase, ↑ MOG
Shirazi et al. 2017	C57BL/6J mice MOG_{35-55} EAE	1,25(OH)2D3 - 0.1 µg - 15 days - i.p., every second day from day 15 to 30 p.i.	0.1% EtOH	Immunohistochemistry	↓ Demyelination, ↑ NG2 (OPCs), ↑ GalC (oligodendrocytes), ↑ Nestin (neural stem cells), ↑ MBP (myelin sheath),
Nystad et al. 2018	C57BL/6J mice Cuprizone model	Cholecalciferol - 50-500-6200-12500 IU/kg - 9 weeks - Addition to food, every day, starting 2 weeks before cuprizone treatment and stopping at the end of cuprizone treatment (demyelination phase)	Chow w/o Cholecalciferol	Immunohistochemistry	↓ Axonal loss (↑ NFL area) (dose dependent), = Axonal damage (SMI-32 area), = Axonal transection (APP density)
		1,25(OH)2D3 - 2 µg - 5 weeks - i.p., twice weekly starting 2 weeks before the end of cuprizone treatment and stopping 3 weeks after cuprizone treatment 3 weeks (remyelination phase)	Placebo	Immunohistochemistry	= Axonal loss (↑ NFL area), = Axonal damage (SMI-32 area), = Axonal transection (APP density)
Gomez-Pinedo et al. 2019	Wistar rats Surgical lesions to the corpus callosum and lysolecithin injection	Cholecalciferol - 5,000 IU/kg - 30 days - not mentioned, every day, 15 days prior and after to lysolecithin injection	None	Immunohistochemistry	Subventricular zone: ↑ BrdU+ cells, ↑ DCX+ cells Corpus callosum (lesion site): ↑ BrdU+ cells, ↑ DCX+ cells, ↑ Olig2+ cells, ↓ NeuN+ (Cas3+), ↑ MBP expression, = LRP2 expression, ↑ PLP expression, ↑ NG2+ cells, ↓ Iba1+ cells
				Weighting	= Body weight
MS patients / derived samples					
Smolders et al. 2013	Postmortem brain tissue of MS and control patients (NAWM)	N/A	N/A	Immunohistochemistry	VDR+ cells (both MS and control patients), many positive nuclei lined up in linear conformations, suggestive of oligodendrocytes.
de la Fuente et al. 2015	Post-mortem brain tissue of MS and control patients (NAWM, active and chronic lesions, PPWM)	N/A	N/A	Immunohistochemistry	GFAP+VDR+ cells (astrocytes) ↑ VDR+ cell in active lesions, the only remyelinated lesion identified was full of VDR+ cells, ↑ nuclear VDR staining in active lesions, = cytoplasmic VDR staining in all lesions

Supplementary Table 6. The effects of vitamin D on oligodendrocytes.
The effects of Vitamin D are given as ↑ increased; ↓ decreased; = not affected. Changes in protein and mRNA expression levels are indicated respectively with normal and *italic* text. Combined data on protein and mRNA are written with normal text.

Reference	Study Model/Type	Vitamin D supplementation		Effects of Vitamin D	
		Isoform - Dose - Duration - Administration	Control	Method/Type of experiment	Observation
In Vitro					
Neveu et al. 1994	Primary microglial cells from albino rats (LPS or INFγ stimulated)	N/A	N/A	Immunocytochemistry	OX42+ microglia
		[^{3}H]-25(OH)D3 - 3 nM - 2 hours - Addition to medium	Untreated cells	HPLC	↑ [^{3}H]-1,25(OH)2D3
Lefebvre d'Hellencourt et al. 2003	EOC13 murine microglial cell line (LPS or IFNγ stimulation)	1,25(OH)2D3 - 1-10-100 nM - 12 hours - Addition to medium	Untreated cells	Cytokine bioassays	↓ TNFα, ↓ IL-6
				Griess assay	↓ NO production
				RT-qPCR	*VDR+*
				Northen blot	= *Tnfα*, = *IL-6*, = *iNos*, = *Tgf-β1*
				ELISA	↑ TGF-β1 (not significant)
Hur et al. 2014	Primary microglia from Sprague-Dawley rats (LPS stimulation)	25(OH)D3 - 1-10-100 nM - 24 hours - Addition to medium	Untreated cells	Griess assay, siRNA	↓ NO production (CYP27B1 and VDR dependent, siRNA)
				MTT assay	= Cell viability
				RT-qPCR	*Cyp27b* + (↑ upon LPS stimulation)
	BV-2 murine microglial cell line (LPS stimulation)	25(OH)D3 - 1-10-100 nM - 24 hours - Addition to medium	Untreated cells	Griess assay, siRNA	↓ NO production (CYP27B1 and VDR dependent, siRNA)
				MTT assay	= Cell viability
				RT-qPCR	*Cyp27b1*+ (↑ upon LPS stimulation)
				Western blot	↓ p-p38 (VDR dependent, siRNA)
Djukic et al. 2014	Primary microglia from pups (p0-p2) of C57BL/6J mice (Vitamin D deficient diet) TLR agonists stimulation	25(OH)D3 - <100 IU/kg or 1500 IU/kg - 6 weeks - Addition to food, 6 weeks before mating	Primary microglial cell culture from pups (p0-p2) of C57BL/6J mice (Vitamin D deficient diet)	FACS	= F4/80 expression
				Phagocytic assay	↓ Phagocytosis of E. coli
				Intracellular survival assay	↓ Bacteria killed
				Griess assay	= NO production
				ELISA	↓ IL-6, ↓TNFa, = CXCL1
Smolders et al., 2013	Primary microglia from human brain tissue (TNFα + INFγ stimulation)	1,25(OH)2D3 - 10 nM - 48 hours - Addition to medium	EtOH	RT-qPCR	↓ *Cyp27b1*, ↑ *Cyp24a1*,
Kurtys et al. 2016	BV-2 murine microglial cell line (LPS stimulation)	25(OH)D3 - 0.1-1 µg/ml - 24 hours - Addition to medium	EtOH	XTT assay	= Cell viability
				Griess assay	↓ NO production
				ELISA	↓ IL-6
Boontanrart et al. 2016	Primary microglia from SJL/J mice (IFNγ or LPS stimulation or TMEV infection)	25(OH)D3 or 1,25(OH)2D3 - 10 nM - 24 hours - Addition to medium	Untreated cells	RT-qPCR	↑ *Vdr*, ↑ *Cyp27b1*, ↑ *Cyp24a1*, ↑ *Socs3* (upon IFNγ or LPS stimulation or TMEV infection, 1,25(OH)2D3 further increases the levels) ↑ *Il-10* (25(OH)D3 and 1,25(OH)2D3 in unstimulated cells) ↓ *Il-6*, ↑ *Il-10*, ↓ *Il-12*, ↓ *Tnfα*, ↓ *iNos*, ↓ *Mip-1α* (25(OH)D3 and 1,25(OH)2D3 in IFNγ stimulated cells) (IL-10 and SOCS3 dependent) ↑ Infα, ↑ *Infβ*, ↓ *Il-6*, ↑ *Il-10*, ↓ *Il-12*, ↓ *Tnfα*, ↓ *iNos*, ↓ *Mip-1α* (25(OH)D3 and 1,25(OH)2D3 in TMEV infected cells) (IL-10 and SOCS3 dependent)
Dulla et al. 2016	BV-2 murine microglial cell line (LPS stimulation and/or RXR antagonist HX630 treatment)	1,25(OH)2D3 - 1 µM - 24 hours - Addition to medium	Untreated cells	RT-qPCR	↓ *Il-1β*, ↓ *Il-6*, = *Tnfα*, ↓ *iNos* (also by HX630 alone, apart for IL-1 β) (combination with HX630 stronger effect for *iNos* and *Il-6*)
				WST-8 Assay	↑ Cell viability
				Griess Assay	↓ NO production (also by HX630 alone and even stronger effect in combination with HX630)
				Western blot	↓ iNOS (only when combined with HX630), ↓ pERK1/2 (in combination with HX630 stronger effect)
				ELISA	↓ Il-6 (only when combined with HX630)
				Immunocytochemistry	↓ NF-κB nuclear translocation (also by HX630 alone and in combination with HX630) (ERK dependent)
He et al. 2017	Primary microglia from WT C57BL/6J, VDR$^{-/-}$ and IL-10$^{-/-}$ mice (SEB infected)	1,25(OH)2D3 - 10 nM - 48 hours - Addition to medium	Untreated cells	RT-qPCR, Western blot, shRNA and IL-10$^{-/-}$ or VDR$^{-/-}$ cells	↑ VDR, ↓ TNFα (VDR and IL-10 dependent, shRNA and IL-10$^{-/-}$ cells), ↑ IL-10 (VDR dependent, shRNA or VDR$^{-/-}$ cells)
				ChIP, Western blot, shRNA	↓ LITAF binding to TNFα promotor (VDR dependent, shRNA)
Landel et al. 2018	Primary microglia from Wistar rats	N/A	N/A	RT-qPCR	*Cyp27a1*+, *Cyp27b1*+, *Cyp24a1*+, *Pdia3*+, *Vdr*+ (low)
		1,25(OH)2D3 - 20 nM - 12 hours - Addition to medium	EtOH	RT-qPCR	= *Cyp27a1*, = *Cyp27b1*, ↑ *Vdr*, ↑ *Cyp24a1*, = *Pdia3*
Cui et al. 2019		1,25(OH)2D3 - 1 µM - 30 minutes - Addition to medium, just before 24 hour exposure to Ang II	BV2 murine microglial cells	Western Blot	↑ VDR (Ang II stimulation > without), = AT1, = ACE, ↑ ACE2 (Ang II stimulation > without), ↑ MasR (Ang II stimulation only)

	BV2 murine microglial cell line (Stimulated or not with Ang II)		(Stimulated or not with Ang II)		With Ang II stimulation only: ↓ Nox2 (MasR dependent), = Nox4, ↓ p-p47phox (MasR dependent), ↓ p47phox (MasR dependent). ↓ NADPH oxidase activity (MasR dependent), ↓ ROS production (MasR dependent)
				RT-qPCR	↓ *Il-1β (MasR dependent)*, ↓ *Il-6 (MasR dependent)*, ↓ *Tnfa (MasR dependent)*, ↓ *Cd86 (MasR dependent)*, ↓ *iNos (MasR dependent)*, ↑ *Arg-1 (MasR dependent)*, ↑ *CD206 (MasR dependent)* (from M1 to M2 phenotype)
				Immunocytochemistry	With Ang II stimulation only: ↓ CD86 (MasR dependent), ↑ CD206 (MasR dependent)
Lee et al. 2020	BV-2 murine microglial cell line (LPS stimulation)	Conditioned medium from the N2a cell line treated with 1,25(OH)2D3 - 10-100-1000 nM - 24 hours - Addition to medium (8 hours)	EtOH	RT-qPCR	↓ *MhcII*, ↓ *iNos*, ↑ *Hmox1*, ↑ *Arg1*
				ELISA	↓ IL-6 (IL-34 dependent)
	Primary microglia from P1 mice (LPS stimulation)	Conditioned medium from primary neurons treated with 1,25(OH)2D3 - 10-100-1000 nM - 24 hours - Addition to medium (24 hours)	EtOH	ELISA	↓ IL-6, ↓ IL-1β, = TNFα
Clarke et al. 2020	Primary human microglia from patients undergoing surgery for intractable epilepsy	1,25(OH)2D3 - 100 nM - 6 days - Addition to medium	Vehicle (not mentioned)	Bulk RNAseq (PCA, ORA)	Microglia cluster along the first principal component based on their cellular phenotype (MG_0 and MG_{GMcsf}) and based on treatment with calcitriol (PCA) Differentially expressed genes in "inflammatory response" and "cytokine production/secretion" (ORA)
				RT-qPCR	↓ *MerTK* (MG_0 and MG_{GMcsf})
				FACS	↓ MerTK (MG_0 only)
Alessio et al. 2021	Primary microglia from pups (p2-p4) of C57BL/6J mice (Vitamin D deficient diet)	Cholecalciferol - <5 IU/kg or 1500 IU/kg - 3 weeks - Addition to food (3 weeks before mating, 3 weeks till pups delivery, 1 week after pup delivery)	Primary microglial cultures from pups (P2-4) of C57BL/6J mice (Vitamin D suficient diet)	Immunocytochemistry	↑ Total cell number, ↑ Activated cells (↑ Soma size, ↑ Process lengths), ↑ Iba1+ cells
				FACS	↑ Ki67+ cells, ↑ ATM+ cells
				Cell Counting Kit-8 (CCK-8)	= Cell viability
				DCFH-DA assay	↑ Intracellular ROS production
				D-ROM Test	= Media ROS levels
				Nexin V assay	= Annexin V+ cells, = 7AAD+ cells (= Apoptosis)
				In Situ Senescence-Associated β-Galactosidase Assay	↑ β-Gal+ cells
	Primary microglia from pups (p2-p4) of C57BL/6J mice (Vitamin D deficient diet)	25(OH)D3 or 1,25(OH)2D3 - 100 nM - 24 hours - Addition to medium	DMSO 0.001% or 100 nM PEA	FACS	↓ Ki67+ cells (VitD deficient derived cells only), = Cell cycle state, = ATM+ cells,
				Cell Counting Kit-8 (CCK-8)	= Cell viability
				DCFH-DA assay	↑ Intracellular ROS production
				D-ROM Test	= Media ROS levels
				Nexin V Assay	= Annexin V+ cells, = 7AAD+ cells (= Apoptosis)
				In Situ Senescence-Associated β-Galactosidase Assay	↓ β-Gal+ cells
In Vivo					
Nataf et al. 1996	Lewis rats GPSC induced chronic relapsing EAE	1,25(OH)2D3 - 5 µg/kg and 1 µg/kg - 23 days - i.p., starting with 5 µg/kg on d11 and d13 post immunisation and continuing with 1 µg/kg on d19, d21, d23	Propylene glycol and disodium phosphate	Clinical score	↓ Disease incidence, ↓ Clinal score at d23
				Immunohistochemistry	= MD-1+ microglia/macrophages, ↓ OX42+ microglia/macrophages in the anterior brain (= in the rest of the CNS), ↓ CD4+ T-cells/microglia/macrophages in all CNS parts
Garcion et al. 1997	Lewis rats GPSC induced chronic relapsing EAE	1,25(OH)2D3 - 5 µg/kg and 1 µg/kg - 23 days - i.p., starting with 5 µg/kg on d11 and d13 post immunisation and continuing with 1 µg/kg on d19, d21, d23	Propylene glycol and disodium phosphate	Immunohistochemistry	↓ OX42+ microglia/macrophages in the anterior brain (= in the rest of the CNS)
				In-situ hybridisation	↓ OX42+iNOS+ microglia/macrophage in the cerebellum, brainstem and spinal cord (= in the anterior brain)
Garcion et al. 1998	Lewis rats Hippocampal lesion and stereotactic LPS injection	1,25(OH)2D3 - 1000nM - 5-15-24-48-72 hours - Simultaneous stereotactic injection of LPS and VitD in the hippocampus	PBS	Immunohistochemistry	↑ OX42+ microglia/macrophages (72h only)
				TUNEL assay	= Cell death
				In Situ Hybridisation	↓ OX42+iNOS+ microglia/macrophages (at 15-24h)
Nashold et al. 2000	B10.PL(73NS)/Sn mice MBP EAE	1,25(OH)2D3 - 100 ng (injection) + 50 ng (diet) - 72 hours - i.p., every day, starting at moderate EAE severity + Additional food supply every day	Soybean oil (injection), 1,25(OH)2D3 devoid diet	Immunohistochemistry	↑ Mac-1+ macrophages/microglia
Garcion et al. 2003	Lewis rats GPSC induced chronic relapsing EAE	1,25(OH)2D3 - 5 µg/kg and 1 µg/kg - 23 days - i.p., starting with 5 µg/kg on d11 and d13 post immunisation and continuing with 1 µg/kg on d19, d21, d23	Propylene glycol and disodium phosphate	Clinical score	↓ Incidence of score 4 first paralytic attack, earlier second paralytic attack (Similar kinetics in Vitamin D3 deprived animals)
				Immunohistochemistry	↓ OX42+ cells, ↓ MHCII+ cells, ↓ CD4+ cells, ↓ iNOS+ cells
				In situ hybridization	↓ *iNos+*, = *Tgf-β1*
Kim et al. 2006	Sprague-Dawley rat 6-OHDA model of Parkinson's disease	1,25(OH)2D3 - 1 µg/ml at 1 ml/kg/day - 7 days - i.p., every day, starting 7 days before 6-OHDA injection	Ethanol 0.1%	Immunohistochemistry	↑ Tyrosine hydroxylase+ cells, ↓ CD11b+ activated microglia
	C57BL/6J mice MPTP model of Parkinson's disease	1,25(OH)2D3 - 1 µg/ml at 1 ml/kg/day - 7 days - i.p., every day, starting 7 days before MPTP injection	Ethanol 0.1%	Immunohistochemistry	↓ CD11b+ activated microglia
				RT-qPCR	↓ *Tnfα*, ↓ *Infγ*

Wergeland et al. 2011	C57BL/6J mice Cuprizone Model	Not mentioned (Vitamin D3) - 50, 500, 6200, 12'500 IU/kg - 10 weeks - Addition to food, every day, starting 2 weeks before cuprizone model	Cuprizone untreated C57BL/6J mice	Immunohistochemistry	↓ Number of Mac-3+ activated microglia/macrophages
Nystad et al. 2014	C57BL/6J mice Cuprizone Model	1,25(OH)2D3 - 0.2 ug/mouse - 4 weeks - i.p., twice weekly, starting 5 weeks after cuprizone treatment	Vehicle (not mentioned)	Immunohistochemistry	↓ Mac-3+ activated microglia/macrophages (only after 1-week remyelination)
Calvello et al. 2017	C57BL/6N mice MPTP model of Parkinson's disease	1,25(OH)2D3 - 1 µg/kg - 10 days - Intragastric gavage, starting 3 days before MPTP injection	Saline solution	Immunohistochemistry	↓ Tyrosine hydroxylase+ cells, ↓ Iba1+ cells On Iba-1+ cells: ↑ CD206, ↑ CD163, ↑ CD204
				Western blot	↓ Tyrosine hydroxylase, ↓ Iba1, ↓ TLR-4, ↓ iNOS, ↑ CD206, ↑ CD163, ↑ CD204
				RT-qPCR	↓ *Tnfα*, ↓ *Il-1β*, ↑ *Il-10*, ↑ *Tgfβ*, ↑ *Il-4*
Evans et al. 2018	C57BL/6J mice SAH model	1,25(OH)2D3 - 100 ng/kg/day - 5 days - i.p., every day, starting 5 days prior SAH	Sterile water, propylene glycol and ethanol (5:4:1)	Immunohistochemistry	↑ M2 microglia/macrophages (CD206+ cells)
Cui et al. 2019	Wistar–Kyoto (WKY) rats and Spontaneous Hypertensive (SHR) rats	1,25(OH)2D3 - 100 ng/kg - 6 weeks - Oral gavage, every day	Saline solution	Body weighting	= Body weight
				Immunohistochemistry, Western blot	↑ VDR (WKY>SHR) In SHR rats brain only: ↓ Apoptosis, ↑ Neuron viability, = ACE, ↓ Ang II, = AT1 (VDR co-localized), ↑ ACE2, ↑ Ang (1–7), ↑ MasR (VDR co-localized), ↓ Nox2, ↓ p-p47phox, = p47phox, ↓ Nox4, ↓ total Nox activity, ↓ DHE, ↓ MDA, ↑ SOD activity, ↑ CAT activity
de Oliveira et al. 2020	C57BL/6J mice MOG_{35-55} EAE	1,25(OH)2D3 - 5 µg/kg - 16 days - i.p, every two days, starting 24 hours after immunization	Saline solution	FACS	↓ $CD45^{High}CD11b^{+}$ cells (activated microglia), = $CD45^{Low}CD11b^{+}$ cells (resting microglia), ↓ MHC-II on activated and resting microglia, = PD-L1 on activated and resting microglia
Spanier et al. 2020	C57BL/6 (B6) mice (Vitamin D deficient diet)	1,25(OH)2D3 - 0 or 1 µg/day - 7-8 weeks - Food supply, starting 3-4 weeks before EAE induction	C57BL/6 (B6) mice (Vitamin D sufficient diet)	Clinical score analysis	↑ Disease incidence, ↑ Peak disease score, ↑ Cumulative disease index
				FACS	↓ CTLA-4+ Conventional T cells and Tregs, ↓ CTLA-4 MFI on Conventional T cells and Tregs (trend)
	C57BL/6 (B6) mice lacking exon 8 of the Cyp27b1 gene in myeloid lineage cells (M-$Cyp27b1^{0}$) (Vitamin D deficient diet) MOG_{35-55} EAE	1,25(OH)2D3 - 0 or 1 µg/day - 7-8 weeks - Food supply, starting 3-4 weeks before EAE induction	C57BL/6 (B6) mice lacking exon 8 of the Cyp27b1 gene in myeloid lineage cells (M-$Cyp27b1^{0}$) (Vitamin D sufficient diet)	Clinical score analysis	= Disease incidence, = Peak disease score, = Cumulative disease index
				FACS	= CTLA-4+ Conventional T cells and Tregs, = CTLA-4 MFI on Conventional T cells and Tregs (trend)
Alessio et al. 2021	C57BL/6J mice (Vitamin D deficient diet)	Cholecalciferol - <5 IU/kg or 1500 IU/kg - 7 weeks - Food supply (3 weeks before mating, 3 weeks till pups delivery, 1 week after pup delivery)	C57BL/6J mice (Vitamin D sufficient diet)	Quantitative chemiluminescent microparticle immunoassay	↓ Serum 25(OH)D3 levels
				D-ROM Test	= Serum ROS levels
				Simplified Up-Down method	↓ Tactile withdrawal threshold
				Immunohistochemistry	= Number of Iba-1+ cells, ↑ Activated Iba-1+ cells (more prominent in females), ↑ Dystrophic Iba-1+ cells (more prominent in females)
MS patients / derived samples					
Smolders et al. 2013	Postmortem brain tissue of MS and control patients (NAWM and active lesions)	N/A	N/A	Immunohistochemistry	HLA+VDR+ macrophages/microglia (both MS and control patients, in both NAWM and active lesions), HLA+CYP24A1- macrophages/microglia (both MS and control patients, in both NAWM and active lesions)
				RT-qPCR	↑ *Vdr*, ↑ *Cyp27b1* (in chronic active lesions vs NAWM)

Supplementary Table 7. The effects of vitamin D on microglia.
The effects of Vitamin D are given as ↑ increased; ↓ decreased; = not affected. Changes in protein and mRNA expression levels are indicated respectively with normal and *italic* text. Combined data on protein and mRNA are written with normal text.

Objectifs

Mon projet fait partie d'un plus vaste projet collaboratif financé par l'ANR et l'ARSEP dont le but est de déterminer le rôle de la vitamine D dans la restauration des voies de régulation immunitaires défectueuses dans la SEP et de fournir de nouvelles informations moléculaires sur les effets de la vitamine D dans le contrôle de l'inflammation. Il résulte de la collaboration entre trois centres aux expertises complémentaires : l'institut toulousain des maladies infectieuses et inflammatoires (Infinity) de Toulouse, l'institut de Theodor Kocher (TKI) de Bern et l'institut de génomique fonctionnelle (IGF) de Montpellier.

La carence en vitamine D constitue un facteur de risque de SEP potentiellement via ses propriétés connues de la vitamine D en tant que « modulateur de lymphocytes et de l'inflammation ». De nombreuses données ont mis en évidence ces effets immunomodulateurs sur les lymphocytes circulants dans les modèles animaux de SEP (ex : modèle EAE). Cependant, peu de choses sont connues sur les modulations des sous-populations de lymphocytes et sur la régulation de l'expression génique sous l'effet de traitement par la vitamine D *in vivo,* chez des patients atteints de SEP.

Disposant d'échantillons biologiques de patients inclus dans le PHRC national D-lay MS, une étude de phase III, multicentrique, randomisée en double aveugle *versus* placebo, coordonnée par Éric Thouvenot et évaluant l'efficacité d'un traitement par cholécalciférol (Vitamine D) pour retarder la conversion en SEP active après un SCI, mon projet de thèse vise d'une part à étudier l'effet de la supplémentation en vitamine D sur le phénotype et la fonction des lymphocytes circulants, et d'autre part à identifier de nouvelles voies immunitaires régulées par la vitamine D *in vivo* par à une approche transcriptomique.

Dans un premier temps, j'ai entrepris une étape de découverte basée sur une approche CITE-seq couplée à des analyses bio-informatiques pour identifier et quantifier les différentes sous-

populations lymphocytaires afin d'établir une liste de gènes candidats régulés par la vitamine D dans ces sous-populations. Une analyse de la littérature a permis d'établir une deuxième liste de gènes candidats connus pour être modulés par la vitamine D.

Dans un second temps, j'ai validé la modulation des gènes issus de l'analyse CITE-seq et vérifié les gènes de la littérature comme étant modulés par la vitamine D en PCR quantitative à haut débit (HT-qPCR) sur les sous-populations triées par FACS d'une nouvelle cohorte de patients issus de l'étude D-lay MS.

Enfin, j'ai identifié les voies régulées par la vitamine D *in vivo* et leur implication biologique dans le contexte de la SEP.

Partie II – Etude des modulations des lymphocytes périphériques de patients SEP traités avec de fortes doses de vitamine D.

Chapitre 1 : Article de recherche « CITE-seq reveals inhibition of NF-κB pathway in B cells from vitamin D-treated multiple sclerosis patients » Galoppin et al.

CITE-seq reveals inhibition of NF-κB pathway in B cells from vitamin D-treated multiple sclerosis patients

Manon Galoppin[1], Manon Rival[2], Anaïs Louis[3], Saniya Kari [4], Sasha Soldati [5], Britta Engelhardt [5], Anne Astier [4], Philippe Marin[1], Eric Thouvenot[1,2]

[1]IGF, University of Montpellier, CNRS, INSERM, Montpellier, France

[2]Department of Neurology, Nîmes University Hospital, University of Montpellier, Nîmes, France

[3]MGX-Montpellier GenomiX, University of Montpellier, CNRS, INSERM, Montpellier, France

[4]Toulouse Institute for Infectious and Inflammatory Diseases (Infinity), University of Toulouse, CNRS, INSERM, France

[5] Theodor Kocher Institute (TKI), University of Bern, Switzerland

Corresponding author:

Prof. Éric Thouvenot

Service de Neurologie, Hôpital Carémeau

4 place du Professeur Robert Debré

30029 Nîmes cedex 9, France.

Phone : +33 6 19 55 74 42

E-mail: eric.thouvenot@chu-nimes.fr

Conflict of interest: The authors have declared that no conflict of interest exists.

Abstract

Vitamin D deficiency is a risk factor for multiple sclerosis (MS) and is correlated with disease activity and progression. Vitamin D treatment has emerged as potentially protective, even though results from randomized controlled trials are conflicting. Here, we used single-cell RNA-seq (scRNA-seq) coupled with barcoded antibodies targeting surface markers (CITE-seq) to discover candidate genes and pathways regulated in PBMC subpopulations from MS patients treated with high-dose vitamin D (n=5) or placebo (n=5). Best candidates were combined with genes involved in immune function and vitamin D metabolism for validation in a new cohort (n=8 in each group) by high-throughput qPCR (HT-qPCR) in FACS-sorted naive CD4, Th1, Th17, Treg, naive CD8, memory and naive B cells, and MAIT cells. CITE-seq showed no significant changes in the proportions of these subpopulations in vitamin D-treated patients. Of the 92 candidate genes revealed by CITE-seq, differential expression of five genes (*UXT*, *SNRPN*, *SUB1*, *GNLY* and *KLF6*) was validated by HT-qPCR. CITE-seq also revealed the regulation of several pathways by vitamin D in naive and memory B cells, including MAPK, TLR and interleukin pathways, that contribute to counteract EBV-induced resistance to apoptosis through inhibition of the NF-κB pathway.

Abbreviations:

CIS: Clinically Isolated Syndrome; EAE: Experimental Autoimmune Encephalomyelitis; HT-qPCR: High-Throughput qPCR; MS: Multiple Sclerosis; SCPA: Single-Cell Pathway Analysis; UMAP: Uniform Manifold Approximation and Projection; UMI: Unique Molecular Identifier

Introduction

Multiple sclerosis (MS) is a chronic inflammatory, neurodegenerative and autoimmune disease that primarily affects young women. Epidemiological studies have revealed genetic risk factors such as major histocompatibility complex (MHC) loci, especially HLA DRB1*15:01. Additionally, genome-wide association studies (GWAS) have identified over 200 susceptibility variants unrelated to MHC (1). These variants are part of multiple signaling pathways involved in both innate and adaptive immune responses, distributed across immune cells and glial cells, highlighting the potential influence of altered responses in peripheral immune cells and brain-resident cells in the development of MS.

Various environmental factors including smoking, obesity, infection with the Epstein-Barr Virus (EBV), sun exposure, and vitamin D deficiency (2), also contribute to the risk for developing MS. Circulating levels of Vitamin D are influenced by specific genetic variants, skin color and sun exposure, especially during early life. Numerous studies have established a clinical correlation between circulating vitamin D levels and MS disease activity, as well as disability progression (3). Vitamin D binds to the vitamin D receptor (VDR)-retinoid x receptor (RXR) heteromer and the resulting complex translocates to the nucleus and binds to vitamin D response elements (VDREs) in the genomic DNA to modulate the expression of more than 200 genes (4). Most of them are related to vitamin D metabolism and immune function, highlighting the potential role of vitamin D deficiency in MS and other inflammatory diseases. Although a few immunological effects of vitamin D have been reported *in vivo,* studies have demonstrated pleiotropic effects of vitamin D in animal models of MS (EAE) or *in vitro* and suggested the therapeutic potential of vitamin D in MS patients (5). However, therapeutic trials evaluating the

clinical and radiological benefits of vitamin D treatment in MS patients provided conflicting results, potentially because the effect of vitamin D was evaluated in conjunction with MS immunomodulators (6–8).

Transcriptomic studies allow unbiased characterization of immune system regulation and can help understanding MS pathophysiology and identifying new potential therapeutic targets for disease-modifying drugs (DMDs) (9). In-depth analysis of the impact of vitamin D in MS patients might reveal gene under its control in different immune cell subtypes *in vivo* and its impact on the physiopathology of MS. A recent transcriptomics study evaluated the effects of vitamin D on gene expression in only three major immune cell types ($CD4^+$ T-cells, $CD19^+$ B-cells, $CD14^+$ monocytes) from MS patients and identified differences in gene expression (10).

In the present study, we compared gene expression in peripheral lymphocytes from early MS patients, naive of DMDs and treated with high-dose vitamin D (100,000 IU every 2 weeks for three months) or placebo as part of a randomized clinical trial (NCT01817166). We used single-cell RNA-seq (scRNA-seq) coupled with barcoded antibodies targeting surface markers (CITE-seq) to discover candidate genes and pathways regulated in various PBMC subpopulations. Best candidates were combined with genes involved in immune function and vitamin D metabolism for validation in a new cohort by high-throughput qPCR (HT-qPCR) in naive CD4, Th1, Th17, Treg, naive CD8, memory and naive B cells, and MAIT cells.

Results

Characteristics of the cohorts

Demographic and baseline characteristics of MS patients included in the discovery (n=5 patients per group) and validation (n=8) cohorts and treated with placebo or vitamin D are depicted in Table 1. At inclusion (D0), differences in sex, age and for vitamin D plasma levels

were not significant between placebo and vitamin D groups in the discovery (p=1, p=0.88 and p=0.84, respectively) and validation cohorts (p=0.20, p=0.46 and p=0.22, respectively). In addition, the delay since the last corticosteroid infusion was similar in both groups (p=0.60). After three months of treatment (M3), plasma levels of vitamin D were significantly different between placebo and vitamin D group (p=0.0079 and p=0.0002 in the discovery and the validation cohorts, respectively).

Analysis of lymphocyte subpopulations using CITE-seq

To decipher the effects of vitamin D on the immune balance, we used CITE-seq to describe the evolution of the proportion of each lymphocyte subset in PBMCs from MS patients before and after the 3-month placebo or vitamin D treatment (four patient groups). The expression of specific genes and surface markers was used to annotate major PBMC populations (Supplemental Figure 1), including $CD4^+$ T-cells, $CD8^+$ T-cells, B-cells ($CD19^+$), natural killer (NK) cells ($CD56^+$), natural killer T-cells (NKT; $CD3^+CD56^+$), MAIT cells ($CD161^+$), monocytes, platelets, and dendritic cells (DCs). $CD4^+$ subsets were further annotated with a combination of surface markers including $CD45RA^+$ $CCR7^+$ (naive), $CXCR3^+CCR6^-TBX21^+$ (Th1), $CXCR3^-CCR6^-CCR4^+GATA3^+$ (Th2), $CXCR3^-CCR6^+RORC^+$ (Th17), $CD45RA^-CXCR5^+ICOS$ (Tfh) and $CD45RA^-CD25^+FOXP3^+$ (Treg). $CD8^+$ cell subsets were annotated with $CD45RA^+$ $CCR7^+$ (naive), $CD45RA^-$ $CCR7^+$ (central memory, CM), $CD45RA^-CCR7^-$ (effector memory, EM), $CD45RA^+CCR7^-$ (effector, $EMRA^+$), and $\gamma\delta TCR^+$ ($\gamma\delta$ T-cells). B cell subsets were annotated using $CD27^-IgD^+$ (naive), $CD27^+IgD^+$ (transitional) and $CD27^+IgD^-$ (memory). UMAPs showed similar profiles in the four groups studied (Figure 2A).

For each cluster, we extracted a list of cells, counted and normalized them as a percentage, and compared the proportion of each lymphocyte subset before and after Vitamin D or placebo

treatment (Figure 2E). In all groups, we observed an inter-individual variability in the distribution of the various cell subsets. However, for each patient, no significant changes were seen in the proportions of the different lymphocyte subpopulations after the 3-month treatment (Supplemental Figure 2), except for Th2, which was increased after vitamin D treatment (2-way ANOVA, p=0.0446, Figure 2B).

To further explore the regulation of lymphocyte populations by vitamin D, we analyzed changes in main subset ratios. The Th1/Th17 ratio showed no significant difference between the groups. A non-significant decrease (p=0.092) was measured after the treatment in the placebo group but not in the vitamin D group (p=0.79, Figure 2C). On the other hand, the Th1/Th2 ratio was significantly decreased in the vitamin D group (p=0.0027), but not in the placebo group (p=0.57, Figure 2D), consistent with the increase of Th2 subset in the vitamin D group (Figure 2B).

Modulation of gene expression

To identify genes affected by vitamin D treatment in MS patients, we looked for differentially expressed genes (DEGs) in vitamin D and placebo-treated patients using two different methods. We first analyzed the global treatment effect ratio ((M3/D0 Vitamin D) / (M3/D0 placebo)) for each lymphocyte subset. This revealed 16 DEGs in memory B-cells (9 up-regulated genes and 7 down-regulated genes; > 1.2-fold change, $p < 0.05$), 19 DEGs in naive B-cells, (8 up, 11 down) (Figure 3A). *JUN* was the only common DEG between these two B-cell types (Supplemental Table 3). Among $CD4^+$ T-cells, 21 DEGs were identified in naive $CD4^+$ (2 up, 19 down), 77 in Th17 (72 up, 5 down), 13 in Th1 (7 up, 6 down), and 27 in Treg (14 up, 13 down). Two of them were shared by 2 subsets: *SNHG14* in Th1 and Th17 cells and *EEF1B2* in Th17 and Treg. In naive $CD8^+$ T-cells, 20 DEGs (17 up, 3 down) were identified. Finally, 50 DEGs (47 up, 3 down) were identified in MAIT cells.

Among all DEGs identified with this first approach, some were found in several lymphocyte populations: *JUN* (memory B-cells, naive B-cells, naive $CD4^+$), *C9orf78* (memory B-cells, MAIT), *HNRNPDL* (memory B-cells, naive $CD4^+$, naive $CD8^+$), *PSMB3* (memory B-cells, Th17, Treg), *UXT* (memory B-cells, Th17), *B2M* (naive B-cells, Treg), *BOD1L1* (naive B-cells, MAIT), *GCC2* (naive B-cells, naive $CD4^+$), *TUBA1A* (naive B-cells, naive $CD8^+$), *IKZF1* (naive $CD4^+$, naive $CD8^+$, MAIT), *LAMTOR4* (naive $CD4^+$, naive $CD8^+$), *NDUFA1* (naive $CD4^+$, MAIT), *EID1* (naive $CD8^+$, Th17), *RICTOR* (naive $CD8^+$, Th1), *YPEL3* (naive $CD8^+$, Treg), *ENO1*, *HLA-E*, *MTDH*, *PDIA3*, (MAIT, Th17), *KIF2A*, *PABPC1* (MAIT, Th1), *SNRPN* (MAIT, Treg) (Supplemental Table 3).

We then performed DESeq2 analysis, which identified 123 DEGs (p-value < 0.05) in memory B-cells (50 up-regulated, 73 down-regulated), 131 in naive B-cells (63 up, 68 down), 182 in naive $CD4^+$ (85 up, 97 down), 80 in naive $CD8^+$ (39 up, 41 down), 132 in MAIT cells (65 up, 67 down), 89 in Th17 (49 up, 40 down), 98 in Th1 (45 up, 53 down), and 95 in Treg (49 up, 46 down). Among all DEGs identified with this second approach, some were found in several lymphocyte populations (Supplemental Table 3 and Supplemental Figure 3).

Five DEGs were identified in both analyses in memory B-cells (*S100A6, S100A4, IRF8, ZFP36, PSMB3*), 9 in naive B-cells (*IGCL2, B2M, TUBA1A, KLF2, PSME1, JUN, CAST, DUSP1, SET*), 2 in naive $CD4^+$ (*CDC42SE2, JUN*), 5 in naive $CD8^+$ (*TUBA1A, RNF213, SP100, PABPC4, CLEC2D*), 4 in MAIT cells (*NDUFA1, NDUFA11, SAT1, SUB1*) and 3 in Th17 (*CYTIP, IFITM2, NUCKS1*). No common genes were found in Th1 and Treg subsets (Supplemental Table 3).

Among all DEGs, we selected those exhibiting the strongest modulation (>1.2-fold change) upon vitamin D treatment. These include 11 DEGs in memory B-cells, 12 in naive B-cells, 11 in naive $CD4^+$, 10 in naive $CD8^+$, 15 in MAIT and Th17, 13 in Th1 and 17 in Treg were selected for the qPCR validation step (Supplemental Table 3).

Analysis of pathways regulated by vitamin D

We next investigated which pathways are over-represented after 3 months of treatment by Vitamin D (or placebo) using Single Cell Pathway Analysis (SCPA) Package from CITE-seq data and the Reactome Pathway Database.

After three months of placebo treatment, subtle changes were observed: one pathway was over-represented and eight were under-represented in naive $CD4^+$, three were over-represented in naive $CD8^+$, one was under-represented in MAIT and one was under-represented in Th17 (Figure 3B and Supplemental Table 4). No significant pathway modulation was seen in memory B-cells, naive B-cells, Th1 and Treg. The three-months vitamin D treatment induced more robust changes: three pathways were significantly under-represented in memory B-cells, seven were over-represented and 15 were under-represented in naive B-cells, four were under-represented in naive $CD4^+$ cells, one was under-represented in MAIT and one was over-represented in Treg (Figure 3B and Supplemental Table 4). We observed no significant pathway modulation in naive $CD8^+$, Th1 and Th17. Naive B-cell subsets had the larger number of modulated pathways after vitamin D treatment, including several mitogen-activated protein kinase (MAPK) pathways (MAPK6 MAPK4 SIGNALING, RAF INDEPENDENT MAPK 1,3 ACTIVATION, MAPK TARGETS NUCLEAR EVENTS MEDIATED BY MAP KINASES, MAPK FAMILY SIGNALING CASCADES, NEGATIVE REGULATION OF MAPK PATHWAY) (e.g., *JUN, FOS, DUSP1, PI3KCA, PPP2CB,* Supplemental Table 4). In addition, naive B-cells showed modulation of TOLL LIKE RECEPTOR 9 TLR9 CASCADE, TOLL LIKE RECEPTOR TLR1 TLR2 CASCADE, MYD88 INDEPENDENT TLR4 CASCADE, and INTERLEUKIN 1 SIGNALING (e.g., *JUN, FOS, PPP2CB, PSMD2, USP14*), four pathways activated by microbial components including LPS or CpG and involved in IL-1 signaling in lymphocytes. The TRAF6 MEDIATED NF KB ACTIVATION and INTERLEUKIN 1

FAMILY SIGNALING pathways were regulated in memory B-cells (e.g., *TRAF6, PSMB4, PSMD7*).

Moreover, DEFECTIVE INTRINSIC PATHWAY FOR APOPTOSIS was under-represented in naive B-cells, indicating an increased apoptosis in these cells, while SIGNALING BY ROBO RECEPTORS was under-represented in naive CD4 T-cells, indicating a reduction of migration capacity of these cells.

Finally, TOLL LIKE RECEPTOR 9 TLR9 CASCADE and MAPK TARGETS NUCLEAR EVENTS MEDIATED BY MAP KINASES pathways were modulated in both naive B-cells and naive $CD4^+$ T-cells, while TOLL LIKE RECEPTOR TLR1 TLR2 CASCADE was modulated in both naive B-cells and MAIT cells (Supplemental Table 4).

Validation of candidate genes by high throughput qPCR

In order to enlarge the number of genes selected for validation by HT-qPCR, we added 92 genes encoding for cytokines/chemokines and their receptors, as well as proteins involved in adhesion, proliferation, activation, migration, and vitamin D metabolism (Supplemental Table 2)to the DEGs identified in the CITE-seq-based discovery step, leading to a list of 178 genes that were analyzed by HT-qPCR in a new cohort of MS patients (Table 1).

PBMC samples from this cohort were sorted by FACS to select 8 subpopulations of interest (memory B-cells, naive B-cells, naive CD4, naive CD8, Th1, Th17, Treg, MAIT cells). As for the CITE-seq analysis, we observed an important inter-individual variability in the proportions of these lymphocyte subpopulations at baseline (Figure 2F). No significant changes were seen in the proportion of each lymphocyte subpopulation after three months of placebo or vitamin D treatment (Figure 2F).

Comparing placebo and vitamin D patients after three months of treatment identified 4 DEGs in memory B-cells and naive B-cells, 5 DEGs in naive $CD4^+$, 3 DEGs in MAIT cells, 8 DEGs in Treg, 23 DEGs in Th17 and 38 DEGs in Th1 (Figure 4). Some of them were previously identified by the global ratio method in the same population (UXT in memory B-cells and SNRPN in MAIT cells), or the DESeq2 method (ITGA5 in MAIT cells and GNLY in Th17) or both methods (SUB1 in MAIT cells, NEDD4L and MIER2 in Th17, and KLF6 in Th1). Concordant variations were found in the discovery and validation steps for five of these genes (UXT decrease in memory B-cells, KLF6 decrease in Th1, GNLY decrease in Th17, SNRPN and SUB1 increase in MAIT cells). No candidate gene modulated by vitamin D in naive $CD8^+$ was validated by HT-qPCR.

Intriguingly, some regulated genes involved in identified pathways and selected for qPCR validation showed non-significant ratios by HT-qPCR in naive B-cells (*DUSP1,* p=0.10 and *FOS*, p=0.13) and memory B-cells (*JUN*, p=0.28). When a p-value threshold of 0.20 was considered, an opposite regulation of gene expression by vitamin D was found in naive and memory B-cells: whereas most genes were downregulated in memory B-cells in the vitamin D-treated group, they were up-regulated in naive B-cells (Figure 5).

Discussion

We performed the first CITE-seq analysis of PBMCs collected from MS patients before and after three months of treatment with high-dose vitamin D or placebo, and identified candidate genes and pathways modulated by vitamin D *in vivo*. Compared to previous transcriptomic studies (10), our strategy comparing PBMCs samples from the same individuals at D0 and M3 reduces interindividual variability inherent to clinical samples. Comparing vitamin D and placebo groups highlights gene expression changes actually modulated by vitamin D and not

resulting from the recent MS attack and its management with high dose steroid therapy. In addition, single-cell transcriptome analysis allowed a more exhaustive characterization of the different lymphocyte subpopulations affected by vitamin D in MS than previous studies focusing on major lymphocyte populations ($CD4^+$ T-cells, B-cells and monocytes). Using 35 different barcoded antibodies targeting specific surface markers, we did not only identify the main lymphocyte populations ($CD4^+$ T-cells, $CD8^+$ T-cells, B-cells, NK-cells, NKT-cells, MAIT cells), but also several $CD4^+$ (naive, Th1, Th2, Th17, Tfh, Tregs), $CD8^+$ (naive, CM, EM, $EMRA^+$, γδ T-cells) and B-cell (naive and memory) subpopulations. In line with previous studies, vitamin D treatment did not significantly affect the proportion of each cell subset, except for the Th2 population (5). This suggests that vitamin D does not modulate immune cells through important changes in the proportion of pro- or anti-inflammatory cell subsets, but rather through a regulation of the intrinsic properties of these cells (proliferation, adhesion, migration).

Our CITE-seq unsupervised analysis provided a more accurate analysis of gene expression changes in different lymphocyte populations, than classical scRNA-seq performed without barcoded antibodies (11), and is the first to validate candidate genes by HT-qPCR in a different cohort. Using this two-step strategy, we identified and validated five genes: *UXT* in memory B-cells, GNLY in Th17, KLF6 in Th1, and SNRPN and SUB1 in MAIT cells. *UXT* encodes a Ubiquitously Expressed Prefoldin Like Chaperone. Its mRNA undergoes alternative splicing giving rise to multiple isoforms. One of them, UXT-V1, binds to TNF receptor-associated factor 2 and prevents TNF receptor-associated death domain protein from recruiting Fas-associated protein (FADD), and thus protects cells against TNF-induced apoptosis (12). Moreover, Epstein-Barr virus BGLF4 kinase downregulates NF-κB transactivation through engagement of UXT and reduces memory B-cell apoptosis (13). The decreased expression of UXT measured in memory B-cells upon vitamin D treatment suggests that chronic vitamin D treatment might restore B-cell susceptibility to apoptosis and consequently reduces the pro-inflammatory

properties of memory B-cells in MS (14,15). Interestingly, we found an opposite regulation of *UXT* in Tregs, suggesting that vitamin D might protect Tregs from apoptosis induction through the stimulation of death receptors and thus prevent Treg deficiency in MS.

GNLY encodes granulysin, a protein released in cytotoxic granules by activated lymphocytes that displays cytolytic activity against microbial and tumor cells (16). At micromolar concentrations, granulysin is a chemoattractant for monocytes, $CD4^+$ and $CD8^+$ memory cells and actively recruits immune cells to inflammation sites (17). The decreased *GNLY* expression observed in Th17 cells from vitamin D-treated patients is consistent with previous findings indicating that vitamin D decreases the percentage of total granulysin-positive cells, including $CD4^+$, $CD8^+$ and NK-cells in pulmonary tuberculosis patients (18).

KLF6 encodes Krueppel-like factor 6, a DNA binding protein involved in activation of transcription and post-translational regulatory pathways. KLF family members have been implicated in the differentiation, activation, quiescence, or homing of various T-cell subsets. A previous transcriptomic analysis showed an overexpression of *KLF6* gene in activated T-cells from psoriasis patients (19). The decreased expression of *KLF6* in Th1 after vitamin D treatment found in our study supports the notion that vitamin D can modulate Th1 activation in MS (20).

The *SNRPN*-encoded small nuclear ribonucleoprotein polypeptide N (Sm-D) plays a role in gene expression regulation and in pre-mRNA processing. It is the target of auto-antibodies in systemic lupus erythematosus. Expression of genes encoding Sm proteins was positively correlated with the infiltration of Th2 cells, but negatively correlated with the infiltration of mast cells, Th1 cells and NK cells in a model of lung cancer (21). However, the significance of *SNRPN* expression increase in MAIT cells after vitamin D treatment in the context of MS remains unclear.

SUB1 encodes positive cofactor 4 (PC4), a transcription factor involved in Tregs activation through a pathway distinct from *FOXP3* (22). Luo *et al.* have shown that *SUB1* overexpression increases *IL10*, *HMGB2*, and *MKI67* mRNA levels in Tregs (22). In diffuse large B-cell lymphoma, PC4 is overexpressed and acts as an upstream regulator of c-Myc. PC4 is closely related to lymphoma clinical staging and prognosis. PC4 knockdown and the resulting inhibition of c-Myc expression can induce autophagic cell death (23). However, the significance of *SUB1* expression increase in MAIT and Th17 cells observed in our study remains to be explored.

The limited number of vitamin D regulated genes identified in our study compared with previous studies using *in vitro* or *ex vivo* preparations challenged or not with vitamin D might have several reasons. First, gene expression analysis was performed after chronic vitamin D treatment (3 months), which might have precluded the identification of early and transiently regulated genes whereas previous *in vitro* or *ex vivo* studies rely on acute vitamin D treatment (24). Second, vitamin D levels in our patients increased from around 50nM to 150 nM, which differs from main *ex vivo* protocols where cells are cultured in culture media deprived from vitamin D and stimulated by adding various and sometimes higher concentrations of vitamin D (generally 100 to 400nM) (25). These different vitamin D concentration ranges might explain why many genes identified (but not validated) in those studies, such as genes encoding cytokines and chemokines, were not found in our CITE-seq analysis. To further explore the influence of vitamin D on the expression of these genes in MS patients, we decided to add to the DEGs identified by CITE-seq a set of genes potentially involved in the pathophysiology of MS, for validation by HT-qPCR. Hence, we observed a decreased expression of *CXCL13* transcript in memory B-cells after vitamin D treatment. CXCL13 is a chemokine that attracts B lymphocytes and its expression is increased in the CSF of RRMS patients and reflects the

presence of B-cells (26). Our results suggest that vitamin D could reduce memory B-cell chemo-attraction in the CNS, thereby reducing local inflammation.

Likewise, we found a decreased expression of *IL-6, JAK1* and *STAT3 i*n Th1 cells from vitamin d-treated patients. This contrasts with data from Chauss et al. in healthy donors, showing a paradoxical increase in expression of *IL-6* and *STAT3* transcripts in Th1 cells, that peaks at 48 h post-vitamin D stimulation (20). Nevertheless, our data are consistent with the activation of the IL-6/JAK1/STAT3 pathway described in Th17 from EAE mice (5). CD46 and CD69 were also decreased in our study, consistent with previous data showing a negative correlation between CD46 expression on T-cells and the levels of circulating vitamin D in a cohort of MS patients supplemented by vitamin D (27) and a decreased CD69 expression in active T-cells from Crohn's disease patients (28). Finally, the decreased expression in Th1 of two genes involved in vascular cell adhesion, namely *ITGA4* that encodes for CD49d (alpha-4 subunit of VLA4, target of natalizumab) and *CD226* might suggest an inhibitory effect of vitamin D on Th1 ability to cross BBB (27). Notably, CD226 is expressed by both Th1 and Th17 cells and anti-CD226 treatment ameliorates the course of EAE (29,30).

Intriguingly, we observed contrasting regulations of gene expression by vitamin D in naive and memory B-cells, two populations with opposite functions (31). Pathway analysis also showed distinct regulations by vitamin D of several MAPK, TLR and interleukin pathways in naive and memory B-cells. In naive B-cells, we found that vitamin D treatment down-regulates interleukin-1 signaling and TLR 1/2 and TLR9 pathways known to stimulate NF-κB signaling (32). NF-κB, MAP kinase, IRF7, and PI3 kinase pathways are known to be stimulated via latent membrane protein 1 (LMP1), a mechanism favoring B-cell immortalization during chronic EBV infection (33). Thus, vitamin D might restore sensitivity to apoptosis of naive B-cells, consistent with the under-representation of the DEFECTIVE INTRINSIC PATHWAY FOR APOPTOSIS. The under-representation of the TRAF6 pathway after vitamin D treatment also

suggests a reduction of NF-κB activation in memory B-cells. Collectively, these data suggest that chronic vitamin D treatment contributes to reduction of chronic EBV-induced B-cell activation in MS patients. TLR9 and MAPK pathways are also negatively regulated by vitamin D in naive $CD4^+$ T-cells, suggesting similar pro-apoptotic effects of vitamin D in naive B-cells and naive $CD4^+$ T-cells. These pro-apoptotic effects might also explain the synergistic effects of vitamin D and glucocorticoid therapy in EAE (34).

Conclusion

The analysis of gene expression in PBMC sub-populations from MS patients naive from any disease modifying drugs and chronically treated with vitamin D deciphered many subpopulations not investigated by previous transcriptomics studies performed on larger cell populations sorted by FACS (10). It revealed changes in the expression of genes in different lymphocyte subsets that reflect *in vivo* modulation of the immune system by vitamin D in MS patients. It also identified pathways regulated by vitamin D in naive and memory B-cells that potentially prevent EBV-induced resistance to apoptosis through inhibition of the NF-κB pathway, thus highlighting the unexplored regulation by vitamin D of B-cells, a key therapeutic target in MS.

Material and methods

Sample of MS patients included in the discovery cohort.

The cohort of MS patients is part of the D-lay MS study (NCT01817166), a multi-center, phase III, randomized, double-blind, placebo-controlled hospital-based clinical research protocol in early MS patients naive from DMDs. The primary objective of D-lay MS was to assess the efficacy of cholecalciferol (Vitamin D3) monotherapy in reducing the rate of conversion to active MS after a clinical isolated syndrome (CIS) (according to 2005 McDonald criteria (35)),

and its safety over 2 years. Three hundred and sixteen patients, aged between 18 and 55, who had presented a typical CIS less than 90 days ago with the presence of lesions suggestive of MS on brain and spinal cord MRI (36) and low plasma vitamin D levels (< 100 nmol/L) were included and randomized to 100,000 IU cholecalciferol or placebo every 2 weeks. Cerebral and spinal cord MRI, blood sampling for vitamin D, calcium, creatinine, and urinalysis were performed at the initial visit (D0) and after 3 months (M3). Patients underwent follow-up visits at 3, 6, 12, 18 and 24 months and follow-up MRIs at 3, 12 and 24 months to assess safety and disease activity. Five MS patients treated with vitamin D (mean age 42 years-old) and 5-matched MS patients treated with placebo (39 years-old) were selected (Table n°1). Plasmatic vitamin D levels were assayed using the electrochemiluminescence on Cobas 8000 (Roche™) on the e801 module.

Blood sampling and PBMC preparation.

Blood samples of participants of the D-lay MS study was collected in EDTA-containing tubes at D0 and M3 of treatment (placebo or vitamin D). PBMCs were isolated on Ficoll® gradient and cryopreserved in liquid nitrogen at a concentration of 5 X 10^6 cells per ml in PBS solution containing using 4% human albumin and 10% DMSO.

PBMCs were rapidly thawed in a water bath at 37°C, immediately resuspended in cell culture medium (RPMI 1640, 10% FCS, L-glutamine, penicillin (50U/mL), streptomycine (50 µg/mL), sodium pyruvate 1 mM, Hepes 10 mM, and non-essential amino acids (both Life Technologies), centrifuged (350 X g for 7min at 20°C) and washed once with cell staining Buffer (BioLegend). The PBMC pellet was resuspended and cells were stained with Human TruStain FcX Fc Blocking Reagent (BioLegend) for 10 min at 4°C and then with fixable viability dye DAPI

(Merck), Vybrant Ruby (Life Technologies) and 35 TotalSeq-B antibodies (Supplemental Table 1) for 30 min on ice. Live cells were sort purified as a singlet on a BD FACSMelody™.

Sorted cells were washed in 0,4% BSA in PBS. Approximately 5,000 cells per samples were loaded on a 10x chip and run onto the 10x Chromium controller using Chromium Next GEM Single Cell 3' Reagents Kits v3.1 with Feature Barcoding technology for Cell-Surface Protein (10x Genomics), according to the manufacturer's protocol.

Gene expression and cell-surface protein expression libraries were multiplexed using individual Chromium i7 sample indices. Gene expressions libraries were sequenced on 3 lanes of a NovaSeq6000 S4 flow cell using 28-92nt paired-ends reads and 8nt for the i7 index. Cell-surface protein expression libraries were sequenced on a NovaSeq6000 SP flow cell using 28-92nt paired-ends reads and 8nt for the i7 index. Sequencing quality was assessed using SAV (Sequence Analysis Viewer) software from Illumina, raw data quality was assessed using FastQC (v0.11.9, Babraham Institute) and contaminant screening was performed using Fastq Screen (v0.15.1, Babraham Institute).

Single-cell sequencing data processing

Cell Ranger software (10x Genomics, v.6.1.1) was used to demultiplex samples, process raw data, align reads to the GRCh38 human reference genome and summarize unique molecular identifier (UMI) counts. Filtered gene-barcode and cell-surface protein expression-barcode matrices containing only barcodes with UMI counts that passed the threshold for cell detection were aggregated into a single matrix and used for further analysis. Then, the aggregated filtered UMI count matrix was processed using the R package Seurat (version 4.3.0). Cells that expressed fewer than 200 genes and/or >15% mitochondrial reads, and genes expressed in fewer than three cells were removed from the count matrix. After quality control, only raw gene counts

in high-quality cells were subjected to log-normalization, identification of high-variable genes by using the vst method, scaling, and regression against the number of UMIs and mitochondrial RNA content per cell. An unbiased calculation of the k-nearest neighbors, was applied to generate the neighborhood graph and embedding using UMAP.

Annotation of Seurat clusters was manually curated using a combination of surface markers for each cluster and visual inspection of key markers using UMAP visualization. After initial cluster annotation, all clusters were split into $CD4^+$, $CD8^+$ and B-cells subsets and reanalyzed. After sub-setting, integration using reciprocal principal component analysis was performed to remove batch effects and the integrated assay was used for principal component analysis and unsupervised clustering. Seurat subclusters were annotated using a combination of canonical protein and mRNA markers.

Clustering of human PBMCs was performed using the following parameters: 18 PCs and resolution 1. Sub-clustering of human $CD4^+$, $CD8^+$ T-cells and B-cells was performed using the following parameters: 12 PCs and resolution 1.

To assess the quantitative effect of vitamin D, lymphocyte subsets were counted per patient and transformed into percentages relative to each patient. Two-way ANOVA with a false discovery correction according to the Benjamini-Hochberg approach was performed to assess the interaction of time and treatment. A Mann-Whitney test was performed on the M3/D0 percentage ratios.

Differential gene expression between vitamin D and placebo groups

Differentially expressed genes were identified by two methods. The first one calculated the mean expression of each gene per patient and per subset. Means were aggregated by treatment group and time (D0 and M3). A ratio of M3/D0 means was then calculated followed by a Welch

Test. A gene was considered significant with a p value < 0.05. The fold-change was then obtained by vitamin D/placebo ratio. The second method was to transform the scRNA-seq data to a pseudo-bulk RNA-seq data and perform a differential expression analysis using DESeq2 R package (v.1.38.3).

Pathway Analysis

Pathway enrichment analysis was performed using Single Cell Pathway Analysis (SCPA) Package (v.1.5.3) (37) from CITE-seq data in RStudio® (Integrated Development for R. RStudio, PBC, Boston, MA) using Reactome Pathway Database (https://reactome.org) and M3-D0 for each treatment group. A pathway was considered significantly enriched with an adjusted p-value < 0.05.

Validation cohort, Flow Cytometry and Cell Lysis

Eight MS patients treated with vitamin D (mean age 37.4 years-old) and 8-matched placebo (40.6 years-old) were selected (Table n°1). PBMCs were thawed, resuspended in (RPMI 1640, 10% FCS, L-glutamine, penicillin (50U/mL), streptomycine (50 µg/mL), sodium pyruvate 1 mM, Hepes 10 mM, and non-essential amino acids (both Life Technologies) and centrifuged (350 X g for 7min at 20°C). After resuspension in calcium- and magnesium-free PBS, PBMCs were stained with Zombie NIR–APC-Cy7 (viability dye, 1:3000) for 15min at 4°C. After incubation with FcBlock (PBS + 12% human serum AB (Merck)) for 10 min at 4°C, cells were divided into two panels and labelled as follows : *panel 1* : CD4 (BUV496, clone: SK3, Becton Dickinson), CD27 (BUV737, clone: M-T2H, Becton Dickinson), CD8a (Pacific Blue, clone: RPA-T8, BioLegend), CD45RA (BV510, clone: HI100, Becton Dickinson), CCR7 (BV787, clone: G043H7, BioLegend), IgD (FITC, clone: IA6-2, BioLegend), CD19 (PerCP-Cy5.5,

clone: HIB19, BioLegend), CD3 (AF700, clone: OKT3, BioLegend) for memory B-cells, naive B-cells, naive CD4 and CD8 T-cells; and *panel 2* : CD4 (BUV496, clone: SK3, Becton Dickinson), CD8a (Pacific Blue, clone: RPA-T8, BioLegend), CD3 (AF700, clone: OKT3, BioLegend), CD127 (BV650, clone: 7019D5, BioLegend), CCR6 (BV711, clone: MI-15, Becton Dickinson), CD161 (FITC, clone: DX12, Becton Dickinson), CD25 (PE-CF594, clone: BC96, Becton Dickinson), CXCR3 (APC, clone : G025H7, BioLegend) for Th1, Th17, Treg and MAIT cells. For each cell-type and each panel, 100 cells were sorted in 5 µL RT Mix (Superscript® VILO™ cDNA Synthesis Kit, 20 U/ µL SUPERase-In™ RNase Inhibitor, 10% NP-40 Detergent Surfact-Amps Solution, ThermoFisher Scientific) using a BD FACSAria™ Fusion and stored at -80°C.

High-throughput qPCR

PCR Primers

A set of 190 PCR primers (Supplemental Table 2) corresponding to the 178 genes of interest plus 12 housekeeping genes (*ACTB, GAPDH, HPRT1, IPO8, POLR2F, PPIA, RPL10, RPLP0, RPS18, TFRC, UBC, UBE2D2*) was prepared by Fluidigm for the Fluidigm DeltaGene Assay (Fluidigm Co., San Francisco, CA, USA, https://d3.fluidigm.com/account/login). Fluidigm analyses were carried out on several sub-populations at a time. Only the three most robust reference genes per population were used for analysis.

Reverse transcription

Reverse transcription was performed using the SuperScript® VILO™ cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, United States) according to the manufacturer's protocol. The reverse transcription conditions were as follows: 25°C for 5min, 50°C for 30min,

55°C for 25min, 60°C for 5min and 70°C for 10min. Complementary DNAs (cDNAs) were stored at -20°C.

Pre-amplification

A pre-amplification step was included to increase the number of cDNA copies to a detectable level and to allow the concurrent amplification of the different gene expression targets, using the PreAmp Master Mix (Fluidigm Co., San Francisco, CA, USA). The pre-amplified products were treated with exonuclease I to remove the unincorporated primers and diluted 5-fold with the DNA suspension buffer before being assayed by PCR. The pre-amplified DNAs were stored at -20°C until testing.

Analysis of gene expression

Gene expression was analyzed by the Fluidigm qPCR procedure, using the Biomark HD™ microfluidic device. Primer-probe sets and samples were transferred to an integrated fluidic circuits (IFC) plate and loaded into an automated controller that prepares the nanoliter reactions. The IFC plate was run on the Biomark instrument, which uses a thermal cycler for real-time quantitative PCR following this cycle conditions: 70°C for 40 min, 60°C for 30 s, 95°C for 60 s, followed by 30 cycles of 96°C for 5 s and 60°C for 20 s, using the 96.96 dynamic array™ IFC (microfluidic chip). Distilled water, instead of cDNA, was used as negative control. Melting curves were generated for each gene by increasing the temperature from 60°C to 95°C while the fluorescence was measured. Non-detectable expression was considered a Ct of 24. $^{\Delta}$Ct values were calculated relative to the geometric mean of three housekeeping genes in the 96-gene set for each subset. $^{\Delta\Delta}$Ct were calculated using D0 for reference for each gene in each subpopulation. Fold changes were calculated as $2^{-\Delta\Delta Ct}$.

Statistical analysis

The Mann-Whitney test was used to evaluate statistical differences in the gene expression means between placebo and vitamin D group. P-values lower than 0.05 were considered statistically significant. P-values lower than 0.20 were considered as part of the discovery step of genes potentially modulated by vitamin D. Statistical analyses and box plot representation were performed using RStudio®. Heat-map was performed using the free-software Perseus® (Max-Planck-Institute of Biochemistry, Martinsried, Germany, version 2.0.7.0). A gradient coloring scheme was applied to display upregulated (above 2-fold expression (in log2 display above 1, red color)) *vs.* downregulated (below 2-fold expression (in log2 display below -1, blue color)) genes. White color nodes represent changes of non-modulated genes. The heatmap was hierarchically clustered by Pearson's correlation distance using row average linkage.

Study Approval

The study protocol was approved by a national review board, the CPP Sud-Méditerranée III ethics committee (2013.02.08 bis on March, 6th 2013) and health authorities (EudraCT 2013-000910-40, ANSM 130342A-31). Written informed consent was obtained from all the study participants before any study-related procedures were performed (ClinicalTrials.gov identifier NCT01817166). The study was conducted in accordance with the International Conference on Harmonization Guidelines for Good Clinical Practice and the Declaration of Helsinki.

Data and code Availability

Anonymized raw sequencing data and counts matrix have been deposited in the Gene Expression Omnibus (GEO) with accession numbers GEO: GSE239626. The code used for the analysis can be shared on request.

Author Contributions:

M.G conducting experiments, acquiring data, analyzing data, writing and reviewing the manuscript; A.L acquiring, analyzing data and reviewing the manuscript; M.R analyzing data; S.K reviewing the manuscript; A.A reviewing the manuscript; S.S reviewing the manuscript; B.E reviewing the manuscript; P.M reviewing the manuscript; E.T designed research studies, writing and reviewing the manuscript.

Acknowledgments:

This research was supported by Agence Nationale de la Recherche N° ANR-19-CE14-0043, the Swiss National Science Foundation (SNSF grant n° 310030E_189312), and CHU Nîmes.

We thank Brigitte Lafont, Hanane Agherbi for logistic assistance and the ressource biological center from Nîmes University Hospital and Nantes University Hospital for providing PBMC samples.

We thank Amélie Sarrazin, Myriam Boyer-Clavel and Stéphanie Viala from MRI platform for technical assistance in cell sorting experiments.

We thank Laurent Journot, Dany Severac, Simon George from MGX platform for CITE-seq experiments and technical assistance. MGX acknowledges financial support from France

Génomique National infrastructure, funded as part of “Investissement d’avenir” program managed by Agence Nationale pour la Recherche (N° ANR-10-INBS-09).

We thank Christelle Reynes, Manuela Pastore and Lea Besnard from STATABIO platform for statistical R scripts and technical assistance.

Flow cytometry for sort experiments in the validation step were performed at INFINITY INSERM UMR1291 core facility connected to the ‘Toulouse Réseau Imagerie’ network. We thank Anne-Laure Iscache and Hugo Garnier for technical assistance.

We thank Sandra Espeout-Fois and Aurore Vernet from CIRAD for technical assistance in HT-qPCR experiments.

References:

1. International Multiple Sclerosis Genetics Consortium, Patsopoulos NA, Baranzini SE, Santaniello A, Shoostari P, Cotsapas C, et al. Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. Science. 27 sept 2019;365(6460):eaav7188.

2. Ramagopalan SV, Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. The Lancet Neurology. juill 2010;9(7):727-39.

3. Thouvenot E, Orsini M, Daures JP, Camu W. Vitamin D is associated with degree of disability in patients with fully ambulatory relapsing-remitting multiple sclerosis. Eur J Neurol. mars 2015;22(3):564-9.

4. Ramagopalan SV, Heger A, Berlanga AJ, Maugeri NJ, Lincoln MR, Burrell A, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: Associations with disease and evolution. Genome Research. 1 oct 2010;20(10):1352-60.

5. Galoppin M, Kari S, Soldati S, Pal A, Rival M, Engelhardt B, et al. Full spectrum of vitamin D immunomodulation in multiple sclerosis: mechanisms and therapeutic implications. Brain Communications. 4 juill 2022;4(4):fcac171.

6. Hupperts R, Smolders J, Vieth R, Holmøy T, Marhardt K, Schluep M, et al. Randomized trial of daily high-dose vitamin D_3 in patients with RRMS receiving subcutaneous interferon β-1a. Neurology. 8 oct 2019;10.1212/WNL.0000000000008445.

7. Camu W, Lehert P, Pierrot-Deseilligny C, Hautecoeur P, Besserve A, Jean Deleglise AS, et al. Cholecalciferol in relapsing-remitting MS: A randomized clinical trial (CHOLINE). Neurol Neuroimmunol Neuroinflamm. sept 2019;6(5):e597.

8. Cassard SD, Fitzgerald KC, Qian P, Emrich SA, Azevedo CJ, Goodman AD, et al. High-dose vitamin D3 supplementation in relapsing-remitting multiple sclerosis: a randomised clinical trial. eClinicalMedicine. mai 2023;59:101957.

9. Shevtsov A, Raevskiy M, Stupnikov A, Medvedeva Y. In Silico Drug Repurposing in Multiple Sclerosis Using scRNA-Seq Data. IJMS. 4 janv 2023;24(2):985.

10. Kim D, Witt EE, Schubert S, Sotirchos E, Bhargava P, Mowry EM, et al. Peripheral T-Cells, B-Cells, and Monocytes from Multiple Sclerosis Patients Supplemented with High-Dose Vitamin D Show Distinct Changes in Gene Expression Profiles. Nutrients. 9 nov 2022;14(22):4737.

11. Stoeckius M, Hafemeister C, Stephenson W, Houck-Loomis B, Chattopadhyay PK, Swerdlow H, et al. Simultaneous epitope and transcriptome measurement in single cells. Nat Methods. sept 2017;14(9):865-8.

12. Huang Y, Chen L, Zhou Y, Liu H, Yang J, Liu Z, et al. UXT-V1 protects cells against TNF-induced apoptosis through modulating complex II formation. Heldin CH, éditeur. MBoC. 15 avr 2011;22(8):1389-97.

13. Chang LS, Wang JT, Doong SL, Lee CP, Chang CW, Tsai CH, et al. Epstein-Barr Virus BGLF4 Kinase Downregulates NF-κB Transactivation through Phosphorylation of Coactivator UXT. J Virol. 15 nov 2012;86(22):12176-86.

14. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H, et al. Distinct Effector Cytokine Profiles of Memory and Naive Human B Cell Subsets and Implication in Multiple Sclerosis. The Journal of Immunology. 15 mai 2007;178(10):6092-9.

15. Li R, Rezk A, Miyazaki Y, Hilgenberg E, Touil H, Shen P, et al. Proinflammatory GM-CSF–producing B cells in multiple sclerosis and B cell depletion therapy. Sci Transl Med [Internet]. 21 oct 2015 [cité 28 juill 2023];7(310). Disponible sur: https://www.science.org/doi/10.1126/scitranslmed.aab4176

16. Krensky AM, Clayberger C. Biology and clinical relevance of granulysin. Tissue Antigens. mars 2009;73(3):193-8.

17. Deng A, Chen S, Li Q, Lyu S chen, Clayberger C, Krensky AM. Granulysin, a Cytolytic Molecule, Is Also a Chemoattractant and Proinflammatory Activator. The Journal of Immunology. 1 mai 2005;174(9):5243-8.

18. Afsal K, Selvaraj P, Harishankar M. 1, 25-dihydroxyvitamin D3 downregulates cytotoxic effector response in pulmonary tuberculosis. International Immunopharmacology. sept 2018;62:251-60.

19. Palau N, Julià A, Ferrándiz C, Puig L, Fonseca E, Fernández E, et al. Genome-wide transcriptional analysis of T cell activation reveals differential gene expression associated with psoriasis. BMC Genomics. déc 2013;14(1):825.

20. Chauss D, Freiwald T, McGregor R, Yan B, Wang L, Nova-Lamperti E, et al. Autocrine vitamin D signaling switches off pro-inflammatory programs of TH1 cells. Nat Immunol [Internet]. 11 nov 2021 [cité 23 nov 2021]; Disponible sur: https://www.nature.com/articles/s41590-021-01080-3

21. Liu G, Li F, Chen M, Luo Y, Dai Y, Hou P. SNRPD1/E/F/G Serve as Potential Prognostic Biomarkers in Lung Adenocarcinoma. Front Genet. 3 mars 2022;13:813285.

22. Luo Y, Xu C, Wang B, Niu Q, Su X, Bai Y, et al. Single-cell transcriptomic analysis reveals disparate effector differentiation pathways in human Treg compartment. Nat Commun. 23 juin 2021;12(1):3913.

23. Caldwell RB, Braselmann H, Schoetz U, Heuer S, Scherthan H, Zitzelsberger H. Positive Cofactor 4 (PC4) is critical for DNA repair pathway re-routing in DT40 cells. Sci Rep. 4 juill 2016;6(1):28890.

24. Hanel A, Carlberg C. Time-Resolved Gene Expression Analysis Monitors the Regulation of Inflammatory Mediators and Attenuation of Adaptive Immune Response by Vitamin D. IJMS. 14 janv 2022;23(2):911.

25. Häusler D, Torke S, Peelen E, Bertsch T, Djukic M, Nau R, et al. High dose vitamin D exacerbates central nervous system autoimmunity by raising T-cell excitatory calcium. Brain. 1 sept 2019;142(9):2737-55.

26. Krumbholz M, Theil D, Cepok S, Hemmer B, Kivisäkk P, Ransohoff RM, et al. Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. Brain. 1 janv 2006;129(1):200-11.

27. Killick J, Hay J, Morandi E, Vermeren S, Kari S, Angles T, et al. Vitamin D/CD46 Crosstalk in Human T Cells in Multiple Sclerosis. Front Immunol. 24 nov 2020;11:598727.

28. Bendix M, Greisen S, Dige A, Hvas CL, Bak N, Jørgensen SP, et al. Vitamin D increases programmed death receptor-1 expression in Crohn's disease. Oncotarget. 11 avr 2017;8(15):24177-86.

29. Dardalhon V, Schubart AS, Reddy J, Meyers JH, Monney L, Sabatos CA, et al. CD226 Is Specifically Expressed on the Surface of Th1 Cells and Regulates Their Expansion and Effector Functions. The Journal of Immunology. 1 août 2005;175(3):1558-65.

30. Zhang R, Zeng H, Zhang Y, Chen K, Zhang C, Song C, et al. CD226 ligation protects against EAE by promoting IL-10 expression *via* regulation of CD4+ T cell differentiation. Oncotarget. 12 avr 2016;7(15):19251-64.

31. Leandro MJ. B-cell subpopulations in humans and their differential susceptibility to depletion with anti-CD20 monoclonal antibodies. Arthritis Res Ther. 2013;15(Suppl 1):S3.

32. Gaglia MM. Anti-viral and pro-inflammatory functions of Toll-like receptors during gamma-herpesvirus infections. Virol J. déc 2021;18(1):218.

33. Ersing I, Bernhardt K, Gewurz B. NF-κB and IRF7 Pathway Activation by Epstein-Barr Virus Latent Membrane Protein 1. Viruses. 21 juin 2013;5(6):1587-606.

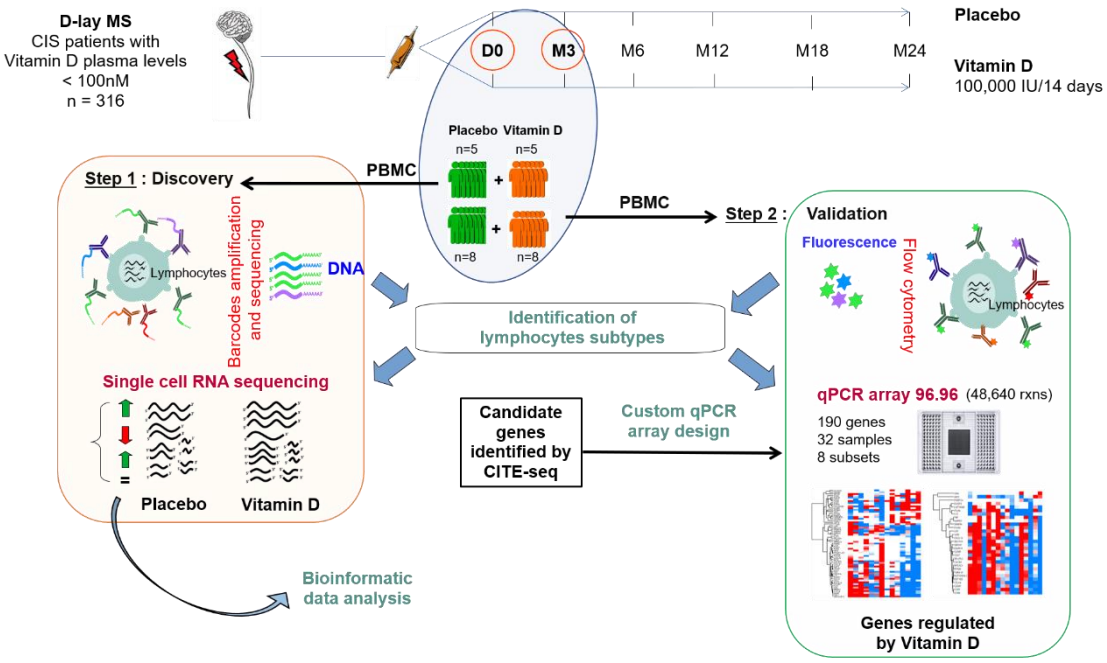
34. Hoepner R, Bagnoud M, Pistor M, Salmen A, Briner M, Synn H, et al. Vitamin D increases glucocorticoid efficacy via inhibition of mTORC1 in experimental models of multiple sclerosis. Acta Neuropathol. sept 2019;138(3):443-56.

35. Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Ann Neurol. déc 2005;58(6):840-6.

36. Swanton JK, Rovira A, Tintore M, Altmann DR, Barkhof F, Filippi M, et al. MRI criteria for multiple sclerosis in patients presenting with clinically isolated syndromes: a multicentre retrospective study. The Lancet Neurology. août 2007;6(8):677-86.

37. Bibby JA, Agarwal D, Freiwald T, Kunz N, Merle NS, West EE, et al. Systematic single-cell pathway analysis to characterize early T cell activation. Cell Reports. nov 2022;41(8):111697.

Figures and figure Legends :



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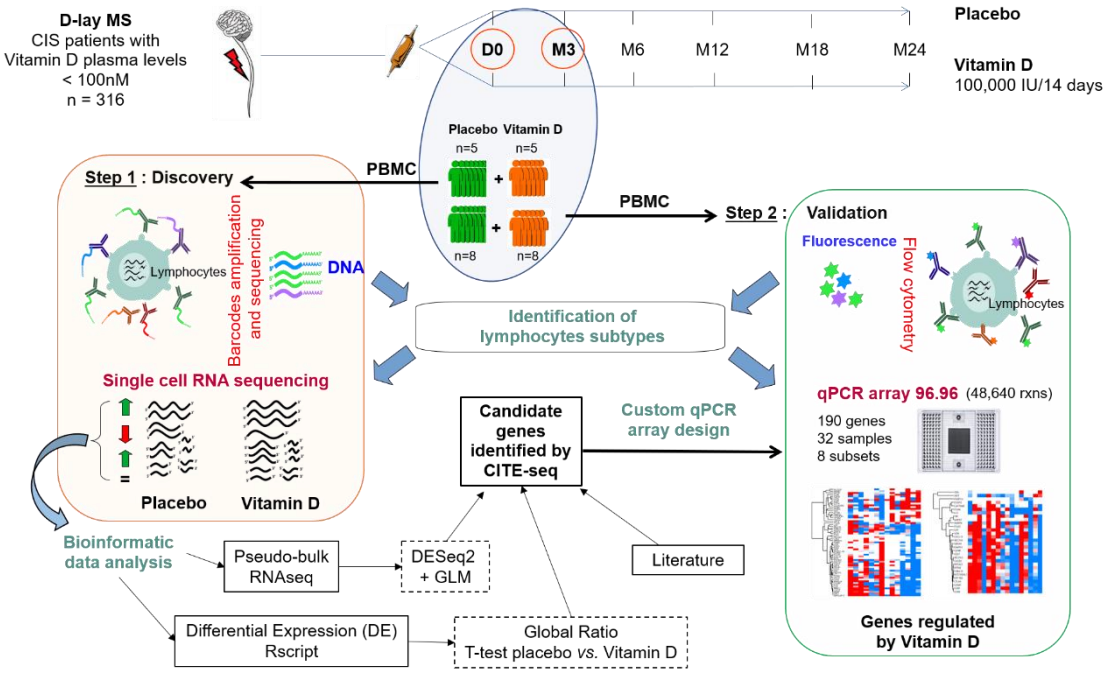


Figure 1. Overview of the study design and analysis method.

	Discovery			Validation		
	Placebo group (n = 5)	Vitamin D group (n = 5)	p-value between groups	Placebo group (n = 8)	Vitamin D group (n = 8)	p-value between groups
Age, y, mean (SD)	35.8 (10.3)	34.6 (7.70)	0.881	40.6 (8.62)	37.4 (8.83)	0.458
Women, %	80.0	80.0	> 0.999	87.5	50	0.282
Conversion rate (%)	100	100	> 0.999	62.5	50	> 0.999
Days from last steroid infusion (SD)	35.7 (8.08)	42.7 (20.5)	0.600	42.3 (22.6)	30.3 (15.9)	0.394
Serum 25(OH)D ng/mL, mean (SD) D0	56.8 (29.2)	58.4 (14.0)	0.841	56.4 (23.8)	46.7 (14.3)	0.487
Serum 25(OH)D ng/mL, mean (SD) M3	52.6 (26.6)	155 (21.1)	0.0079	53.8 (18.0)	143.3 (17.5)	0.0002
p-value serum 25(OH)D D0 *vs.* M3	> 0.999	0.0079		0.898	0.0002	

Table 1. Demographic characteristics of MS patients included in discovery and validation cohorts.

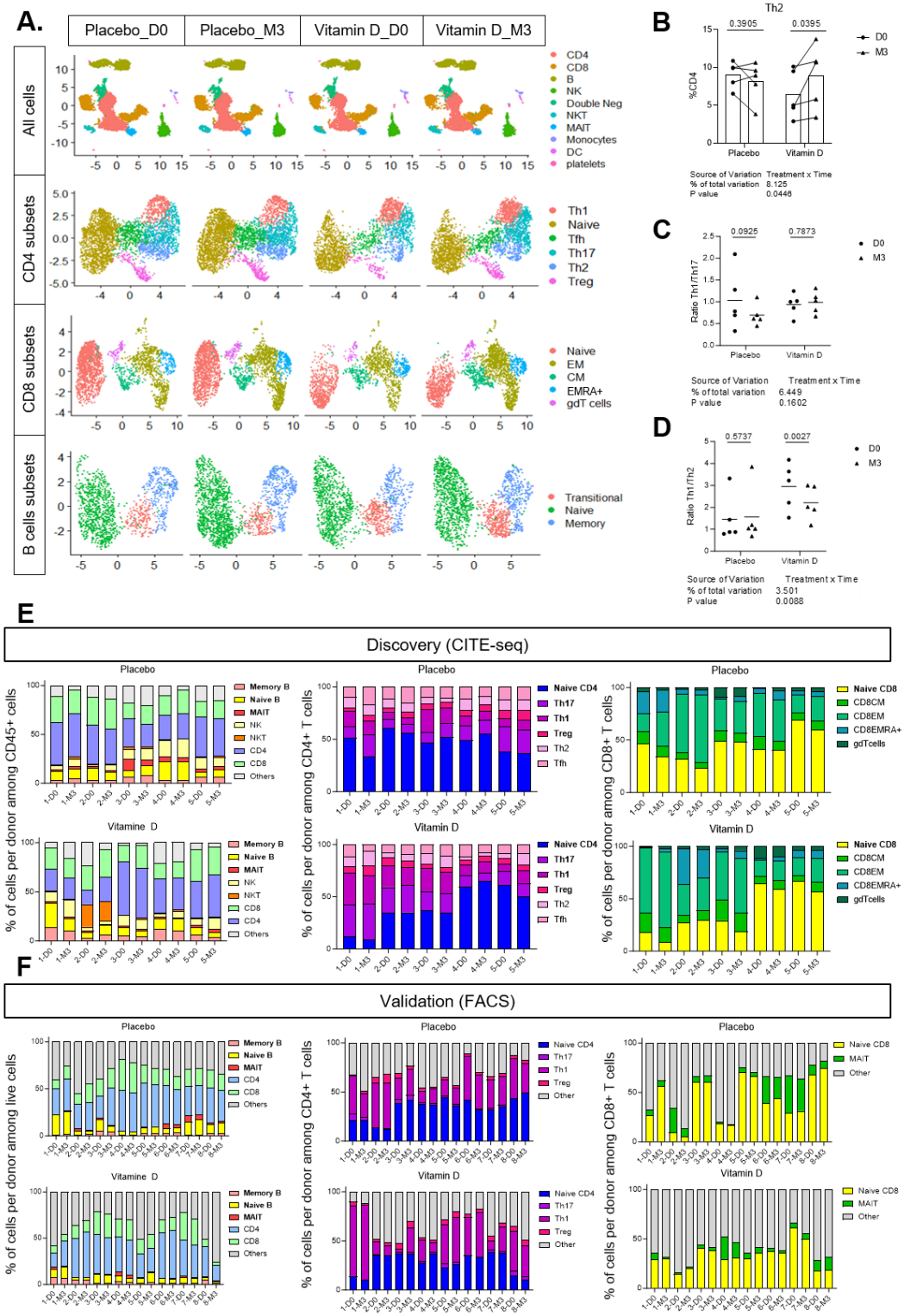


Figure 2. Characterization of circulating lymphocytes subsets in PBMCs from MS patients using CITE-seq.

(A) UMAP and clustering of different lymphocytes subsets of PBMCs. NK, natural killer; NKT, natural killer T-cells; MAIT, mucosal-associated invariant T-cells; EM, effector memory; CM, central memory, EMRA+, terminally differentiated effector memory cells re-expressing CD45RA.

(B) Change in the proportion of Th2 cells before/after treatment in the placebo and vitamin D groups.

(C and D) The Th1/Th17 and Th1/Th2 cell ratio were expressed as the ratio of the percentage of Th1 and Th17 or Th2 subsets in each patient.

(E) Proportion of each lymphocyte subset before and after Vitamin D or placebo treatment in the discovery step.

(F) Proportion of each lymphocyte subset before and after Vitamin D or placebo treatment in the validation step.

Significance was determined by 2way-ANOVA (B, C, D) with original FDR BH correction.

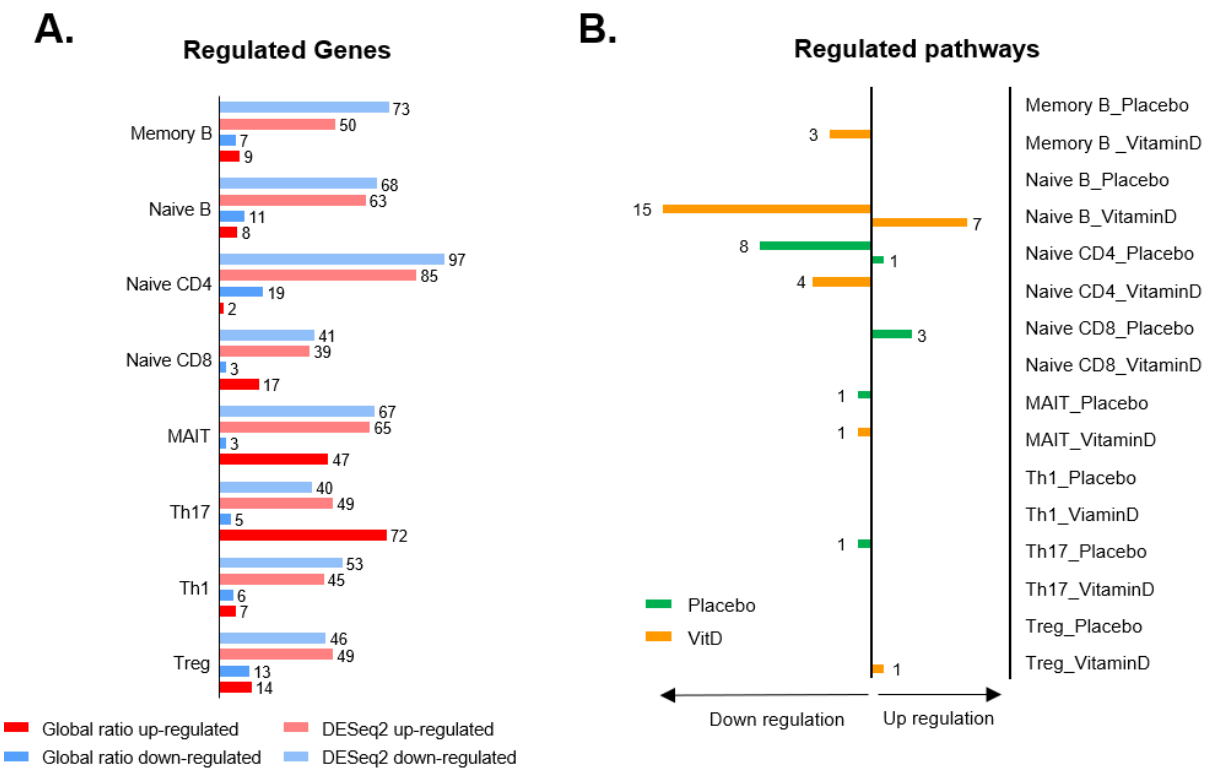


Figure 3. Regulation of gene expression and pathways by 3-months placebo or vitamin D treatment.

(A) Number of differentially expressed genes (DEGs, $p<0.05$) in lymphocytes subsets according to the method used for analysis (global ratio or DESeq2 analysis).

(B) Number of significantly overrepresented and underrepresented pathways ($p<0.05$) from the Reactome Pathway Database in lymphocytes subsets assessed by Single Cell Pathway Analysis (SCPA) Package from RStudio.

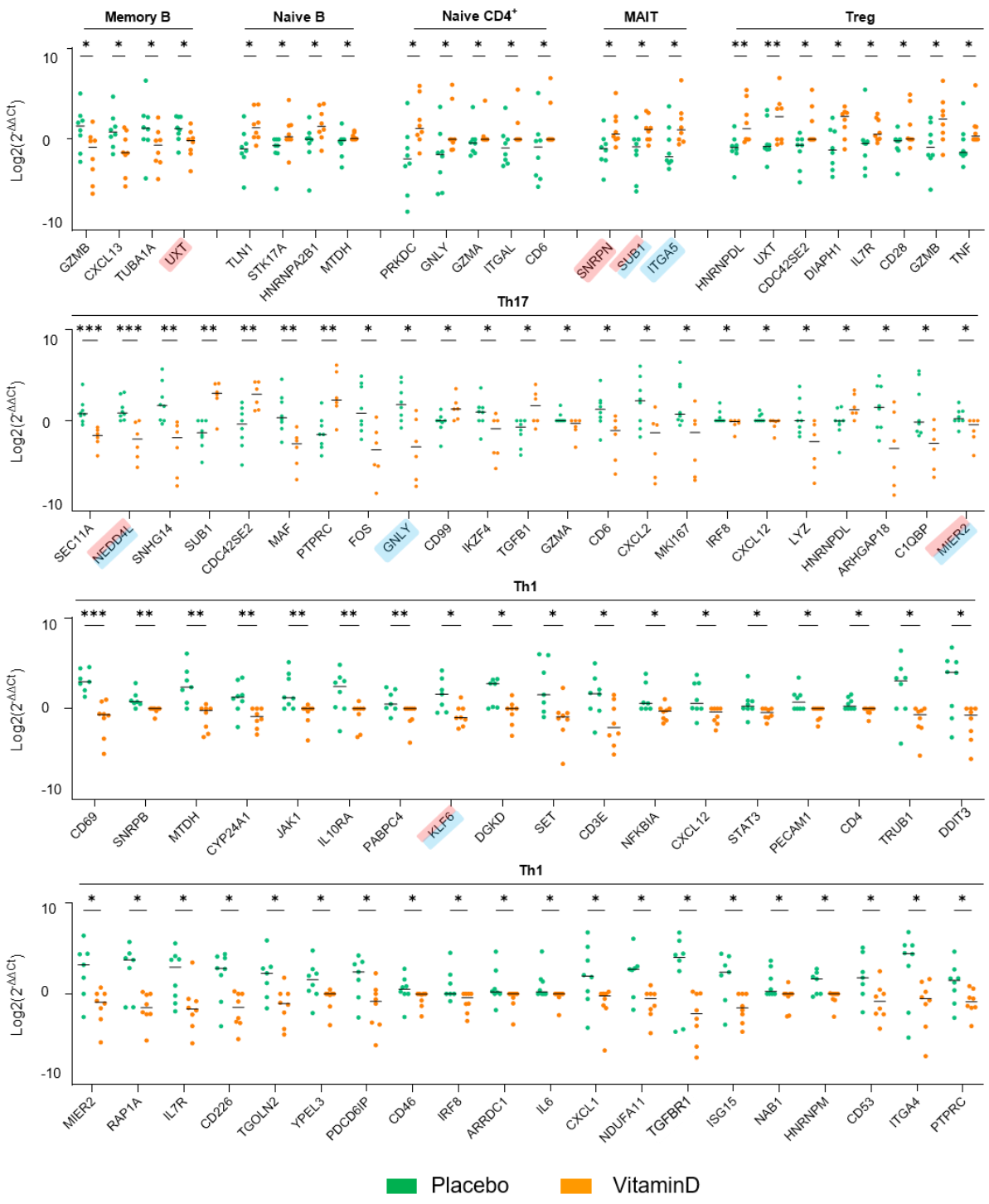


Figure 4. Validation of candidate genes by high-throughput qPCR in lymphocyte subset.

Genes identified by the global ratio method are highlighted in red. Genes identified with the DESeq2 method are highlighted in blue. Expression of each gene is plotted as Log2($2^{-\Delta\Delta Ct}$). Significance was determined by multiple Mann-Whitney tests, *p < 0.05, **p < 0.01, ***p<0.001.

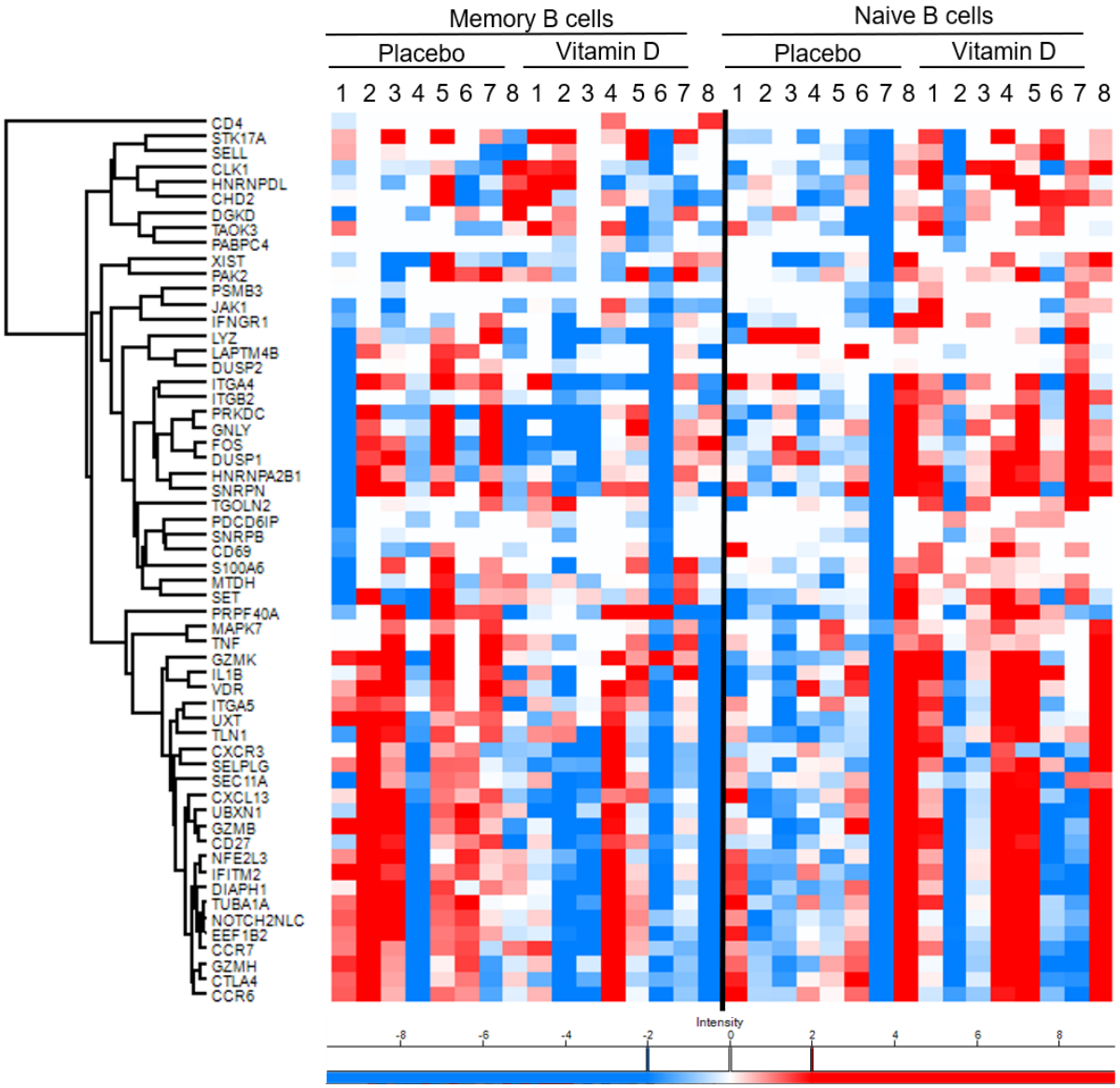


Figure 5. Heat-map of the DEGs (p<0.20) between memory and naive B-cells.

For each group (vitamin D and placebo), eight patients (1 to 8) of the validation cohort are represented. Heat-map was performed using Perseus® (Max-Planck-Institute of Biochemistry, Martinsried, Germany, version 2.0.7.0). A gradient coloring scheme was applied to display upregulated (above 2-fold expression (in log2 display above 1, red color) *vs.* downregulated (below 2-fold expression (in log2 display below -1, blue color) genes. White color nodes represent changes of non-modulated genes. Heatmap was hierarchically clustered by Pearson's correlation distance using row average linkage.

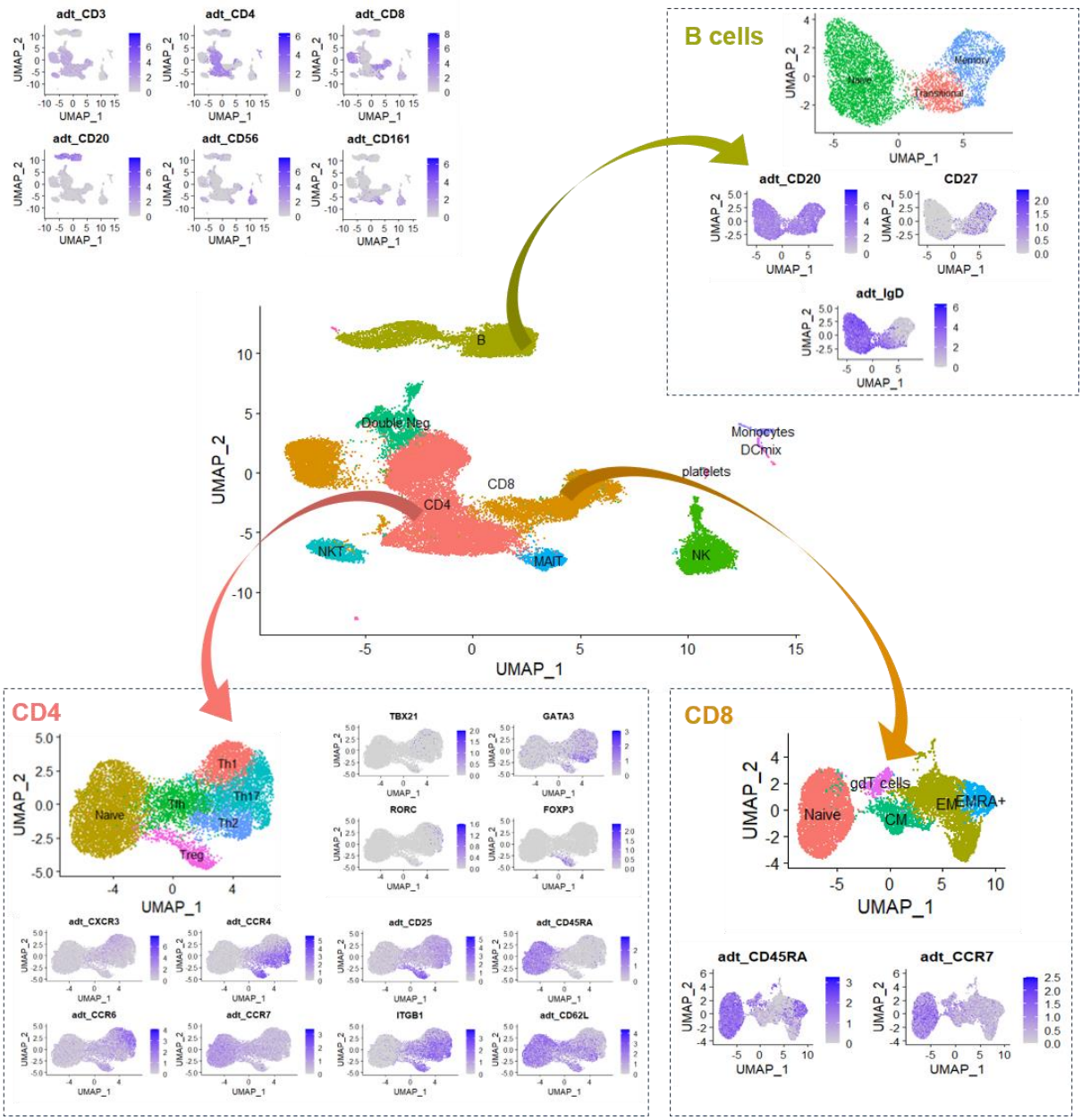
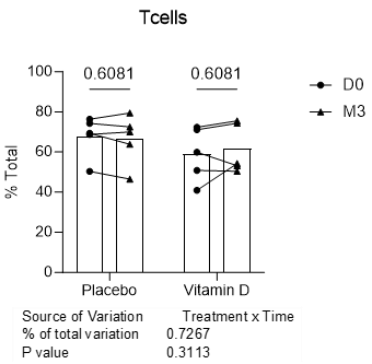
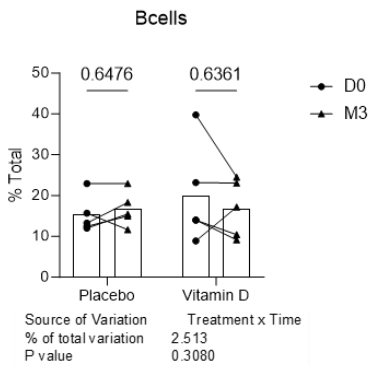


Figure Sup 1. **Annotation of Seurat clusters using a combination of surface markers and gene expression and visual inspection of key markers using UMAP visualization.**

Expression of characteristic surface markers and gene expression was used to annotate major PBMC populations including $CD4^+$ T-cells ($CD4^+$), $CD8^+$ T-cells ($CD8^+$), B-cells ($CD19^+$), natural killer (NK) cells ($CD56^+$), natural killer T-cells (NKT; $CD3^+CD56^+$), MAIT cells ($CD161^+$), monocytes, platelets, and dendritic cells (DCs). $CD4^+$ subsets were further annotated with a combination of surface markers and gene expression including $CD45RA^+$ $CCR7^+$ (naive), $CXCR3^+CCR6^-TBX21^+$ (Th1), $CXCR3^-CCR6^-CCR4^+GATA3^+$ (Th2), $CXCR3^-CCR6^+RORC^+$ (Th17), $CD45RA^-CXCR5^+ICOS$ (Tfh), $CD45RA^-CD25^+FOXP3^+$ (Treg). $CD8^+$ T cells subsets were annotated with $CD45RA^+$ $CCR7^+$ (naive), $CD45RA^-$ $CCR7^+$ (central memory, CM), $CD45RA^-CCR7^-$ (effector memory, EM), $CD45RA^+CCR7^-$ (effector, $EMRA^+$), and $\gamma\delta TCR^+$ ($\gamma\delta$ T cells). B-cells subsets were annotated using $CD27^-IgD^+$ (naïve), $CD27^+IgD^+$ (transitional) and $CD27^+IgD^-$ (memory).


Tcells
0.6081
0.6081
D0
M3
% Total
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
0.7267
P value
0.3113


Bcells
0.6476
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M3
% Total
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
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P value
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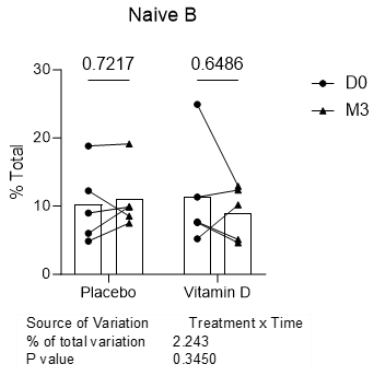
Naive B
0.7217
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D0
M3
% Total
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
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P value
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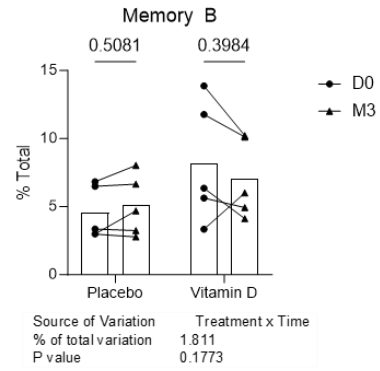
Memory B
0.5081
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D0
M3
% Total
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
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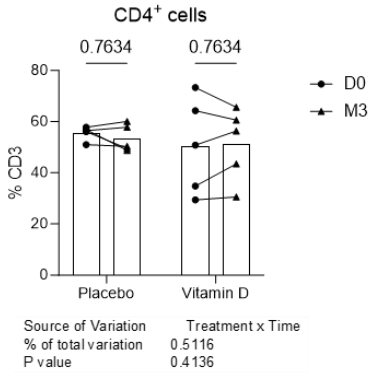
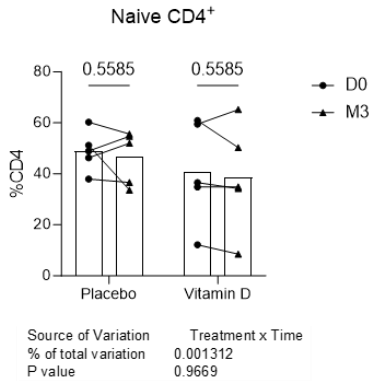
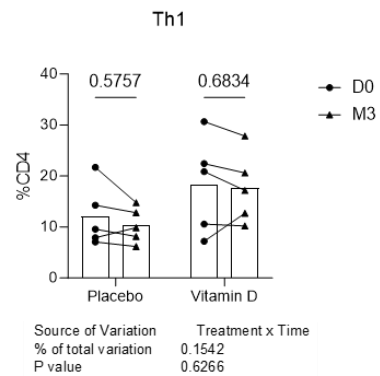
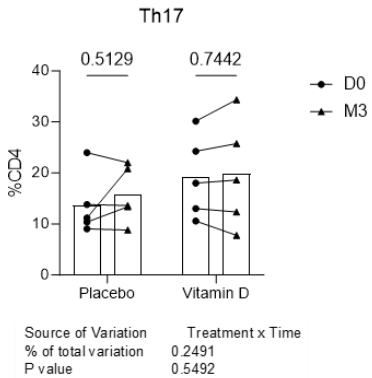
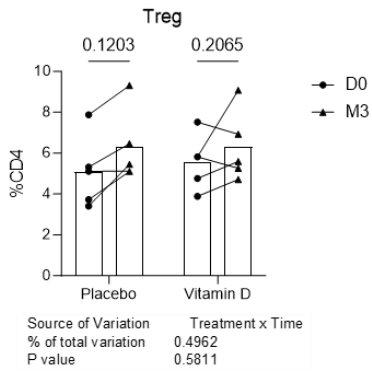
P value
0.1773


CD4+ cells
0.7634
0.7634
D0
M3
% CD3
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
0.5116
P value
0.4136


Naive CD4+
0.5585
0.5585
D0
M3
%CD4
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
0.001312
P value
0.9669


Th1
0.5757
0.6834
D0
M3
%CD4
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
0.1542
P value
0.6266


Th17
0.5129
0.7442
D0
M3
%CD4
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
0.2491
P value
0.5492


Treg
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D0
M3
%CD4
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
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P value
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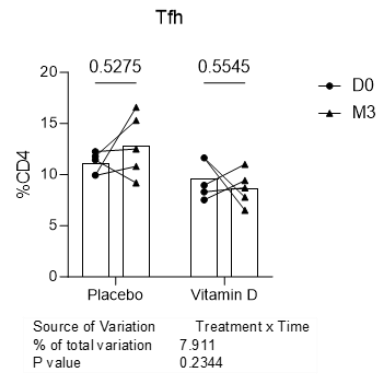
Tfh
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M3
%CD4
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
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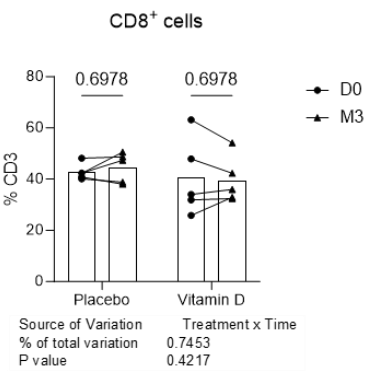
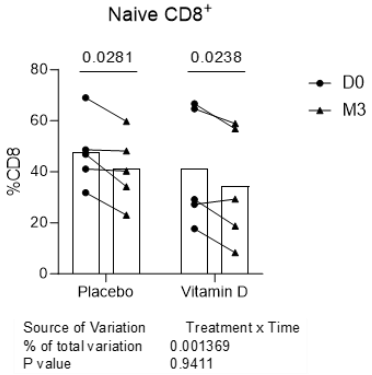
P value
0.2344


CD8+ cells
0.6978
0.6978
D0
M3
% CD3
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
0.7453
P value
0.4217


Naive CD8+
0.0281
0.0238
D0
M3
%CD8
Placebo
Vitamin D
Source of Variation
Treatment x Time
% of total variation
0.001369
P value
0.9411

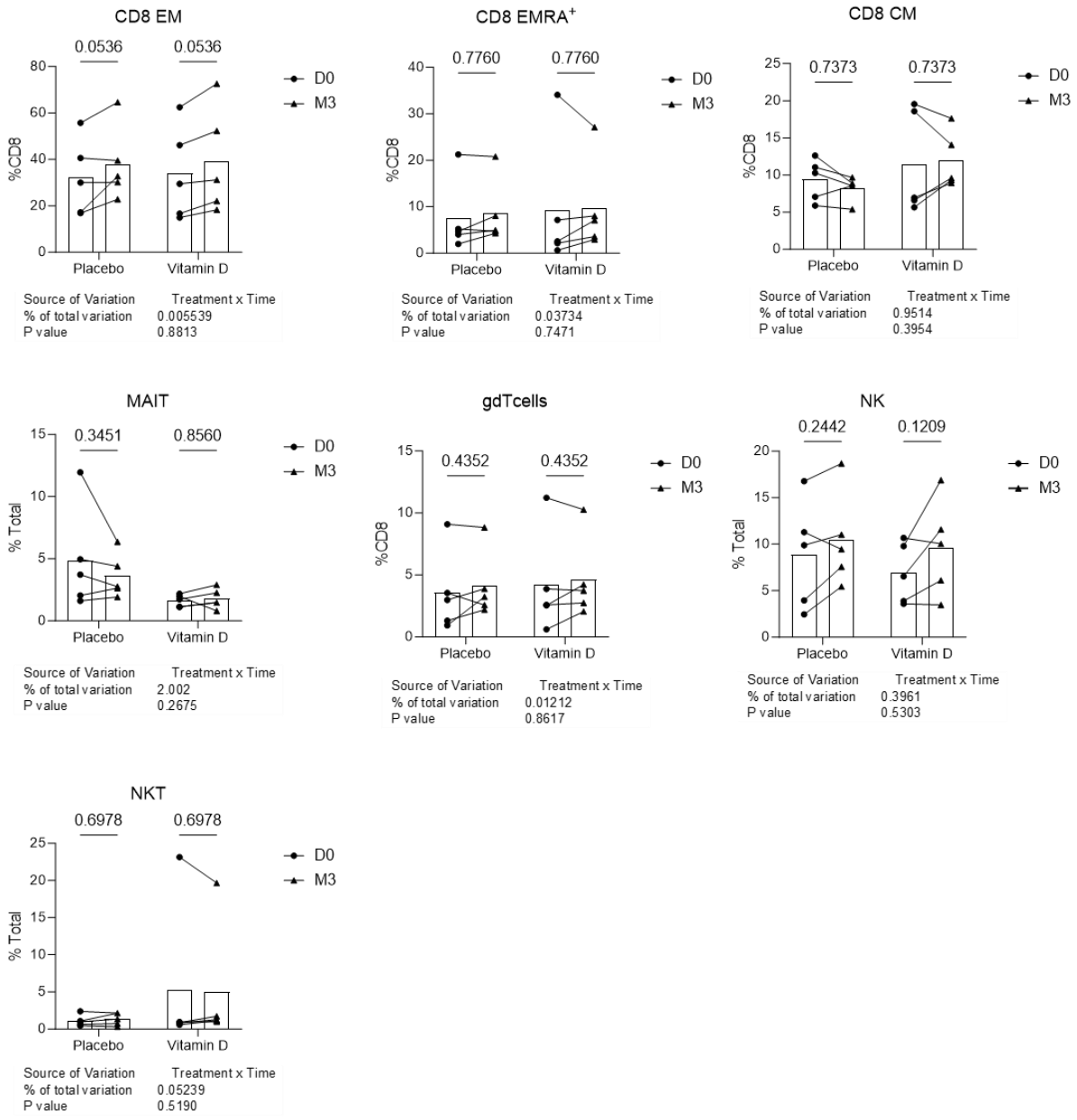
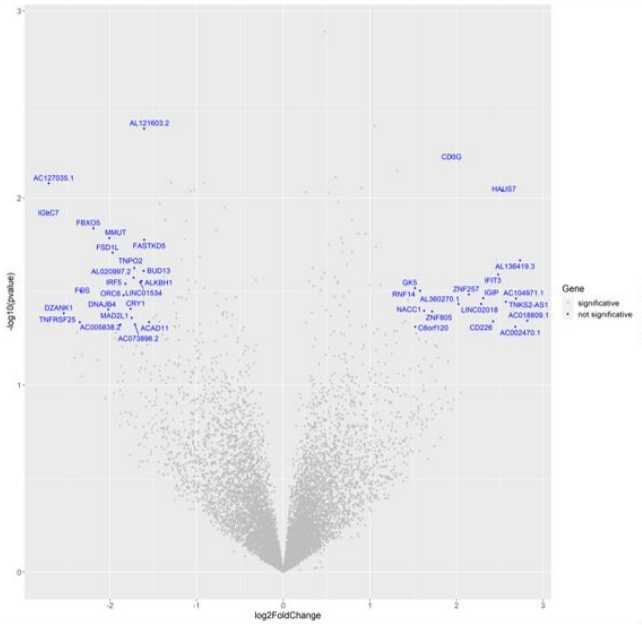


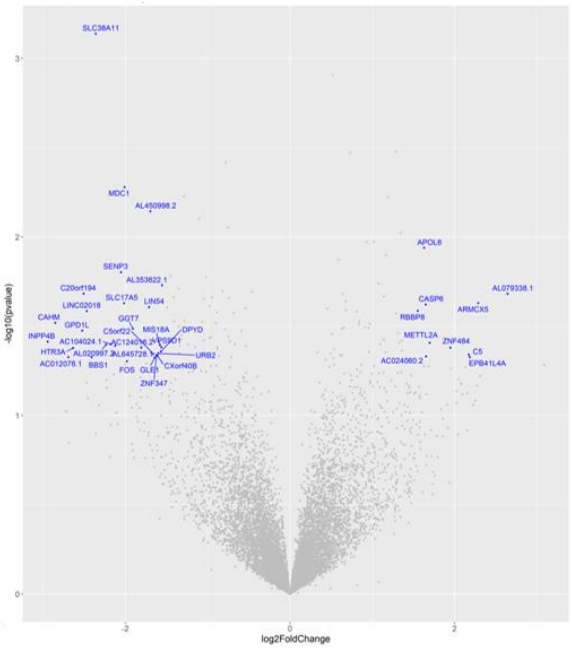
Figure Sup 2. Changes in the proportions of lymphocytes subsets after treatment with placebo or vitamin D.

Significance was determined by 2way-ANOVA with original FDR BH correction.

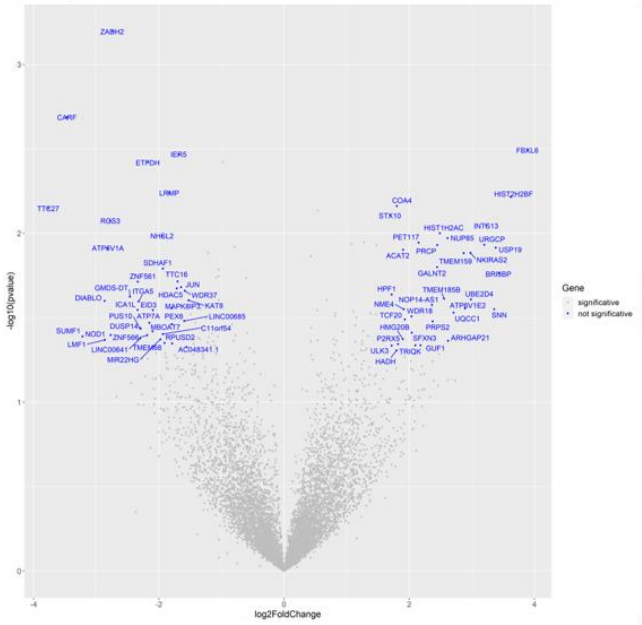
Naive B-cells



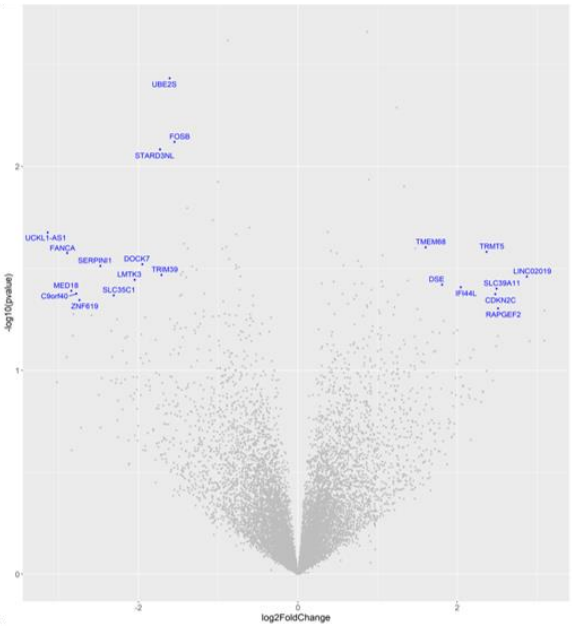
Memory B-cells



MAIT cells



Naive CD8 T-cells



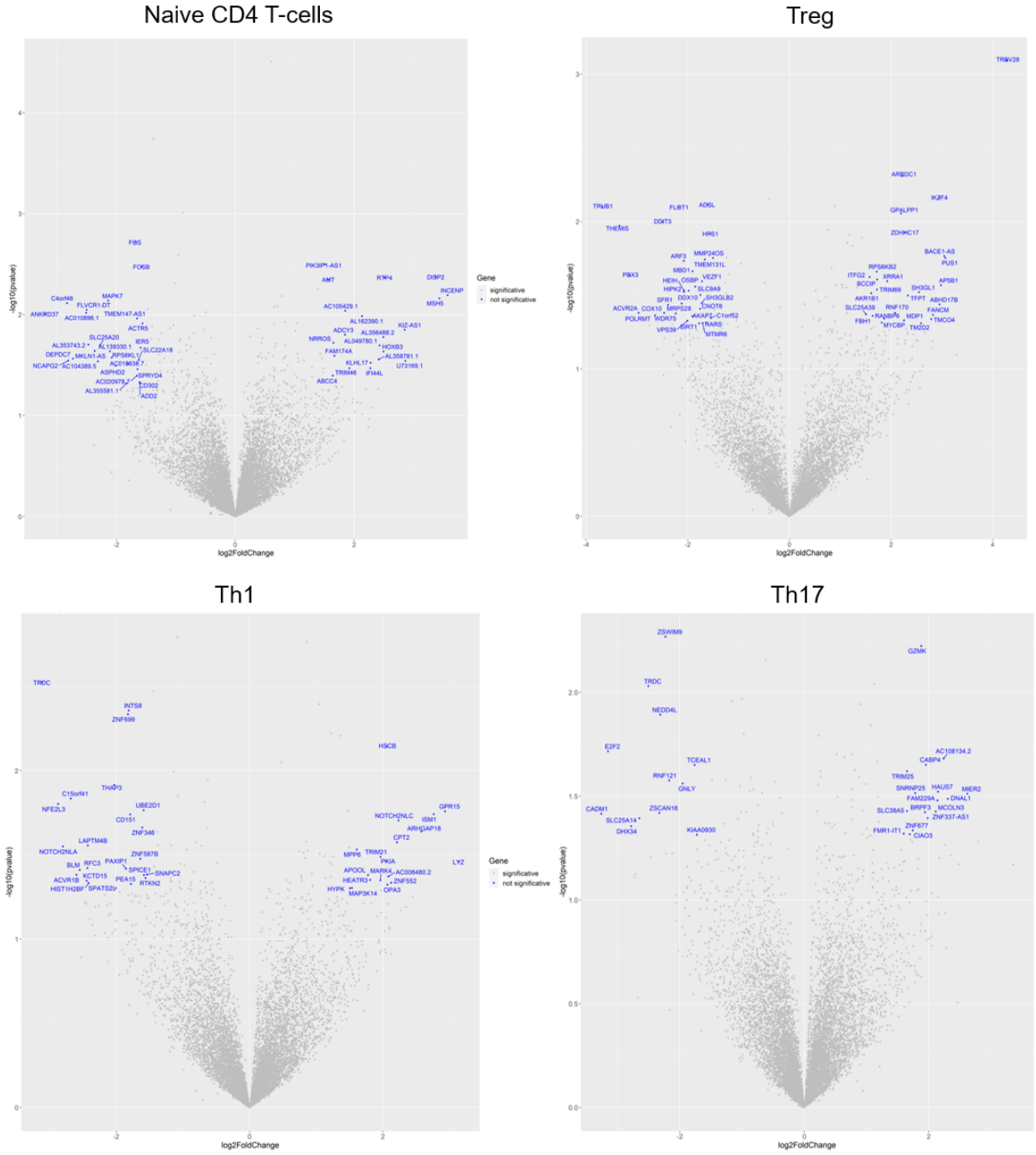


Figure Sup 3. Volcano plot representation of regulated genes identified by DESeq2 analysis.

Antibody	Clone	CITE-seq Barcode
CCR4	L291H4	AGCTTACCTGCACGA
CCR5	J418F1	CCAAAGTAAGAGCCA
CCR6	G034E3	GATCCCTTTGTCACT
CCR7	G043H7	AGTTCAGTCAACCGA
CD11a	TS2/4	TATATCCTTGTGAGC
CD127	A019D5	GTGTGTTGTCCTATG
CD138	MI15	ACTCTTTCGTTTACG
CD161	HP-3G10	GTACGCAGTCCTTCT
CD20	2H7	TTCTGGGTCCCTAGA
CD226	11A8	TCTCAGTGTTTGTGG
CD24	ML5	AGATTCCTTCGTGTT
CD25	BC96	TTTGTCCTGTACGCC
CD27	O323	GCACTCCTGCATGTA
CD3	SK7	TATCCCTTGGGATGG
CD38	HIT2	TGTACCCGCTTGTGA
CD4	RPA-T4	TGTTCCCGCTCAACT
CD45RA	HI100	TCAATCCTTCCGCTT
CD49b	P1E6-C5	GCTTTCTTCAGTATG
CD49d	9F10	CCATTCAACTTCCGG
CD56	5.1H11	TCCTTTCCTGATAGG
CD62L	DREG-56	GTCCCTGCAACTTGA
CD71	CY1G4	CCGTGTTCCTCATTA
CD8	SK1	GCGCAACTTGATGAT
CTLA-4	BNI3	ATGGTTCACGTAATC
CXCR3	G025H7	GCGATGGTAGATTAT
CXCR4	12G5	TCAGGTCCTTTCAAC
CXCR5	J252D4	AATTCAACCGTCGCC
GITR	108-17	ACCTTTCGACACTCG
HLA-DR	L243	AATAGCGAGCAAGTA
ICOS	C398.4A	CGCGCACCCATTAAA
IgD	IA6-2	CAGTCTCCGTAGAGT
IgM	MHM-88	TAGCGAGCCCGTATA
LAG3	11C3C65	CATTTGTCTGCCGGT
TCRVa7.2	3C10	TACGAGCAGTATTCA
TCRγ/δ	B1	CTTCCGATTCATTCA

Table Sup 1. Total Seq-B antibodies used for CITE-seq (human, BioLegend).

Gene symbol	Accession number	Forward Primer	Reverse Primer
ACTB	NM_001101.3	CCAACCGCGAGAAGATGAC	TAGCACAGCCTGGATAGCAA
RPLP0	NM_053275.3	GCGACCTGGAAGTCCAAC	CACATTGTCTGCTCCCACAA
UBE2D2	NM_181838.1	ACAGTGGTCTCCAGCACTAA	GGATCATCTGGATTGGGATCAC
HPRT1	NM_000194.2	GCTTTCCTTGGTCAGGCAGTA	ACTTCGTGGGGTCCTTTTCAC
UBC	NM_021009.	GGGGTGTCTAAGTTTCCCCTT	GGGAATGCAACAACTTTATTGAAAGG
RPS18	NM_022551.2	CGGAAAATAGCCTTTGCCATCA	TCCTCAACACCACATGAGCATA
PPIA	NM_001300981.1	TCTGGTTCCTTCTGCGTGAA	CCAGGGAATACGTAACCAGACA
GAPDH	NM_002046.	GAGAACGGGAAGCTTGTCATCA	TGGACTCCACGACGTACTCA
POLR2F	NM_001301129.	AGCGAATCACCACACCATACA	ACCATCACAGGGGCACAC
RPL10	NM_001303625.	TGCTCATGAGGCAGCAAAC	GAGGTCCAAGTCAAGCTCCA
IPO8	NM_001190995.1	TTCAGTGCAAAGGAAGGGGAA	ACCCCTCGAGTTAATCTCTCCA
TFRC	NM_001128148.1	CCAGTTGGAGTGCTGGAGAC	AGCCTTTAAATGCAGGGACGAA
ALCAM	NM_002228.3	AAGAACTCGGACCTCCTCAC	TGGATTATCAGGCGCTCCA
ARHGAP18	NM_033515.N	TACCAGGAATGCGAATACCC	AAGAGGCCTTCTGTTTCCAA
ARRDC1	NM_001317968.N	CCTGAACAGCATCCCAGACA	CCCGTCTTCACCAGCTTGTA
BCL2	NM_000633.2	ATGTGTGTGGAGAGCGTCAA	GTGCCGGTTCAGGTACTCA
C1QBP	NM_001212.N	GGGCAGAAGGTTGAAGAACA	GGCCTTCTTGCCATCATCA
CADM1	NM_001301043.N	CAGACCCAGCGGTATCTAGAA	TTCCCGGGTTAAGCCTTGTA
CAST	NM_001190442.1	TGTCCCTCCACTACAGAAACC	TAGGTGCTTTGGAGCTGGAA
CCL2	NM_002982.3	TAGCAGCCACCTTCATTCCC	CCTCTGCACTGAGATCTTCCTA
CCR4	NM_005508.	TGCCTCACAGACCTTCCTCA	AGGCTCCTCAAGGCAGGT
CCR5	NM_001100168.1	TGAGACATCCGTTCCCCTACA	TGGCAGGGCTCCGATGTATA
CCR6	NM_004367.5	AGGCAGCGATGTCTGTGAA	AGCTCAAGCCCCAACATCA
CCR7	NM_001301716.1	TGAGGTCACGGACGATTACA	CGTCCTTCTTGGAGCACAAA
CD151	NM_139029.N	ATCGCTGGTATCCTCGCCTA	GCTTGGTCATGGTGTCCTTCA
CD19	NM_001178098.1	GCAACCTGACCATGTCATTCC	TCCAGCCACCAGTCCTCA
CD226	NM_006566.2	CGTGATGAGATTGACTGTAGCC	CTCCAGCCACAAAGAGGGTA
CD27	NM_001242.4	CACTACTGGGCTCAGGGAAA	TGCTGGTCACAGTCCTTCA
CD28	NM_001243077.1	GTGGAGTCCTGGCTTGCTATA	GAGCCTGCTCCTCTTACTCC
CD302	NM_014880.N	TCTACCTAATGGCAGCTCTTCC	GCCTCTTGGCCTGTTGAAAA
CD3E	NM_000733.3	GCAAACCAGAAGATGCGAAC	CATCACATCCATCTCCATGCA
CD4	NM_000616.4	AAAGTTGCATCAGGAAGTGAACC	CCCACACCTCACAGGTCAAA
CD46	NM_172352.2	CCATGGTGTTGCTGCTGTAC	ACCAATGAGCTCCATAGCTTCA
CD53	NM_001040033.	GGCTGCATGGGCTCTATCA	GTCACCTCAGCAAGGAGGATAA
CD6	NM_006725.3	CTGGCGGTTCAACAACTCC	GGAGTGGACAGATTGTGCAAA
CD69	NM_001781.2	TCACCCATGGAAGTGGTCAA	ACACACTTGTCAGACCCTGTA
CD8A	NM_001768.6	CCATCATGTACTTCAGCCACTTCG	GCTGCGACGCGATGGT
CD99	NM_001321367.	TGGCGCTGCTGCTCTT	AGGAAGGGCATCGGATAAATCG
CDC42SE2	NM_020240.N	TCAACTGCTGTATTGCAGAAC	TGTGGGCTCTCCAATCATAC
CDK13	NM_003718.4	AGGCCACCTCCAGAACCTA	TGGCGTTAGCTCCAAGATCC
CHD2	NM_001271.N	GACAGTTCGCTACACAGCAA	ACTGACTGCCTGAGTCTGAA
CLEC2D	NM_013269.4	CAAGCTGCATGCCCAGAAA	TGATGTCCAGTTCTTGGTGTCA
CLK1	NR_027856.N	AGGATTCTTGGACCTCTACCAA	CAGTCTAATCGATCGTGGTGAA
CTLA4	NM_005214.4	CATGGACACGGGACTCTACA	AATCTGGGTTCCGTTGCCTA
CXCL1	NM_001511.2	CTTGCCTCAATCCTGCATCC	AGCCACCAGTGAGCTTCC

CXCL12	NM_199168.3	GCTGGTCCTCGTGCTGAC	GAATCGGCATGGGCATCTGTA
CXCL13	NM_006419.2	GGACCCTCAAGCTGAATGGATA	ACACTGGAACTGGTAGAGTTGAA
CXCL2	NM_002089.3	CCAAAGTGTGAAGGTGAAGTCC	CGATGCGGGGTTGAGACA
CXCR3	NM_001504.1	AACTGTGGCCGAGAAAGCA	TTGAGGCAGCAGTGCATGTA
CYP24A1	NM_001128915.1	CGGGCAGAAGATTTGAGGAA	GTAAATGGTACACTCGGCGTAA
CYP27B1	NM_000785.	CTCGTGTCCCAGACAAAGACA	TCCCTTGAAGTGGCATAGTGAC
CYTIP	NM_004288.4	CCTGATCAGATCGTCCGGAAA	GCTTCAAGCTCCGTTCTTTTCA
DDIT3	NM_001195056.1	GAGCCAAAATCAGAGCTGGAAC	TGCTTTCAGGTGTGGTGATGTA
DGKD	NM_152879.N	CGTAGCTGAATCCAGTACCA	GCACACAAGATGAGCTTCC
DIAPH1	NM_001079812.2	TCCCTTCGTGTGTCTCTCAA	TAAGGAGGCCAAGCCTTCA
DUSP1	NM_004417.3	AGACATCAGCTCCTGGTTCA	CAGTGGACAAACACCCTTCC
DUSP2	NM_004418.N	ACTCCAGGGCTCCTGTCTA	TGCAGGTCTGACGAGTGAC
E2F2	NM_004091.N	TTCAAGCACCTGACTGAGGAC	AGTTGCCAACAGCACGGATA
EEF1B2	NM_001037663.N	AGTGCGTCAGAAGCATTCAA	TCCTCCAGCATATCTGTTCCA
FGL2	NM_006682.2	AGTGTCCCAGCCAAGAACAA	CCTATTGCGTAGTAGTCAGAGCAA
FKBP1A	NM_001199786.N	AAGCGCGGCCAGACCT	TGGCACCATAGGCATAATCTGGAG
FOS	NM_005252.	CTACCACTCACCCGCAGAC	GCAGTGACCGTGGGAATGAA
FOSB	NM_006732.N	CCCTCTGCCGAGTCTCAATA	GAAGGAACCGGGCATTTCC
FOXP3	NM_014009.3	TGTGGGGTAGCCATGGAAA	GGGTCGCATGTTGTGGAA
GC	NM_001204306.	GGCGTTCAGGAAAGATCCAA	ACAGAGGAGCTTGTCCGTAA
GCC2	NM_181453.3	GAGTGACAGCACTACAGGAA	CGGACTTTGTAGCTCTCGAA
GNLY	NM_006433.3	GACCTGTCTGACGATAGTCCAA	GGTCGCAGCATTGGAAACA
GPR15	NM_005290.N	GTGCAAAGGGAGCTCCTACA	TAGCGGTCAACACTCATGCA
GZMA	NM_006144.3	GAAGCCTCCGAGGTGGAA	GAAAACACCCTCGCACAACA
GZMB	NM_004131.4	CCCCATCCAGCCTATAATCCTAA	CTGGGCCTTGTTGCTAGGTA
GZMH	NM_033423.4	CCCCTACATGGCCTTTGTTCA	TGAGCAGCTGTCAGCACAAA
GZMK	NM_002104.2	CACATTTCATCTGGGCTTCTTAAA	GTGACACTTCTTTCCCTCCA
HAVCR2	NM_032782.3	GGATCCAAATCCCAGGCATAA	CTTGGAAAGGCTGCAGTGAA
HDAC2	NR_033441.1	ACCAGAACACTCCAGAATATATGGAA	ACACCAGGTGCATGAGGTAA
HLA-DMB	NM_002118.4	TTCTGGGGATCACTGACCAA	CAGGCTCCCTCGTGTTAAAA
HNRNPA2B1	NM_031243.	TTGAGCCAAAACGTGCTGTA	GCCAACAAACAGCTTCTTCAC
HNRNPDL	NM_031372.2	GGAATTCGCAGAGGGATCCAA	CCCAGCTCAAGCCTCCAATAA
HNRNPM	NM_005968.N	TGGCCAGCTGCTATTTGATA	CTCAGGAGGGAAGAAATCTCC
ICAM1	NM_000201.2	CCCCTACCAGCTCCAGACC	TGCGTGTCCACCTCTAGGAC
IFI44L	NM_006820.N	GTTTTATGGCCACCGTCAGTA	CCTCTTTATGTCGTCTAGGTTATCC
IFITM2	NM_006435.2	ACTGAGAACCATCCCGGTAAC	CCGCTGTTGACAGGAGAGAA
IFNG	NM_000619.2	ACTGCCAGGACCCATATGTAA	GTTCCATTATCCGCTACATCTGAA
IFNgR1	NM_000416.2	AAGCCAGGGTTGGACAAAA	GATATCCAGTTTAGGTGGTCCAA
IKZF1	NM_006060.5	ATACTCCAGATGAGGGCGATG	TCTTGGAGCTTTGCTGTCCT
IKZF4	NM_001351089.N	AACAGCCAGCACTCTTCTCC	GCTTGACTCCTCATCGCTGTA
IL10	NM_000572.2	CCGTGGAGCAGGTGAAGAA	GTCAAACTCACTCATGGCTTTGTA
IL10RA	NM_001558.3	CGCCGAAAGAAGCTACCC	CGCTGGCTGATGAAGATGAA
IL17A	NM_002190.2	ACTACAACCGATCCACCTCAC	ACTTTGCCTCCCAGATCACA
IL17RA	NM_014339.4	CCAAACCACCAGTCCAAGAA	CTCATGCATGGCGTGGTTA
IL1B	NM_000576.2	GACCTGAGCACCTTCTTTCC	CGTGCACATAAGCCTCGTTA
IL2	NM_000586.3	ACCCAGGGACTTAATCAGCAA	GCATATTCACACATGAATGTTGTTTCA

IL2RA	NM_000417.2	TCCTGGGACAACCAATGTCA	GTCACTTGTTTCGTTGTGTTCC
IL6	NM_000600.3	AGAGCTGTGCAGATGAGTACAA	GTTGGGTCAGGGGTGGTTA
IL6R	NM_000565.2	GTAGTGTCGGGAGCAAGTTCA	ATGTTGGCAGGCGGATCA
IL7R	NM_002185.2	GGAGAAAGTGGCTATGCTCAA	CTGCGATCCATTCACTTCCA
IRF8	NM_002163.2	TGGACATTTCCGAGCCATACA	AGCAGTTGCCACGCCTA
ISG15	NM_005101.3	CTGAGAGGCAGCGAACTCA	GCTCAGGGACACCTGGAA
ISM1	NM_080826.N	GCGGGAAGCGAGGAGTTTAA	TCGCTTTTGCAGCTCATCCA
ITGA2	NM_002203.3	TGCCCCGAGCACATCATTTA	CTGATGTCCACACGCAAATCC
ITGA4	NM_000885.	GCCAACGCTTCAGTGATCAA	CAAGTCTTTCCACAAGGTTCTCC
ITGA5	NM_002205.2	ACCCGAATTCTGGAGTATGCA	TGAAGCCTCCTTGGCAGTAA
ITGA6	NM_000210.2	ATATCAGGCTGCCAAATGCA	ACTCCCGAATACTGAGCTACA
ITGAL	NM_001114380.1	CAACAGCAGCAAGCATTTCC	CTTCTCATACACCACGTCAACC
ITGB1	NM_002211.3	GAATGTATACAAGCAGGGCCAAA	CGTGCAGAAGTAGGCATTCC
ITGB2	NM_000211.3	TCAACGAGATCACCGAGTCC	CTTATCAGGGTGCGTGTTCAC
ITGB7	NM_000889.1	CAGCCCATCTTTGCTGTCAC	CCCCAACTGCAGACTTAGGAA
JAK1	NM_002227.2	CCCACAAACACATCGTGTACC	CCCCTTCCACAAACTCTTCCA
JUN	NM_002228.3	AAGAACTCGGACCTCCTCAC	TGGATTATCAGGCGCTCCA
KIF2A	NM_001243952.N	TCGACGGACTGTAGCTTCTA	TGCACGTGCTGAACCAA
KLF6	NM_001300.5	TGCCGTCTCTGGAGGAGTAC	GGGCTCGCTCTGGAGGTA
KLRB1	NM_002258.2	GGACATTCAACAGAGCAGGAA	CTCGGAGTTGCTGCCAATATA
KLRG1	NM_005810.3	GCCTAGCCAGAGACTCACA	AATCCAGCAAAAGGCCTCAC
LAG3	NM_002286.5	TGGAGCCTTTGGCTTTCAC	GAGGGTGAATCCCTTGCTCTA
LAPTM4B	NM_018407.N	TGGGAGGTGACTTTGAGTTCA	AGCCATAGCACATATCAGGATCA
LYST	NM_000081.2	AATCCTGAAGATGGCGAAACC	GGACTGCTTGATGCACTGAA
LYZ	NM_000239.2	GGAGCAGTTAATGCCTGTCA	CCCTCTTTGCACAAGCTACA
MAF	NM_001031804.2	TCGACGACCGCTTCTCC	ATCACCTCCTCCTTGCTGAC
MALT1	NM_006785.N	TGAATTCAGCCAGTGGTCAC	TTCAGAGACGCCATCAACAC
MAPK7	NM_139033.N	TTGTGACCCAGCAGCTATCTA	CTTTGGTGTGCCTGAGAACA
MIER2	NM_017550.N	GGACTCTCTTCCTGCCAACAA	TTGCTGAGGTCAGCTTGGAA
MKI67	NM_001145966.1	AGAGTAACGCGGAGTGTCA	CTTGACACACACATTGTCCTCA
MTDH	NM_001363137.N	ACTAGTGATCCAGCCGAAGTA	ATGGCTCGGTAGAAGTAGCA
NAB1	NM_005966.3	GGAAGGATGGGAAACATCTCAC	GCAGGGCATTATCCTTCACA
NDUFA1	NM_004541.	GATAGGCGCATCTCTGGAGT	TCATCAATCAGGAAAATGCTTCCTT
NDUFA11	NM_001193375.1	AGCCTACAGCACCACCAGTA	CCCGGAGGATTGAGTGTGAC
NEDD4L	NM_001144967.N	GAATTCACTTGGATGGCCGAAC	TTCTGCAGTCTTGGGTCTTCC
NFE2L3	NM_004289.N	CCTGACTGGGAGGCAGAAAA	GTCTTCCAGAGAGAAAGAAGTATCTGT
NFKBIA	NM_020529.2	CACCTCCACTCCATCCTGAA	GGTAGCCATGGATAGAGGCTAA
NOTCH2NLC	NM_001364012.N	AGAGACTATGCGTGGCACAA	TGGGTTGTGTGTGCACTCTA
NUCKS1	NM_022731.N	ATGCCCAAACCCAGACTAAA	CTTTTCTGGCGGGCTTTCC
OPTN	NM_001008211.N	AGCTGGCAGTTCTGCTGAAA	GACGACTCTGCATCTCCATCAA
PABPC4	NM_001135653.N	GCTATGCCTACGTCAACTTCC	GCGGATTGGCTTTCCCTTAA
PAIP2	NM_016480.3	GCAGAGTACATGTGGATGGAA	CATTTCTTGGAAACAGCGTTCA
PAK2	NM_002577.N	CTACGACTCCAACACAGTGAA	GCTGGTGTTCCAGAAGGAA
PCNA	NM_182649.1	TCTGAGGGCTTCGACACCTA	CATTGCCGGCGCATTTTAGTA
PDCD1	NM_005018.2	GCAGCCTGGTGCTGCTA	GTGCGCCTGGCTCCTA
PDCD6IP	NM_001162429.N	TTGTTCTATTGAACCCAAATTCCC	CGAAAGCATCCTTCCAGGTA

PECAM1	NM_000442.	ATGCCGTGGAAAGCAGATA	CATCTGGCCTTGCTGTCTA
PIK3IP1-AS1	NR_110542.N	TGCATCCATCCTGCCATCTG	GCAGAGGCAGTTCCGTTCT
PRDX5	NM_012094.N	GTTCGGCTCCTGGCTGAT	CAAAGATGGACACCAGCGAATC
PRKDC	NM_006904.N	GCTTGGATCTCTAGGAGGACAA	AGGCCACATAGCTCTTCATCA
PRPF40A	NM_017892.3	AACAAGCTGCTCCTCCGATA	TCCTCAAATGCTGGCTCTTTTAC
PSMB3	NM_002795.2	ATGGTGGCCAACCTCTTGTA	CTTAAAGGTCTTCGGGTCCAAC
PSMB9	NM_002800.N	TTGTGATGGGTTCTGATTCCC	GGGGACAGCTTGTCAAACA
PSME1	NM_006263.2	CGCTTGAAGCCTGAGATCA	ACCATCCTCAATCCGAGGTA
PTPRC	NM_002838.4	GTGGCTTAAACTCTTGGCATTT	GGGAAGGTGTTGGGCTTT
RAP1A	NM_001010935.1	CCAACAGTGTATGCTCGAAATCC	CAAAACCTTGGCCGTTCTTCA
RICTOR	NM_152756.3	CTTCCGTGTCGGAGGTTCATA	ACACAGCCTCTGCTTCTTCA
RNF213	NM_020954.2	AGGAAGCAGATGTCCAGGAA	AGAGAGATGATGGCGTGGAA
RORC	NM_001001523.1	CAAGACTCATCGCCAAAGCA	TTTCCACATGCTGGCTACAC
RSF1	NM_016578.N	TGGCAAATCTGTTACTGCAGACA	TCCCATGCCCAGGTACTGTTA
RTKN2	NM_145307.N	AAGAACGAACAGCATGTAAAGGAA	GCTGAAGTGATCAGAGTCTTTCC
RXRA	NM_002957.4	AGGAAACATGGCTTCCTTCAC	TCGCAGCTGTACACTCCATA
S100A4	NM_002961.2	AGGGTGACAAGTTCAAGCTCAA	TTGTCCCTGTTGCTGTCCAA
S100A6	NM_014624.3	AAGCACACCCTGAGCAAGAA	AGCCTTGCAATTTCAGCATCC
SEC11A	NM_001271918.	TCATCGGCACTAATGATCTGGAA	CACCACTACAATCGGACTTTCAC
SELL	NM_000655.4	CACCTGTGGACCATTTGGAA	GTGCTGATAGAGGCTCACAC
SELPLG	NM_001206609.1	AGAAACGGAGCCTCCAGAAA	TGGTAGACTCAGGGGTTCCA
SET	NM_001122821.1	GGTGATCCATCTTCGAAGTCC	TGACTCGAACGTTTCGTCAA
SNHG14	NR_146177.N	AACATGTTTGGCATGGTCTCC	ACAGACACAGTCCTTCCTTCC
SNRPB	NM_003091.	AGGGGAGAATCTGGTCTCAA	AGTGGAACTCGAGCAATACC
SNRPN	NM_003097.3	AGCCCCAACACAGTACCC	TACCAGGTGGAGGAGCCATA
SP100	NM_001080391.1	CTTCATCTCAGCACCGAGAA	CTTGGCCCTCCTTTTCATCA
SRI	NM_003130.2	ACACTGACAGGAGTGGAACA	GCCTGGGGACTCAACCTAA
STAT3	NM_003150.3	GGAAATAATGGTGAAGGTGCTGAAC	CCGAGGTCAACTCCATGTCAAA
STAT4	NM_003151.2	CAGTGCTGGAGGTAAAGGAA	AGAGGCAGATCTGTGTTTCAA
STAT5A	NM_003152.3	CCCAGGCTCCCTATAACATGTA	ATGGTCTCATCCAGGTCGAA
STK17A	NM_004760.2	CTGAGTCGGCTGTTGATTTCA	AACCAGGGGTGCTTTAGACA
SUB1	NM_006713.3	CAGCAGCAGCAGAGATGATAA	ATCGCGAACACTAACGTACC
TAOK3	NM_016281.3	CCAAGAAGCAAGTGGCTATCA	GCCAAGATCTGTTGCTGGAA
TBX21	NM_013351.1	GGGCGTCCAACAATGTGAC	CCGTCGTTCACCTCAACGATA
TGFB1	NM_000660.4	CGTCTGCTGAGGCTCAAGTTA	TCGCCAGGAATTGTTGCTGTA
TGFbR1	NM_004612.2	GAAATTGCTCGACGATGTTCC	ACTGATGGGTCAGAAGGTACA
TGOLN2	NM_001206840.	TTGCCTTGGTCCTCCTGAAC	TTCCTGCAGAAGGCCGTAC
THEMIS	NM_001164685.N	CCCTGATTCTCAAGCCTGTTTA	AGCTGAAGAAACCAGTTAGCA
TIGIT	NM_173799.3	GTGGTGGTCGCGTTGACTA	TCCTGTCCAGCTGATTTTCTCC
TLN1	NM_006289.3	GCTGGGAAAGCTTTGGACTAC	TTCAGGGGTCTCTGTTTCTTCC
TNF	NM_000594.2	CCCAGGGACCTCTCTCTAATCA	ATGGGCTACAGGCTTGTCAC
TNFRSF18	NM_148901.1	GCTGCTGCCGCGATTA	GAATTCAGGCTGGACACACA
TNFRSF9	NM_001561.4	GGGGCAGAAAGAAACTCCTGTA	TCTGGAAATCGGCAGCTACA
TRIM22	NM_006074.N	AGCTGACAGATGTCCAGTAC	ATGTGCAGGTGCGTACA
TRUB1	NM_139169.N	GTTGGAATTGGAAGCGGAAC	TAGCTTTCCCCAGTTCTCCA
TUBA1A	NM_001270399.	GCCAAACGTGCCTTTGTTCA	CACGGGCCTCTGAAAACTCA

UBXN1	NM_015853.4	AGATCGAGAGGGACAAAGCA	GCTGGGAGAAGAGGGAACA
UCKL1-AS1	NR_027287.N	CCACAATGCCAAGGAAACCA	TCCTCAGTGTCGGTTTCACA
UXT	NM_153477.N	CTCACCAAGGACTCCATGAA	GCAGGCCTTGTAGTTCTCTA
VDR	NM_000376.2	ATAAGACCTACGACCCCACCTA	GCTCCCTCCACCATCATTCA
XIST	NR_001564.2	TTGGATGGGTTGCCAGCTA	TCTCCACCTAGGGATCGTCAA
YPEL3	NM_031477.4	GGATGATTGTCACCGGAGGTA	CCCTGACTGCCCTGGAA
ZFP36	NM_003407.3	CCTCATGGCCAACCGTTACA	AGCCTGGGCTGGACTCA

Table Sup 2. Sequences of PCR Primers used for validation step by HT-qPCR using Biomark HD.

GENES	**Memory B**	**Naive B**	**Naive CD4**	**Naive CD8**	**MAIT**	**Th17**	**Th1**	**Treg**	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
INPP4B*	-2,944															
CAHM*	-2,851															
AC012076.1*	-2,692															
HTR3A*	-2,637															
GPD1L*	-2,525															
C20orf194*	-2,508															
LINC02018*	-2,468	2,286														
BBS1*	-2,412															
SLC38A11*	-2,361															
AL020997.2*	-2,235	-1,726														
AC104024.1*	-2,181															
AL645728.1*	-2,123		-1,057													
SENP3*	-2,054															
SLC17A5*	-2,016															
MDC1*	-2,011															
FOS*	-1,981	-2,328	-1,684													
GGT7*	-1,909															
AC124016.2*	-1,806															
LIN54*	-1,713															
AL450998.2*	-1,696															
C5orf22*	-1,662															
GLE1*	-1,644															
ZNF347*	-1,622															
CXorf40B*	-1,610															
URB2*	-1,602															
MIS18A*	-1,580															
DPYD*	-1,569															
VPS9D1*	-1,563															
AL353622.1*	-1,554															
SPATA20*	-1,491															
TRIM65*	-1,453															
NIT1*	-1,413															
ATP8B2*	-1,408															
IMMP2L*	-1,352															
DNASE1L1*	-1,351															
UTP20*	-1,347															
CD69*	-1,346	-1,244														
TRAF6*	-1,341															
MPP5*	-1,320															
TNFRSF10B*	-1,319															
NPTN*	-1,290															

GENES	**Memory B**	**Naive B**	**Naive CD4**	**Naive CD8**	**MAIT**	**Th17**	**Th1**	**Treg**	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
FOSB*	-1,281		-1,575	-1,547			-0,842									
ADARB1*	-1,236															
C2orf69*	-1,151															
MYC*	-1,103															
ODR4*	-1,068															
APPBP2*	-1,066															
KNOP1*	-1,048															
AC044849.1*	-1,046	-1,173														
EHMT2*	-1,022															
MCM7*	-0,976															
CEP295*	-0,959															
ZC3H6*	-0,944															
PEA15*	-0,898						-1,778									
PPP1R15A*	-0,889		-1,376	-1,428												
ZMYM4*	-0,888															
JOSD1*	-0,880															
PRXL2C*	-0,864															
DDX50*	-0,818															
FAM172A*	-0,790															
JUNB*	-0,782															
BLOC1S6*	-0,756															
GNL1*	-0,750															
NARS*	-0,744															
RNF167*	-0,731															
OTUD1*	-0,709															
BACH2*	-0,682															
GNA13*	-0,666															
CYSLTR1*	-0,586															
PDCD10*	-0,540															
SERBP1*	0,335															
ISG20*	0,452															
CPNE5*	0,494															
PSMB4*	0,525															
JUN	0,594	0,523/-1,091*	0,403/-1,468*		-1,646*		-1,383*						0,469			
WAS*	0,594															
MPG*	0,608															
MHENCR*	0,629				1,245											
RAB14*	0,629															
PSMD7*	0,641															
THUMPD3-AS1*	0,660															

GENES	**Memory B**	**Naive B**	**Naive CD4**	**Naive CD8**	**MAIT**	**Th17**	**Th1**	**Treg**	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
ANXA4*	0,679															
HSH2D*	0,679															
PDCD6IP	0,718															
WIPF1	0,726															
GTF2B*	0,815															
UXT	0,828					1,210										
CTSC*	0,838															
SLC35A3*	0,845															
HNRNPDL	0,865		0,827	0,840												
HIST1H1D*	0,911	0,866			-0,776											
TYMP*	0,936	0,958					1,477									
TIMM8B*	0,954															
MAU2*	0,962															
POP4*	0,971															
FBXO21*	1,020															
PARP9*	1,074	1,000				0,933										
RGP1*	1,098															
ARMH3*	1,141															
OAS1*	1,142															
TP53BP1*	1,165															
IFI35*	1,168															
IGHA1	1,168															
MLEC*	1,187															
BDP1	1,204															
CDKN2D	1,219											1,508				
HLA.DMB	1,250															
DPYSL2*	1,289															
KLHL14*	1,289															
AC007384.1*	1,315															
CD68*	1,332															
C9orf78	1,337				1,531							1,489				
SNRPB	1,343															
TTBK2*	1,344															
ITFG2-AS1*	1,357															
SOX4*	1,363															
RBBP8*	1,553															
APOL6*	1,631															
CASP6*	1,650															
AC024060.2*	1,654															
METTL2A*	1,698															
ZNF484*	1,949															

GENES	**Memory B**	**Naive B**	**Naive CD4**	**Naive CD8**	**MAIT**	**Th17**	**Th1**	**Treg**	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
C5*	2,173															
EPB41L4A*	2,186															
ARMCX5*	2,289															
AL079338.1*	2,644															
SELENOW/*	0,514*			0,832												
PSME2/*	0,533*	0,594*						1,330								
ZFP36/*	0,696-0,540*															
IRF8/*	0,758/-0,462*															
DGKZ/*	-0,98*								1,491							
PSMB3/*	1,428/0,431*					1,224		1,503								
S100A6/*	1,522/0,518*															
S100A4/*	1,655/0,734*															
IGLC7*		-2,710														
AC127035.1*		-2,706														
DZANK1*		-2,532														
TNFRSF25*		-2,348														
FBXO5*		-2,190														
DNAJB4*		-2,018														
MMUT*		-2,007														
FSD1L*		-1,970														
AC005838.2*		-1,880														
ORC6*		-1,843														
IRF5*		-1,821														
CRY1*		-1,763														
MAD2L1*		-1,750														
TNPO2*		-1,718														
AC073896.2*		-1,710														
LINC01534*		-1,651														
ALKBH1*		-1,635														
BUD13*		-1,610														
FASTKD5*		-1,605														
AL121603.2*		-1,604														
ACAD11*		-1,549														
TRMT12*		-1,499														
EHD4*		-1,469				-0,973										
TRIM41*		-1,430														

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
ATG4A*		-1,430														
FTO*		-1,426														
ABCB1*		-1,376														
SOCS5*		-1,361														
REXO1*		-1,292														
EXD3*		-1,260														
FAN1*		-1,259														
ARHGAP32*		-1,234														
SREBF2-AS1*		-1,234	-1,243													
LNPK*		-1,215														
NUP155*		-1,199														
R3HCC1L*		-1,134														
ORC3*		-1,120														
PPP2CB*		-1,118														
CSTF2T*		-1,097														
DEDD2*		-1,070														
SAMD4B*		-1,024														
TUBB4B*		-1,009														
LIMS1*		-0,963														
TMEM260*		-0,962														
RAB11FIP1*		-0,912														
MPHOSPH9*		-0,910														
GTF2H5*		-0,782														
NBPF19*		-0,767														
HDAC8*		-0,753														
SIN3A*		-0,699														
TMEM179B*		-0,695														
DENND4B*		-0,687														
SUN2*		-0,676														
LIMD1*		-0,655														
SERPINB9P1*		-0,653														
NLRP1*		-0,649														
ITPR2*		-0,599														
SAT2*		-0,586														
AHNAK/*		-0,550								0,717						
VDAC2*		-0,457														
PNRC1		-0,314			-0,550											0,615
HLA-C*		0,332														
DNAJC10*		0,446														
HHEX*		0,478														
TRIM56*		0,498				0,649										

GENES	**Memory B**	**Naive B**	**Naive CD4**	**Naive CD8**	**MAIT**	**Th17**	**Th1**	**Treg**	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
PARP14*		0,528														
RESF1/*		0,530	0,380			1,244										
BST2*		0,548														
SNRPC*		0,594														
C17orf49*		0,605														
AC004687.1/*		0,629											1,810		2,150	
STMN3*		0,631														
DENND11*		0,634														
RAB18*		0,653														
LPP*		0,668														
CXCR5*		0,682														
SAMD9*		0,686														
PSMD2*		0,696														
IRF9*		0,737				0,908										
ZBED1*		0,738														
ZNF292		0,755														1,630
NUAK2*		0,759														
PWWP3A*		0,759														
GCC2		0,772	0,891													
UROS*		0,776														
TBCC*		0,792														
USP14*		0,797														
LYST		0,805														
DGKD		0,806														
ADD1		0,825										1,548				
CTSZ		0,864														
BOD1L1		0,874			1,420											
CHD2		0,899														
PIK3CA*		0,905														
PLSCR1*		0,913														
ARRDC3*		0,953		0,873												
GNLY*		1,060				-1,955										
UNC45A*		1,067														
MBD4		1,122														
STAT1*		1,126	0,691	1,062		0,768										
N4BP2L2		1,155														
KDM3A*		1,315														
AC010618.3*		1,380														
WARS*		1,411														
MAGEH1*		1,444														
GK5*		1,520														

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
C6orf120*		1,524														
RNF14*		1,579														
NACC1*		1,627														
ZNF805*		1,719														
CD3G/*		1,941							1,245							
AL360270.1*		2,025														
ZNF257*		2,144														
IGIP*		2,311														
CD226*		2,422														
IFIT3*		2,480														
HAUS7*		2,525				2,148										
TNKS2-AS1*		2,571														
AC002470.1*		2,680														
AC104971.1*		2,689														
AL136419.3*		2,734														
AC018809.1*		2,819														
CLEC2D/*		0,343*		1,276/0,300*												
DUSP1/*		0,446/-1,174*														
TUBA1A/*		0,638/-0,773*	-0,525	0,571/-0,876*		-0,465*										
B2M/*		1,182 / 0,267						1,165								
SET/*		1,215/0,265*														
KLF2/*		1,226/0,304*						0,477*								
PSME1/*		1,242/0,351*														
CAST/*		1,333/0,433														
IGLC2/*		1,379/0,482*														
ANKRD37*			-3,206													
C4orf46*			-2,830													
NCAPG2*			-2,809													
DEPDC7*			-2,728													
AC010896.1*			-2,507													
FLVCR1-DT*			-2,499													
AL353743.2*			-2,469													

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
MKLN1-AS*			-2,362													
AC104389.5*			-2,309													
SLC25A20*			-2,274													
MAPK7*			-2,136													
AL139330.1*			-2,107													
RPS6KL1*			-2,076													
ASPHD2*			-2,008													
AL355581.1*			-1,830													
AC018638.7*			-1,803													
AC020978.7*			-1,653													
TMEM147-AS1*			-1,651													
SPRYD4*			-1,645													
SLC22A18*			-1,622													
CD302*			-1,607													
ADD2*			-1,605													
IER5*			-1,592		-1,685		-1,051									
ACTR5*			-1,560													
IFT22*			-1,495													
ADPRHL2*			-1,408													
PEX5*			-1,397													
DENND1A*			-1,298													
ISOC1*			-1,288													
KLHL22*			-1,282													
MXD1*			-1,269													
CBR3-AS1*			-1,263													
MOSPD1*			-1,193													
RABIF*			-1,137													
SEPTIN11*			-1,094													
NXT2*			-1,071													
PI4K2B*			-1,053													
NRBF2*			-1,037													
DDX41*			-1,028	-1,270												
CBWD2*			-1,026													
HAUS2*			-1,020													
FBLN5*			-1,012													
SOCS2*			-0,956													
AC009061.2*			-0,956													
PIK3CG*			-0,946													
SEH1L*			-0,942													
TXNDC17*			-0,942													
SPIN2B*			-0,920													

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
ASPSCR1*			-0,920				1,245									
POLR3F*			-0,903													
ETFDH*			-0,900		-2,172											
HSPBAP1*			-0,894													
ODC1*			-0,880	-0,774												
PPP1R21*			-0,823													
RCC1L*			-0,821													
A1BG*			-0,815													
GTF3C5*			-0,811													
SMAD3*			-0,796													
AC253572.2*			-0,794													
NDEL1*			-0,788													
ZNF737*			-0,776													
TUBD1*			-0,773													
OSER1-DT*			-0,769													
PIGT*			-0,697													
DERL2*			-0,696													
SPAG9*			-0,687													
AC007952.4*			-0,681													
DNAJB2*			-0,656													
BCL7B*			-0,651													
KPNA3*			-0,624													
NAA35*			-0,621													
PPP1R18*			-0,614													
GTF2F2*			-0,601													
FAM76B*			-0,552													
SETD5*			-0,520													
LZIC*			-0,516													
CLDND1*			-0,507													
C1orf162*			-0,494													
SF3A3*			-0,486													
DNM1L*			-0,476													
SMARCB1*			-0,475													
ACAA1*			-0,471													
NSMCE3*			-0,468													
THAP12*			-0,431													
MRPL34*			-0,406													
SF3B5*			-0,402													
HSPA5*			-0,354													
UFC1*			-0,352													
TRAPPC1*			-0,327													

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
OGT*			0,330													
ITK*			0,363													
DOCK10*			0,365													
FRG1*			0,384													
ESYT2*			0,396													
TMEM161B-AS1*			0,429													
DDHD1*			0,438													
TOP2B*			0,441													
CREBZF*			0,444													
NMT2*			0,447													
CITED2*			0,462													
U2AF1L4*			0,480													
AP2B1*			0,483													
PPP6R3*			0,484													
SETD2*			0,516													
LPXN*			0,566													
CERT1*			0,588													
SNHG25*			0,608	0,489												
CEP68*			0,636													
MCFD2*			0,651													
GIGYF1*			0,652													
SLC25A42*			0,653													
MATR3.1*			0,656													
EDEM3*			0,656			-0,821										
GPR183*			0,660													
IFRD2*			0,660													
TMX3*			0,669													
HNRNPA1P48*			0,710													
AC090948.1*			0,776													
TRA2B/*			0,788			-0,508*										
TAGLN2			0,803							0,826				0,753		
FAM226B*			0,815													
WDR20*			0,815													
C12orf57			0,815													
MKNK1*			0,832													
CERS6*			0,835													
TCIRG1*			0,837													
DNAJC5*			0,840													
NME6*			0,841													
REX1BD			0,846													
KMT2A			0,851													

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
C1QBP			0,853													
ARHGEF7*			0,855													
DDX5			0,856													
TPR			0,858													
DGKA			0,865													
KAT2B*			0,870													
KHDRBS1			0,875													
DUSP2/*			0,880				1,924/ 0,858*									
LAMTOR4			0,884	1,230								1,410				
LMBR1*			0,884													
HMGN1			0,893													
NDUFA1/*			0,897		1,776/0, 531*											
ARPC3			0,900													
COMMD6			0,916						1,263							
RPLP1			0,917													
SLC25A23*			0,999													
AC003102.1*			1,015													
TGFBR1*			1,030													
WASL*			1,038													
TRIM11*			1,045													
ZNF66*			1,054													
NEDD9*			1,062													
IKZF1			1,115	1,243	0,824											
PALM2-AKAP2*			1,123													
LRRC7*			1,157													
AL161457.2*			1,170													
AC068888.2*			1,294													
GTDC1*			1,296													
MSANTD3*			1,393													
ZHX3*			1,430													
CST7*			1,464													
HEXD-IT1*			1,482													
AC093227.1*			1,487													
PIK3IP1-AS1*			1,505													
AMT*			1,566													
ABCC4*			1,641													
NRROS*			1,654													
FAM174A*			1,671													

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
ADCY3*			1,848													
AC105429.1*			1,853													
TRIM46*			1,921													
AL162390.1*			2,134													
IFI44L*			2,273	2,044												
KLHL17*			2,278													
AL358781.1*			2,417													
AL049780.1*			2,430													
AL356488.2*			2,492													
HOXB3*			2,496													
RTP4*			2,514													
KIZ-AS1*			2,850													
U73169.1*			2,865													
DISP2*			3,375													
MSH5*			3,437													
INCENP*			3,575													
RCAN3/*			0,230*			1,224										
SEMA4D/*			0,378*						1,614							
CYTIP/*			0,407*			1,527/ 0,520*										
KLF6/*			-0,743*				0,652/- 0,631*									
GPRIN3/*			0,779*						1,588							
CDC42SE2/*			1,168/0,255													
UCKL1-AS1*				-3,137												
FANCA*				-2,892												
MED18*				-2,841												
C9orf40*				-2,779												
ZNF619*				-2,740												
SERPINI1*				-2,477												
SLC35C1*				-2,309												
LMTK3*				-2,046												
DOCK7*				-1,950												
STARD3NL*				-1,728												
TRIM39*				-1,710												
UBE2S*				-1,607												
CASTOR1*				-1,478												
AP3S2*				-1,467												

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
AC124016.1*				-1,456												
ZNF561*				-1,443	-2,337											
GALNT4*				-1,400												
SNAPC3*				-1,388												
TAB1*				-1,380												
TTC16*				-1,324	-1,702											
AC005224.2*				-1,173												
C15orf61*				-1,064												
STRN*				-1,000												
ELMSAN1*				-0,946												
RNF5*				-0,934												
PLPBP*				-0,896												
OXR1*				-0,861												
C4orf48*				-0,776												
KMT2B*				-0,773												
TSPYL2*				-0,743												
HIKESHI*				-0,740												
CNST*				-0,729												
RNF187*				-0,719												
NAE1*				-0,608												
DHPS*				-0,561												
SARS*				-0,558												
FCMR*				0,390												
PAN3*				0,562												
TTC39C*				0,574												
CDKN2AIP*				0,631												
TBRG1*				0,707												
MIS18BP1*				0,711												
FAM117B*				0,733												
KPNA6*				0,755												
SLC5A3*				0,797												
FBXW4*				0,806												
RALGAPB*				0,868												
LIPA*				0,871												
IFI6*				0,889												
CAMK1D*				0,917												
PDE4D*				0,925												
CCL5*				1,051												
LRIF1*				1,066												
GPBP1				1,100							1,157					
AC104532.2*				1,128												

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
FBL				1,155												
YPEL3				1,173				1,471		0,842						
RICTOR				1,208			0,744									
MIGA2*				1,214												
ICAM3				1,224												
DPP4*				1,241												
CMPK1				1,250					1,340							
RELL1*				1,258												
EID1				1,272		1,246										
PPP2R5C				1,281												
CD53/*				1,298				0,896								
MZT2A				1,323												
ROCK2*				1,334												
TRAPPC9*				1,382												
TLN1				1,412												
METTL6*				1,474												
TMEM68*				1,603	-1,910											
DSE*				1,809												
TRMT5*				2,367												
CDKN2C*				2,477												
SLC39A11*				2,494												
RAPGEF2*				2,512												
LINC02019*				2,875												
SP100/*				1,299/0,418*												
RNF213/*				1,307/0,377*			0,478*							1,420		
PABPC4/*				1,316/0,450*												
TTC27*					-3,764											
CARF*					-3,469											
SUMF1*					-3,220											
DIABLO*					-2,867											
LMF1*					-2,862											
ATP6V1A*					-2,823											
RGS3*					-2,776											
NOD1*					-2,770											
ZADH2*					-2,743											
GMDS-DT*					-2,462											
ICA1L*					-2,338											
ITGA5*					-2,325											

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
PUS10*					-2,301											
LINC00641*					-2,299											
DUSP14*					-2,289											
ZNF566*					-2,186											
ATP7A*					-2,152											
MBOAT7*					-2,099											
EID3*					-2,096											
MIR22HG*					-1,977											
NHSL2*					-1,950											
C11orf54*					-1,937											
SDHAF1*					-1,935											
LRMP*					-1,837											
PEX6*					-1,797											
MAPK8IP3*					-1,794											
RPUSD2*					-1,790											
HDAC5*					-1,714											
LINC00685*					-1,597											
WDR37*					-1,587											
AC048341.1*					-1,557											
KAT8*					-1,525											
SPRY1*					-1,494											
ATE1*					-1,441											
TCF12*					-1,440											
KLHL28*					-1,417											
RING1*					-1,373											
SLC33A1*					-1,347											
TARSL2*					-1,325											
KIF21A*					-1,324											
MRPL18*					-1,245											
ZFYVE28*					-1,217											
TM2D1*					-1,211											
GOPC*					-1,203											
SNX14*					-1,169											
CEP290*					-1,153											
PIEZO1*					-1,133											
VCPIP1*					-1,100											
BAG5*					-1,086											
GAK*					-1,059											
TMEM147*					-1,045											
SSBP3*					-1,042											
POU2F2*					-1,041											

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
SLC38A2*					-0,986											
MIDN*					-0,836											
EIF2S2*					-0,801											
CD27*					-0,796											
EIF4A1*					0,426											
LCP1*					0,435											
LDHA*					0,680											
ITGB7*					0,791											
SRP19*					0,810											
DNAJC1*					0,814											
TXN*					0,815											
BUD31*					0,842											
RNF216*					0,935											
SECISBP2*					1,074											
TFAM*					1,106											
AP3B1*					1,140											
CAPRIN1*					1,142											
ZNF331*					1,154											
TMEM258					1,172											
GNAS					1,183											
CD52					1,210											1,418
KIF2A/*					1,221		0,672/-0,487*									
PPA2*					1,228											
PLEKHB2*					1,243											
RPS17					1,246					0,834						
ATP8A1*					1,246											
PHACTR2					1,249											
UPF3B*					1,263											
POLR2L					1,265											
SUPT16H*					1,280											
GMFG					1,282											0,777
VIM					1,289											
ANKRD13A*					1,294											
HLA.E					1,311	1,232										
GPSM3					1,316											
RNF220*					1,320											
ANKRD28*					1,343											
GZMM					1,362											
ATRX					1,364											

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
ARPC5L					1,367											
MTDH					1,375	1,311										
CAPZB					1,378							1,803				
ITFG2*					1,380			1,579								
SLC38A1					1,395											
COX8A					1,396											
TGOLN2					1,398											
TMED2					1,412											
TRAM1					1,447								1,458			
STK17B					1,447											
MGAT4A					1,449											
COPS4*					1,466											
SUMO1					1,475											
DDX39B					1,479											
CCNL2*					1,480											
DAZAP2					1,486									0,867		
SEC11A					1,490											
PDIA3					1,506	1,204										
PAIP2					1,527							1,461			1,520	
C4orf3					1,546											
RBMX					1,558											
SRI					1,620											
ISG15/*					1,627			1,017								
H2AFZ					1,649											
SNRPN					1,681			0,746								
STK17A					1,686											
STX10*					1,695											
ULK3*					1,718											
HPF1*					1,720											
MAF					1,726											
ENO1					1,727	1,205										
HADH*					1,796											
DIAPH1					1,797											
COA4*					1,803											
P2RX5*					1,821											
HMG20B*					1,897											
ACAT2*					1,901											
NME4*					1,912											
TAOK3					1,916				1,692							
TCF20*					1,929											
WDR18*					2,035											

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
SFXN3*					2,039											
PRPF40A					2,046											
TRIQK*					2,098											
PET117*					2,149											
GUF1*					2,180											
NOP14-AS1*					2,363											
PRPS2*					2,374											
GALNT2*					2,444											
PRCP*					2,448											
HIST1H2AC*					2,486											
TMEM185B*					2,556											
NUP85*					2,613											
ARHGAP21*					2,620											
UQCC1*					2,707											
TMEM159*					2,870											
ATP6V1E2*					2,892											
NKIRAS2*					2,978											
UBE2D4*					2,986											
URGCP*					3,200											
INTS13*					3,230											
SNN*					3,357											
USP19*					3,382											
BRI3BP*					3,430											
HIST2H2BF*					3,623											
FBXL6*					3,888											
UBXN1/*					0,699/-0,601*											
NDUFA11/*					0,708/-0,593*				1,334							
SAT1/*					1,495/0,534*											
PABPC1/*					1,588/0,539*		1,217					1,630				
SUB1/*					1,663/0,426*											
CADM1*						-3,261										
E2F2*						-3,153										
DHX34*						-2,781										
SLC25A14*						-2,651										
TRDC*						-2,506	-3,112									
ZSCAN16*						-2,330										

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
NEDD4L*						-2,315										
ZSWIM9*						-2,232										
RNF121*						-2,170										
TCEAL1*						-1,762										
KIAA0930*						-1,726										
MED31*						-1,481										
INTS3*						-1,284										
WDR36*						-1,260										
ITGAE*						-1,160										
GTF2H3*						-1,065										
ACLY*						-1,033										
EXOC5*						-1,015										
SUDS3*						-1,007										
ATF1*						-0,931										
PTRHD1*						-0,875										
KIAA0586*						-0,859										
PTTG1IP*						-0,822										
WDFY2*						-0,820										
MYADM*						-0,748	-1,136									
HPS1*						-0,743		-1,560								
ICE2*						-0,743										
OGDH*						-0,732										
WDR43*						-0,676										
UCP2*						-0,621										
TMEM256*						-0,607										
CCNH*						-0,580										
MYO1G*						-0,494										
NOSIP*						-0,392										
ITGB1*						-0,363										
MT-ND1*						0,205										
MT-ATP6*						0,222										
MT-ND2*						0,229										
RPL38*						0,260										
HLA-E*						0,262										
RBMS1*						0,450										
TNFRSF14*						0,487										
SNRNP70*						0,534										
N4BP2L1*						0,656										
GATAD2A*						0,662										
MALT1						0,729										
PPM1G						0,752										

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
NELL2*						0,756										
PRDX5						0,757										
TAP2*						0,766										
APOL3*						0,766										
FNIP1*						0,767										
SYF2						0,801										
TRPM7*						0,811										
CRIP1						0,852			1,457			0,792				
GBP5*						0,884										
PRR12*						0,931										
MAPKAPK3*						0,987										
AP2A1*						1,005										
AASDH*						1,035										
FAU						1,041					1,105					
RPS13						1,048										
RPL32						1,064										
RPS19						1,068										
RPS9						1,068										
CSDE1						1,069						1,441				
RPS23						1,070										
RPL12						1,071										
SH2D3A*						1,071										
PHYH*						1,072										
RPL30						1,072										
EEF1A1/*						1,075		-0,184/*								
NACA						1,077										
RPS12						1,087										
RPL9						1,088					1,103					
RPL5						1,089										
EEF1B2/*						1,091		0,727/-0,401*								
RPL17						1,092										
RPS5						1,093										
UBA52						1,093										
TPT1						1,093										
RPS6						1,095										
RPL41						1,097										
RPS15						1,100										
RPL13A						1,100										
RPL19						1,101										
RPL29						1,101										

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
RPS25						1,103										
RPL11						1,103										
RPL13						1,103										
TOMM7						1,104										
RPLP2						1,105										
RPS2						1,106										
RPL35						1,106										
RPS18						1,108										
EEF1G						1,110										
RPL18A						1,114						1,215				
EEF1D						1,116										
RPL24						1,123										
XAF1*						1,125										
TRIR						1,135										
PTMA						1,137										
RPL27A						1,141										
RPS20						1,145										
RPL31						1,159										
YWHAB						1,160							1,615			
GNAI2						1,167										
KTI12*						1,168										
SNX10*						1,184										
MT.ND1						1,185								1,118		
MT.ND2						1,187										
MT.CYB						1,191										
CD48						1,199										
SREBF2*						1,199										
MT.ATP6						1,205										
ACTR3						1,243										
PPIA						1,244										
FKBP11						1,279										
GPS2						1,282										
PSMB9						1,299										
HNRNPM						1,314										
PRKDC						1,337										
RAP1A						1,349										
LSM8						1,409										
SNHG14/*						1,418	0,592/-0,676*			0,611						
TMEM14B						1,477										

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
FMR1-IT1*						1,598										
SLC38A5*						1,648										
TRIM25*						1,651										
CIAO3*						1,695										
ZNF677*						1,743										
SNRNP25*						1,783										
GZMK*						1,879										
BRPF3*						1,941										
CABP4*						1,953										
ZNF337-AS1*						1,984										
MCOLN3*						2,110										
FAM229A*						2,138										
AC108134.2*						2,237										
DNAL1*						2,306										
MIER2*						2,619										
IFITM2/*						1,317/ 0,336*										
NUCKS1/*						1,403/ 0,394*										
NFE2L3*							-2,870									
NOTCH2NLA*							-2,800									
C15orf41*							-2,682									
ACVR1B*							-2,597									
BLM*							-2,548									
KCTD15*							-2,443									
RFC3*							-2,432									
LAPTM4B*							-2,425									
HIST1H2BF*							-2,415									
THAP3*							-2,033									
SPATS2L*							-2,006									
PAXIP1*							-1,852									
ZNF699*							-1,823									
INTS8*							-1,814									
CD151*							-1,790									
ZNF587B*							-1,638									
ZNF346*							-1,609									
UBE2D1*							-1,589									
SPICE1*							-1,584									
SNAPC2*							-1,540									

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
SNX27*							-1,442									
NTPCR*							-1,431									
DLGAP1-AS1*							-1,377									
CCDC65*							-1,181									
LINC00824*							-1,180									
UBN2*							-1,178									
MBD1*							-1,166	-1,905								
THEMIS*							-1,081	-3,341								
AC103591.3*							-1,077									
CNIH4*							-1,049									
TRNAU1AP*							-1,033									
RBM14*							-0,976									
HMGXB4*							-0,936									
AL021155.5*							-0,880									
NDUFV3*							-0,868									
AATF*							-0,810									
ZNF33A*							-0,731									
AL499604.1*							-0,721									
SCML4*							-0,676									
PUF60*							-0,661									
NCOR1*							-0,629									
LCP2*							-0,594									
SMARCA5*							-0,562	-0,852								
PSMA6*							0,557									
ATF6B*							0,610									
SUMO3*							0,750									
SF3B1							0,767									
DNAJB9*							0,777									
SDHAF2*							0,780									
MED13*							0,784									
DR1*							0,808									
GABARAP							0,810									
PSME4*							0,836									
ZNHIT3*							0,845									
EBPL*							0,873									
TTC39B*							0,935									
TRIAP1*							0,946									
MFHAS1*							0,948									
TNFRSF4*							1,042									
CSKMT*							1,051									
TPST2*							1,125									

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
KLHL20*							1,142									
GABARAPL1*							1,209									
CFH*							1,219									
IFI44*							1,222									
C19orf53							1,230									
GUSB*							1,255									
RAN							1,323									1,700
RBM38*							1,369									
MGST3							1,384		1,410							
SSU72							1,443									
URI1							1,467									
NR2C2AP*							1,493									
HYPK*							1,506									
MAP3K14*							1,536									
MPP6*							1,609									
APOOL*							1,777									
HEATR3*							1,810									
MARK4*							1,961									
TRIM21*							1,965									
PKIA*							2,050									
HSCB*							2,057									
OPA3*							2,068									
AC006480.2*							2,083									
ZNF552*							2,125									
CPT2*							2,212									
NOTCH2NLC*							2,236									
ARHGAP18*							2,577									
ISM1*							2,763									
GPR15*							2,934									
LYZ*							3,138									
RTKN2/*							-1,560*	1,953/0,763*								
TRUB1*								-3,682								
PBX3*								-3,140								
ACVR2A*								-2,968								
POLRMT*								-2,645								
DDIT3*								-2,505								
COX10*								-2,465								
SFR1*								-2,387								
WDR75*								-2,236								
FLOT1*								-2,173								

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
MRPS28*								-2,121								
ARF3*								-2,078								
HEIH*								-2,078								
HIPK2*								-2,075								
VPS39*								-2,007								
DDX10*								-1,986								
AKAP7*								-1,906								
OSBP*								-1,854								
SIRT1*								-1,774								
CNOT6*								-1,756								
SLC9A9*								-1,751								
SH3GLB2*								-1,723								
VEZF1*								-1,719								
MTMR6*								-1,706								
TMEM131L*								-1,664								
RARS*								-1,648								
ADSL*								-1,620								
C1orf52*								-1,543								
MMP24OS*								-1,503								
BLCAP*								-1,474								
LSM10*								-1,308								
MOAP1*								-1,259								
LPIN2*								-1,245								
CCR7*								-1,234								
MTR*								-1,217								
TRABD2A*								-1,180								
PIM1*								-1,035								
MSN*								-0,699								
ZFAS1*								-0,482								
ACTG1*								-0,418								
HLA-A*								0,304								
SYNE2*								0,516								
RSF1								0,555								
MYH9*								0,578								
CD2*								0,583								
UBB*								0,598								
PAK2								0,659								
FKBP1A								0,668								
TRA2A								0,693								
BPTF								0,697				1,680				
HNRNPA2B1								0,746							1,307	

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
TPM3								0,751								
FUS								0,763				0,802				
NSA2								0,791								
RPL4								0,824				1,236				
CD7*								0,866								
HERPUD1*								0,902								
FOXP3*								0,910								
SLU7*								1,034								
UBE2K*								1,128								
APH1A*								1,135								
BAX*								1,142								
INPP5D*								1,184								
LONP2*								1,244								
HLA.A								1,255								
KDM5B*								1,327								
JAK1								1,364								
FAM120A*								1,426								
ARHGEF1								1,458								
SLC25A39*								1,508								
NDUFB8								1,574								
AKR1B1*								1,612								
GLRX								1,627								
FBH1*								1,639								
CNOT6L								1,648								
RPS6KB2*								1,720								
TRIM69*								1,724								
BCCIP*								1,727								
MYCBP*								1,818								
RANBP6*								1,867								
XRRA1*								1,924								
RNF170*								2,082								
GPALPP1*								2,198								
ARRDC1*								2,220								
MDP1*								2,258								
ZDHHC17*								2,275								
TFPT*								2,332								
SH3GL1*								2,551								
TM2D2*								2,592								
TMCO4*								2,790								
FANCM*								2,824								
IKZF4*								2,950								

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
ABHD17B*								2,956								
AP5B1*								2,984								
BACE1-AS*								3,049								
PUS1*								3,073								
TRBV28*								4,282								
CLK1/*								0,420/-0,953								
OPTN/*								1,803/0,717*					1,575			
CDK13/*								1,828/0,831								
TRIM22/*								2,158/1,053*								
IRF3									0,711							
UBE2D2									0,743							
SPCS1									1,245							
EIF3F									1,268							
YTHDC1									1,286							
YWHAZ									1,288							
PDIA6									1,327							
RNASET2									1,332							
PSMC5									1,347							
UBE2V1									1,349							
NDUFB11									1,389							
JTB									1,454							
KAT6A									1,468							
AKAP9									1,484							
GYPC									1,490						1,759	
HP1BP3									1,538				1,366			
EVI2B									1,552							
SPTBN1									1,552							
DOK2									1,563							
DUT									1,577							
ZMYM2									1,594							
SFXN1									1,630							
XRN2									1,632	0,734						
PIK3R1									1,674							
TAF15									1,827							
DYNLT1									2,268				1,914			
MYCBP2									1,464*				1,420			
EIF5B										0,607						

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
UBALD2										0,707						
MDM4										0,709						
PSMA7										0,737						
NDUFS5										0,742						
JPX										0,752						
HCLS1										0,753						
FKBP8										0,753						
RPS27L										0,776						
RAD21										0,776						
NSD3										0,783						
EIF4A2										0,829						
GSTK1										1,241					1,715	
SMDT1										1,311						
HLA.F										1,441		1,461	1,372			
SF3B2										1,553						
CD47										1,593						
PDE3B										1,896						
COX7A2L											0,684					
ROCK1											0,690					
PSAP											0,721					
ERBIN											0,739					
CFLAR											0,777					
DNAJC8											0,810					
EIF3K											0,836					
KRTCAP2											0,844					
MAP2K2											1,180					
KLRB1												0,485				
RAB5IF												0,605				
CAPNS1												0,610				
RHOA												1,254				
PNISR												1,293				
MEAF6												1,461				
RNASEK												1,537				
BCL11B												1,552				
PCED1B.AS1												1,582				
TAPBP												1,616				
SCP2												1,638				
UBXN4												1,706				
TMED4												1,768				
CSNK1A1												1,879				
CREBRF												1,937				

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
PEBP1												1,957				
GSTP1												2,084				
SETX												2,230				
PRDX6												2,240				
COX6C													1,156			
PDCD4													1,243			0,592
GAPDH													1,291			
ATP5F1D													1,293			
SPCS3													1,352			
HIGD2A													1,354			
UBE2I													1,364			
SOD1													1,382			2,286
LITAF													1,389			
ARPC2													1,390			
CCND3													1,396			
CCDC85B													1,425			
GNB1													1,455			
RCSD1													1,465			
GLG1													1,488			
AC092821.3													1,494			
TSPO													1,534			
P4HB													1,551			
RBL2													1,565			
ARHGDIA													1,569			
ATP2B1													1,576			
XBP1													1,577			
DOCK8													1,614			
SH3KBP1													1,690			
STK10													1,691			
WDR83OS													1,807			
SNHG7													1,907			
SLC9A3R1													2,359			
MIF														1,138		
CBX3														1,299		
NDUFC2														1,390		
SNRPG														1,449		
CYTH1															0,509	
ATP5MD															0,649	
ANKRD11															0,662	
NDUFA6															0,723	
RPL10A															1,132	

GENES	Memory B	Naive B	Naive CD4	Naive CD8	MAIT	Th17	Th1	Treg	Th2	Tfh	NK	NKT	CD8CM	CD8EM	CD8EMRA+	gdTcells
RPL28															1,172	
ZEB2															1,370	
EFHD2															1,507	
KMT2E															1,574	
CCSER2															1,774	
RSL24D1															1,827	
CWC15															2,118	
ARGLU1																0,620
MALAT1																0,855
PIK3IP1																1,468
U2SURP																1,883
DBI																2,153

Table Sup 3. Differentially expressed genes (DEGs) according to two methods in each lymphocyte subsets.

Genes with * were identified in the DESeq2 analysis and are expressed as Log2(fold-change). Other genes were identified by the global ratio analysis and the value of the global ratio ((M3 vitamin D / D0 vitamin D) / (M3 placebo / D0 placebo)) is reported in the table.

Significant genes from both analyses ($p<0.05$) are quoted ‘gene/*’ and values are reported are as such: global ratio / DESeq2*. Genes in bold were selected for the qPCR validation step.

	Pathway	pval	padj	qvalue	Fold Change
Bmemory Vitamin D	POSITIVE_EPIGENETIC_REGULATION_OF_RRNA_EXPRESSION	6,74E-08	6,53E-05	2,05	-0,77
	TRAF6_MEDIATED_NF_KB_ACTIVATION	6,87E-06	0,0067	1,48	-0,76
	INTERLEUKIN_1_FAMILY_SIGNALING	1,16E-05	0,0112	1,40	-1,08
Bnaive Vitamin D	TOLL_LIKE_RECEPTOR_9_TLR9_CASCADE	3,86E-12	3,74E-09	2,90	-1,57
	MAPK_FAMILY_SIGNALING_CASCADES	3,10E-10	3,00E-07	2,55	0,61
	NGF_STIMULATED_TRANSCRIPTION	7,08E-10	6,86E-07	2,48	-1,18
	SIGNALING_BY_NUCLEAR_RECEPTORS	3,42E-08	3,32E-05	2,12	-2,55
	SENESCENCE_ASSOCIATED_SECRETORY_PHENOTYPE_SASP	7,06E-08	6,84E-05	2,04	-1,14
	SIGNALING_BY_RECEPTOR_TYROSINE_KINASES	7,06E-08	6,84E-05	2,04	-0,22
	INTERLEUKIN_1_SIGNALING	5,62E-07	0,0005	1,81	1,03
	MYD88_INDEPENDENT_TLR4_CASCADE	5,62E-07	0,0005	1,81	-1,38
	FC_EPSILON_RECEPTOR_FCERI_SIGNALING	7,83E-07	0,0008	1,77	0,11
	ESTROGEN_DEPENDENT_GENE_EXPRESSION	2,06E-06	0,0020	1,64	-2,44
	RAF_INDEPENDENT_MAPK1_3_ACTIVATION	2,06E-06	0,0020	1,64	-0,81
	TOLL_LIKE_RECEPTOR_TLR1_TLR2_CASCADE	2,06E-06	0,0020	1,64	-1,54
	PRE_NOTCH_EXPRESSION_AND_PROCESSING	2,82E-06	0,0027	1,60	-1,03
	DEFECTIVE_INTRINSIC_PATHWAY_FOR_APOPTOSIS	5,22E-06	0,0051	1,52	-0,33
	DISEASES_OF_SIGNAL_TRANSDUCTION_BY_GROWTH_FACTOR_RECEPTORS_AND_SECOND_MESSENGERS	1,70E-05	0,0165	1,34	1,78
	ESR_MEDIATED_SIGNALING	1,70E-05	0,0165	1,34	-2,35
	MAPK6_MAPK4_SIGNALING	1,70E-05	0,0165	1,34	0,45
	LEISHMANIA_INFECTION	2,27E-05	0,0220	1,29	0,79
	MAPK_TARGETS_NUCLEAR_EVENTS_MEDIATED_BY_MAP_KINASES	2,27E-05	0,0220	1,29	-0,97
	NEGATIVE_REGULATION_OF_MAPK_PATHWAY	2,27E-05	0,0220	1,29	-1,27
	NUCLEAR_EVENTS_KINASE_AND_TRANSCRIPTION_FACTOR_ACTIVATION	2,27E-05	0,0220	1,29	-1,16
	AMINO_ACIDS_REGULATE_MTORC1	3,96E-05	0,0384	1,19	0,14
CD4naive Placebo	CELLULAR_RESPONSE_TO_STARVATION	3,29E-07	0,0003	1,87	-0,44
	EUKARYOTIC_TRANSLATION_ELONGATION	8,79E-07	0,0009	1,75	-0,87
	SELENOAMINO_ACID_METABOLISM	1,42E-06	0,0014	1,69	-0,78
	RRNA_PROCESSING	3,59E-06	0,0035	1,57	-0,07
	NONSENSE_MEDIATED_DECAY_NMD	8,76E-06	0,0085	1,44	-0,52
	RESPONSE_OF_EIF2AK4_GCN2_TO_AMINO_ACID_DEFICIENCY	8,76E-06	0,0085	1,44	-0,70
	DNA_REPLICATION	2,54E-05	0,0246	1,27	0,84
	INFLUENZA_INFECTION	4,68E-05	0,0454	1,16	-0,38
	SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	4,68E-05	0,0454	1,16	-0,32

	Pathway	pval	padj	qvalue	Fold Change
CD4naive Vitamin D	TOLL_LIKE_RECEPTOR_9_TLR9_CASCADE	1,81E-07	0,0002	1,94	-1,20
	MAPK_TARGETS_NUCLEAR_EVENTS_MEDIATED_BY_MAP_KINASES	6,88E-06	0,0067	1,48	-0,65
	SIGNALING_BY_ROBO_RECEPTORS	6,88E-06	0,0067	1,48	-1,22
	RESPONSE_OF_EIF2AK4_GCN2_TO_AMINO_ACID_DEFICIENCY	1,20E-05	0,0116	1,39	-1,69
CD8naive Placebo	EUKARYOTIC_TRANSLATION_ELONGATION	2,31E-06	0,0022	1,63	0,05
	CELLULAR_RESPONSE_TO_STARVATION	1,13E-05	0,0109	1,40	0,37
	SELENOAMINO_ACID_METABOLISM	4,89E-05	0,0474	1,15	0,25
MAIT Placebo	TRANSCRIPTIONAL_REGULATION_BY_TP53	4,77E-05	0,0462	1,16	-0,93
MAIT VitD	TOLL_LIKE_RECEPTOR_TLR1_TLR2_CASCADE	8,00E-06	0,0077	1,45	-1,45
Th17 Placebo	RESPONSE_OF_EIF2AK4_GCN2_TO_AMINO_ACID_DEFICIENCY	1,33E-05	0,0128	1,38	-2,20
Treg VitD	MAP2K_AND_MAPK_ACTIVATION	3,74E-05	0,0363	1,20	0,92

Table Sup 4. Analysis of pathways regulated by vitamin D revealed by SCPA Package.

Only pathways with a p-value adjusted <0.05 were represented.

Chapitre 2 : Pour aller plus loin : Analyse Supervisée

Dans ce deuxième chapitre, je présente les résultats obtenus en réalisant une analyse complémentaire non rapportée dans l'article basée sur une analyse supervisée des résultats de l'approche CITE-seq décrite précédemment. Ainsi, la partie de description de la cohorte, la préparation des échantillons et le protocole de CITE-seq sont identiques au chapitre précédent. Néanmoins, cette méthode n'est pas celle la plus couramment utilisée pour analyser les effets d'un traitement sur l'expression des gènes. Ici, elle permet d'offrir une autre analyse pour étoffer la liste de gènes potentiellement modulés par la vitamine D.

Matériel et méthodes

Traitement des données de scRNA-seq

Le logiciel Cell Ranger (10x Genomics, v.6.1.1) a été utilisé pour démultiplexer les échantillons, traiter les données brutes, aligner les lectures sur le génome humain de référence GRCh38 et résumer les nombres d'identifiants moléculaires uniques (UMI). Les matrices filtrées gène-barcode et expression de protéine de surface cellulaire-barcode qui ne contenaient que des codes-barres avec des comptes UMI dépassant le seuil de détection des cellules ont été utilisées pour la suite de l'analyse.

Visualisation des données CITE-seq et clusterisation

Les données des 20 échantillons (5 patients vitamine D J0/M3, 5 patients placebo J0/M3) ont été agrégées puis visualisées en UMAP et analysées avec le logiciel Loupe®. Les différentes sous populations lymphocytaires ont été définies avec une combinaison de marqueurs de surface spécifiques préalablement définie avec des seuils d'expression propre à chaque marqueur. Les seuils ont été déterminés à l'aide de violin plots représentant la répartition d'expression de chaque marqueur dans chaque cluster.

Analyse des populations, des différentiels d'expression des gènes et des protéines de surface entre les groupes vitamine D et placebo

La liste des cellules de chaque cluster a été extraite, importée et analysée dans R Studio à l'aide de plusieurs scripts rédigés et adaptés des vignettes Seurat (https://satijalab.org/seurat/articles/pbmc3k_tutorial.html). Le différentiel d'expression des gènes et des protéines de surface entre les groupes vitamine D et placebo ont été calculés avec la méthode du "global ratio" ((M3/J0 Vitamin D) / (M3/J0 placebo)). En parallèle, pour chaque cluster, nous avons extrait une liste de cellules, les avons comptées et normalisées en pourcentage, et avons comparé la proportion de chaque sous-ensemble de lymphocytes avant et après traitement par vitamine D ou placebo.

Analyse statistique

Une ANOVA à 2 facteurs (2-way ANOVA) avec une correction des FDR (Benjamini-Hochberg) a été réalisée pour évaluer l'interaction entre le temps et le traitement pour l'évaluation des modifications des populations cellulaires. Un test de Mann-Whitney a été effectué sur les "global ratio" pour établir la liste de gènes et des protéines de surface significativement modulées dans le groupe vitamine D par rapport au groupe placebo. Les résultats sont considérés comme significatifs pour des p-value < 0,05.

Résultats

Clusterisation

Afin d'analyser les effets de la vitamine D sur différentes populations immunitaires impliquées dans la physiopathologie de la SEP, nous avons utilisé le CITE-seq pour décrire l'évolution de la proportion de chaque sous-population lymphocytaires chez les patients atteints de SEP après 3 mois de traitement par placebo ou par de fortes doses de vitamine D. Les marqueurs de surface caractéristiques associée à un seuil d'expression ont été utilisés pour annoter les principales

populations de PBMC (Table 1), permettant l'identification de 32 sous-populations lymphocytaires des lignées B (B^+, B naïfs, B transitionnels, B mémoires switches, B mémoires non switches, B régulateurs, Plasmablastes), des lignées T ($CD4^+$, CD4 naïfs, CD4 effecteurs mémoires (EM), CD4 $EMRA^+$, CD4 central mémoires (CM), Th1, Th1* (Th1/Th17), Th17, Th2, T régulateurs, Tr1, $CD8^+$, CD8 naïfs, CD8 EM, CD8 $EMRA^+$, CD8 CM, Tc1, Tc1*, Tc2, Tc17), les NK et NKT, les MAIT, les γδTcells et les lymphocytes T-CD20 ($CD3^+CD20^+$) (Figure 1).

Populations	CD3	CD56	CD20						
CD4+	CD3 >7	CD56<4.5	CD20 <6	CD4 >6.5					
CD4+ naïf	CD3 >7	CD56<4.5	CD20 <6	CD4 >6.5	CD45RA > 6	CCR7 >3			
CD4+ EM	CD3 >7	CD56<4.5	CD20 <6	CD4 >6.5	CD45RA < 6	CCR7 <3			
CD4+ EMRA+	CD3 >7	CD56<4.5	CD20 <6	CD4 >6.5	CD45RA >6	CCR7 <3			
CD4+ CM	CD3 >7	CD56<4.5	CD20 <6	CD4 >7	CD45RA <6	CCR7 >3	CD62L >5		
Th1	CD3 >7	CD56<4.5	CD20 <6	CD4 >7	CXCR3 >4.5	CCR6 <4	CCR5>2.5		
Th1*	CD3 >7	CD56<4.5	CD20 <6	CD4 >7	CXCR3 >4.5	CCR6 >4			
Th17	CD3 >7	CD56<4.5	CD20 <6	CD4 >7	CXCR3 <4.5	CCR6 >4	CCR4>2		
Th2	CD3 >7	CD56<4.5	CD20 <6	CD4 >7	CXCR3 <4.5	CCR6 <4	CCR4 >2	CXCR4>2	
Treg	CD3 >7	CD56<4.5	CD20 <6	CD4 >7	CD45RA >6	CD25 >2.5	GITR >2	CTLA4 >2	
Tr1	CD3 >7	CD56<4.5	CD20 <6	CD4 >7	CD49b >3	LAG3 >1.5	CD226 >4	CTLA4 >2	HLADR >5
CD8+	CD3 >7	CD56<4.5	CD20 <6	CD8 > 7.5					
CD8+ naïf	CD3 >7	CD56<4.5	CD20 <6	CD8 > 7.5	CD45RA >6	CCR7 >3	CD62L>5	CD127>5.5	CD27>8
CD8+ EM	CD3 >7	CD56<4.5	CD20 <6	CD8 > 7.5	CD45RA< 6	CCR7 <3	CD62L<5	CD127>5.5	
CD8+ EMRA+	CD3 >7	CD56<4.5	CD20 <6	CD8 > 7.5	CD45RA >6	CCR7 <3	CD62L<5	CD127>5.5	
CD8+ CM	CD3 >7	CD56<4.5	CD20 <6	CD8 > 7.5	CD45RA <6	CCR7 >3	CD62L >5	CD127>5.5	CD27>8
Tc1	CD3 >7	CD56<4.5	CD20 <6	CD8 > 7.5	CXCR3 >4.5	CCR6 <4			
Tc1*	CD3 >7	CD56<4.5	CD20 <6	CD8 > 7.5	CXCR3 >4.5	CCR6 >4			
Tc2	CD3 >7	CD56<4.5	CD20 <6	CD8 > 7.5	CXCR3 <4.5	CCR6 <4			
Tc17	CD3 >7	CD56<4.5	CD20 <6	CD8 > 7.5	CXCR3 <4.5	CCR6 >4			
B+	CD3 <7	CD56<4.5	CD20 >6						
B naïf	CD3 <7	CD56<4.5	CD20 >6	CD27 <5	IgD >6				

B transitionnel	CD3 <7	CD56<4.5	CD20 >6	CD24 <8	CD38 >6.5				
B mémoire switche	CD3 <7	CD56<4.5	CD20 >6	CD27 >5	IgD <0.5				
B mémoire non switche	CD3 <7	CD56<4.5	CD20 >6	CD27 >5	IgD <5 et >0.5	IgM >1			
B régulateur	CD3 <7	CD56<4.5	CD20 >6	CD27 >5	IgD <6	CD38 >3	CD71 >5		
Plasmablastes	CD3 <7	CD56<4.5	CD20 >6	CD27 >5	IgD <6	CD38 >3	CD138 <2		
NK	CD3 <7	CD56>4.5	CD20 <6						
NKT	CD3 >7	CD56>4.5	CD20 <6						
γδ T cells	CD3 >7	CD56<4.5	CD20 <6	TCRgd >4					
T CD20 +	CD3 >7	CD56<4.5	CD20>6						
MAIT cells	CD3 >7	CD56<4.5	CD20 <6	CD161> 3.5	TCRVa7.2>4.5				

Table 1 : Marqueurs de surface et gènes spécifiques utilisés pour l'identification des sous populations lymphocytaires et leur seuils (log2) associés.

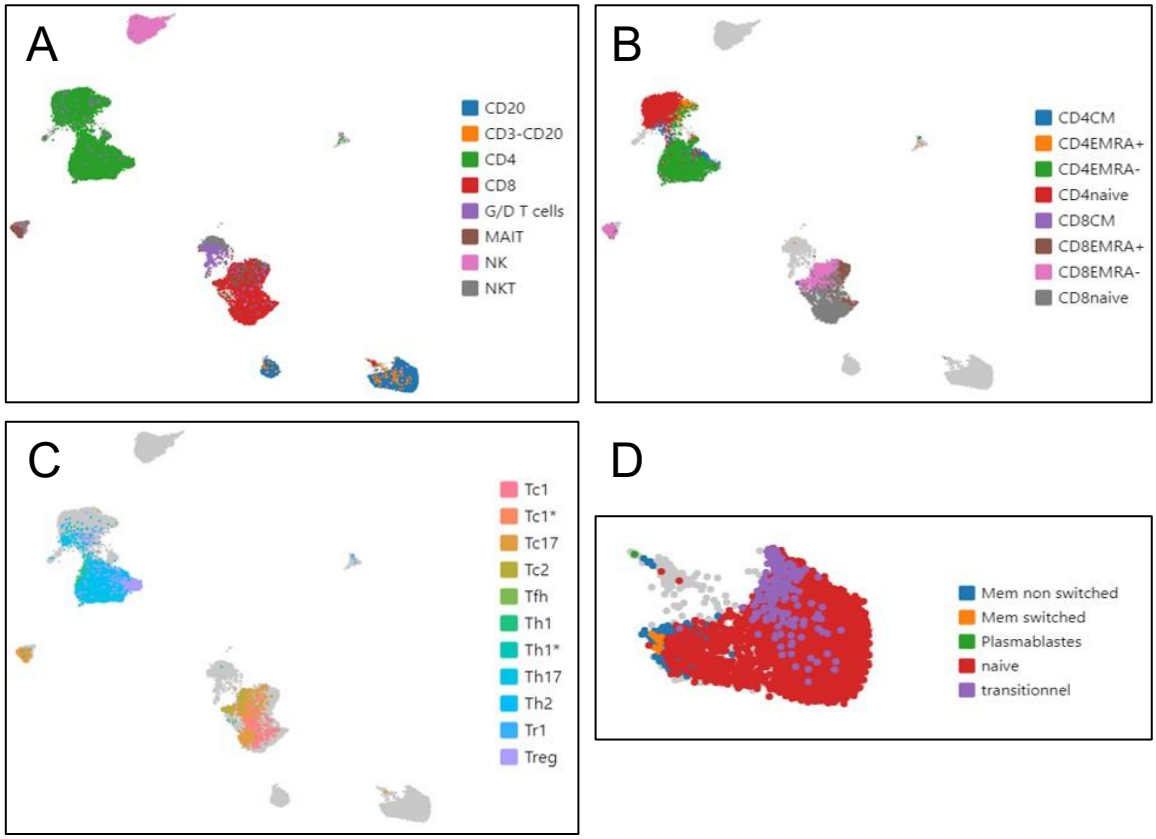
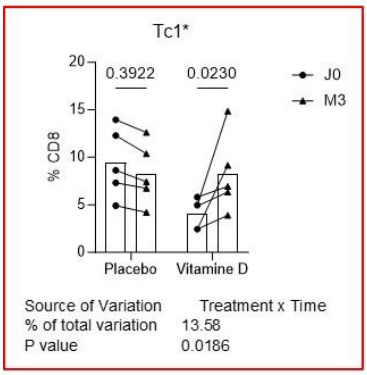
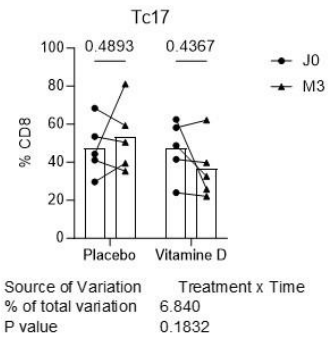


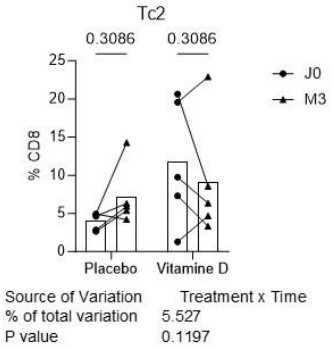
Figure 1 : Annotation des clusters à l'aide d'une combinaison de marqueurs de surface et d'expression génétique et inspection visuelle des marqueurs clés à l'aide de la visualisation UMAP sur Loupe®.

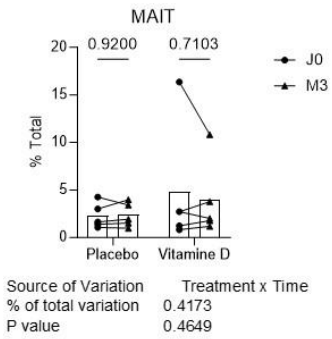
Modulation des populations

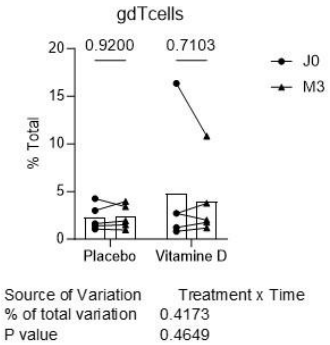
Dans les deux groupes, nous avons observé une variabilité interindividuelle dans la distribution des différentes sous populations. Cependant, pour chaque patient, aucun changement significatif n'a été observé dans les proportions des différentes sous-populations lymphocytaires après 3 mois (Figure 2), à l'exception des Tc1*, sous-type de lymphocytes T $CD8^+$ pro-inflammatoires (sécrétion d'IL-1b), qui ont augmenté après le traitement à la vitamine D (2-way ANOVA, p=0,0186).

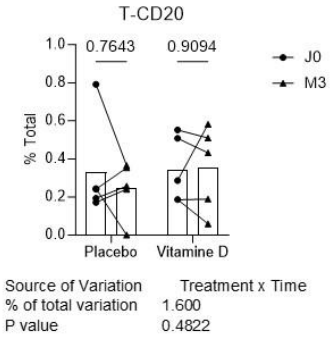


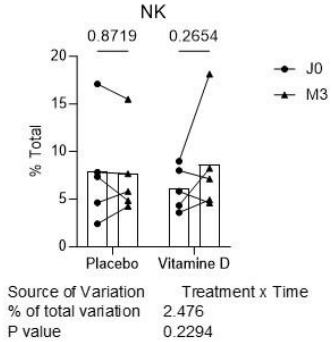


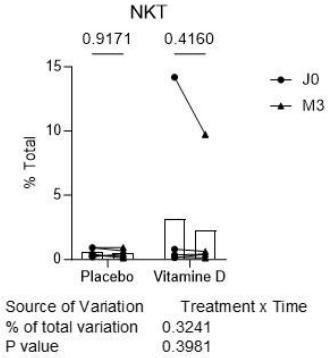


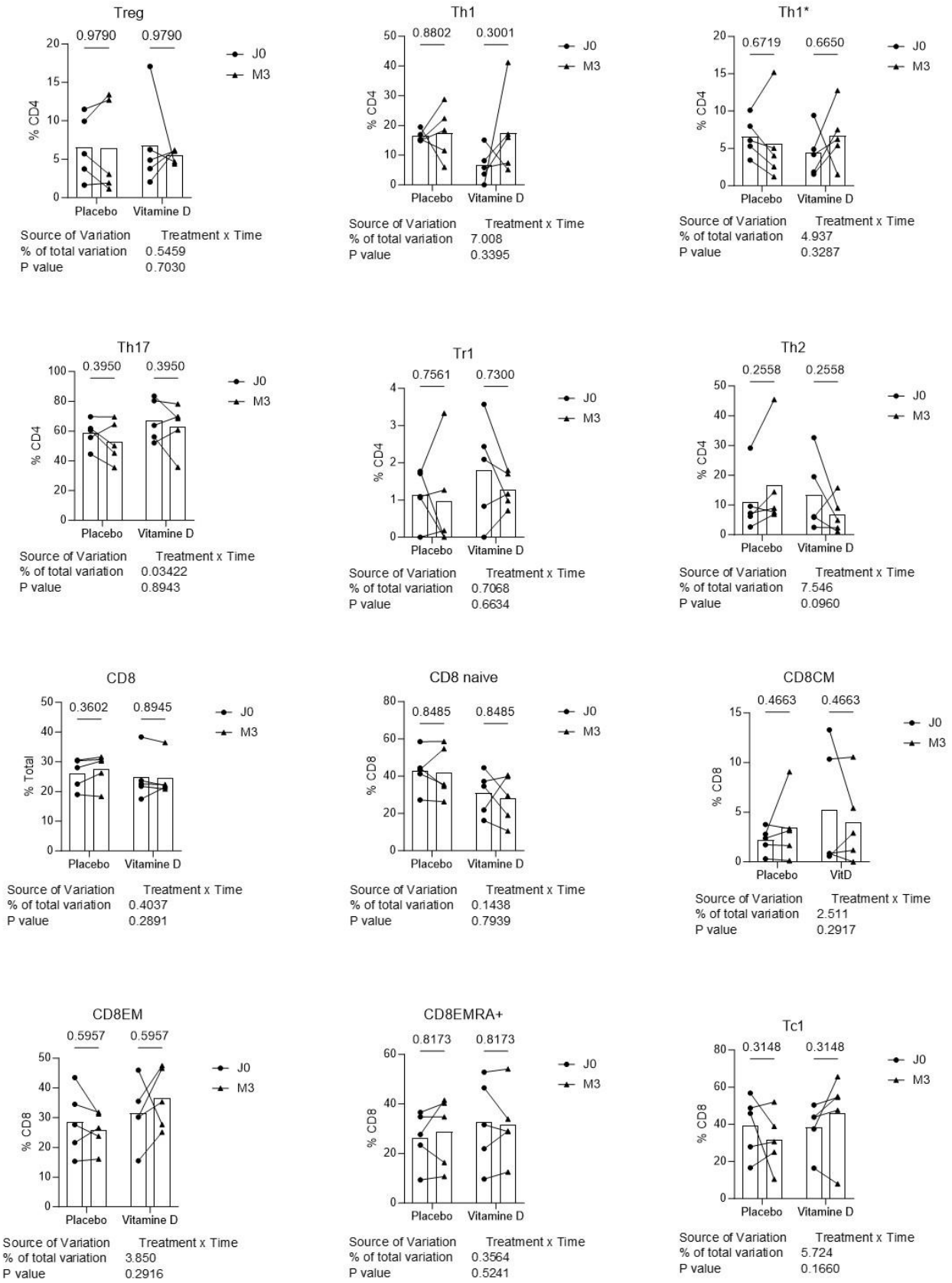












<u>Figure 2</u> : **Changements dans les proportions des populations lymphocytaires après traitement par placebo ou vitamine D.** 2-way-ANOVA avec correction FDR.

Modulation des protéines de surface

Pour aller plus loin dans l'analyse différentielle entre les groupes de traitement, j'ai utilisé la méthode du « global ratio » sur l'expression des marqueurs de surface utilisés en CITE-seq (Table Supp 1 - Chapitre 1) sur les deux types d'analyse : supervisée et non supervisée.

Dans l'analyse non supervisée (chapitre I), la vitamine D a augmenté l'expression de CD71 (B mémoires), CD226 et CD56 (MAIT), CD49d (Th17, CD8CM), CCR6 (Treg), CD8 (NKT) et ICOS (CD8CM) et a diminué l'expression de CD161 (B naïfs), CXCR4 (CD4 naïfs), CCR7 (CD4 naïfs, MAIT et Treg), GITR (Th1) et HLA.DR (NK).

Dans l'analyse supervisée, l'analyse quantitative de l'expression des protéines de surface utilisées pour le CITE-seq a permis de mettre en évidence une augmentation significative de l'expression de CD27 à la surface des lymphocytes B naïfs, de ICOS (CD4 EM), de CD49d (Th17 ; CD8 EMRA^{+}), de CD161 (Tc1) et de CCR5 (Tc17), et une diminution significative de l'expression de TCRVa7.2, LAG3, CD49d, CD226 (CD4 naïfs), de TCRγδ (CD4 naïfs, Treg), de CXCR4 (CD4 naïfs, CD4 EMRA+) CCR7 (γδTcells) et de IgM (NKT) (Table 2).

Analyse non supervisée

	Marqueurs de Surface
B mémoires	CD71 (1.29)
B naïfs	CD161 (0.55)
CD4 naïfs	CXCR4 (0.63) CCR7 (0.84)
MAIT	CCR7 (0.68) CD226 (1.57) CD56 (1.78)
Th17	CD49d (1.44)
Th1	GITR (0.62)
Treg	CCR6 (2.08) CCR7 (0.59)
NK	HLA.DR (0.71)
NKT	CD8 (2.44)
CD8CM	ICOS (2.20) CD49d (1.37)

Analyse supervisée

	Marqueurs de Surface
B naïfs	CD27 (1.19)
CD4 naïfs	TCRVa7.2 (0.50) LAG3 (0.65) CD49d (0.84) CXCR4 (0.68) TCRgd (0.59) CD226 (0.76)
CD4 EM	ICOS (1.25)
CD4EMRA+	CXCR4 (0.63)
Th17	CD49d (1.19)
Treg	TCRgd (0.58)
CD8 EMRA+	CD49d (1.36)
Tc1	CD161 (2.08)
Tc17	CCR5 (1.59)
GdTcells	CCR7 (0.71)
NKT	IgM (0.53)

Table 2 : Analyse de l'expression différentielle des marqueurs de surface.

Seuls les marqueurs de surface dont la p-value < 0,05 sont représentés.

Modulation de l'expression des gènes

Afin d'identifier l'effet de la vitamine D chez les patients atteints de sclérose en plaques, nous avons identifié les gènes différentiellement exprimés (DEGs) après 3 mois de traitement avec de fortes doses de vitamine D par rapport au placebo en utilisant un calcul de ratio global ((M3/J0 Vitamin D) / (M3/J0 placebo)). L'analyse n'a pas pu être effectuée sur l'ensemble des populations lymphocytaires préalablement décrites à cause du fait que certaines populations cellulaires n'étaient pas identifiables à un des deux temps du traitement.

Globalement, la vitamine D module davantage de gènes dans les populations B mémoires switchés, dans les Th1 et les Treg (Table 3, Annexe 1).

	Nombre de gènes	
	Up-régulés	Down-régulés
B naïfs	1	4
B mémoires non switchés	0	11
B mémoires switchés	27	3
CD4 naïfs	1	3
CD4 CM	6	3
CD4 EM	2	2
CD4 EMRA+	2	2
Th1	95	4
Th1*	1	5
Th2	5	0
Th17	2	4
Treg	36	0
CD8 naïfs	11	3
CD8 CM	0	3
CD8 EM	3	1
CD8 EMRA+	3	0
Tc1	6	1
Tc17	2	4
γδTcells	13	0
MAIT	9	2
NK	3	1
NKT	0	3

Table 3 : **Modulation de l'expression des gènes après 3 mois de traitement à la vitamine D.** Nombre de gènes différentiellement exprimés (DEGs) significatifs ($p<0.05$) dans chaque sous-ensemble de lymphocytes.

Certains gènes sont retrouvés dans plusieurs populations : *CAST* (B mémoires non switchés, γδTcells), *DAZAP2* (B mémoires non switchés, Th1), *JUN* (B mémoires non switchés, B naïfs), *ATP5MC3* (B mémoires switchés, Treg), *KMT2A* (B mémoires switchés, CD4 naïfs, Th1), *SEPTIN7* (B mémoires switchés, γδTcells), *SMG1* (B mémoires switchés, Th1), *SUB1* (B mémoires switchés, MAIT), *MT.ND1* (B naïfs, CD4 CM, CD8 EMRA^{+}, MAIT, Tc17), *ARGLU1* (CD4 EM, Th1), *SRSF2* (CD4 EM, Th17), *HNRNPA2B1* (CD8 CM, Th1), *ANRKD12* (CD8EM, CD8 EMRA^{+}, Th1), *KLF13* (CD8 EM, MAIT), *TCF25* (CD8 EM, NKT), *SLC38A1* (CD8 naïfs, Th1), *TAF1D* (CD8 naïfs, Th1), *ARL4C* (γδTcells, Th1), *HLA-F* (γδTcells, MAIT), *IKZF1* (γδTcells, Th17, Th1), *LCP1* (γδTcells, Th1), *STK17B* (γδTcells, MAIT, Th1), *EIF4G2* (Tc1, Th1), *CAPZB* (Th1, Treg) et *DDX17* (Th1*, Treg).

Discussion

La carence en vitamine D dans la SEP et ses effets immunomodulateurs sur le système immunitaire sont connus depuis longtemps sans que l'on ait pu encore mettre démontrer clairement son efficacité thérapeutique. En revanche, son intérêt dans la prise en charge des patients infectés par le SARS-CoV-2, le virus responsable du COVID-19, a suscité de nombreuses études physiopathologiques des effets immunorégulateurs de la vitamine D[113,114]. Toutefois, toutes les études dans la SEP ou dans d'autres contextes pathologiques, ont un impact limité du fait de l'analyse de grandes populations triées en FACS, d'absence de groupe placebo, ou de la présence de traitements de fonds concomitants.

Dans mon travail de thèse, j'ai pu étudier ces effets en effectuant plusieurs types d'analyses sur mes données issues de l'analyse de lymphocytes de patients SEP traités avec de fortes doses de vitamine D en monothérapie pendant 3 mois comparativement à un groupe placebo. J'ai donc pu étudier *in vivo* les modulations des différentes populations lymphocytaires, les changements

transcriptomiques de chaque sous population ainsi que les voies de signalisation impliquées par ces changements transcriptomiques.

Dans un premier temps, j'ai identifié différentes sous populations lymphocytaires soit par un clustering automatique, en utilisant une combinaison de marqueurs de surface et de gènes spécifiques des populations nécessaires à l'identification des populations avec RStudio (analyse non supervisée - chapitre I), la méthode de référence, soit par la combinaison de divers marqueurs de surface en définissant des seuils d'expression avec le logiciel Loupe (analyse supervisée - chapitre II). Ces analyses m'ont permis d'identifier 24 sous populations en analyse non supervisée contre 32 en analyse supervisée. Cette différence peut s'expliquer par le fait de forcer le clustering en analyse supervisée, me permettant ainsi de définir un très grand nombre de sous-populations en associant divers marqueurs de surface. Néanmoins, en comparant les proportions des différentes sous-populations lymphocytaires dans les deux types d'analyse, on remarque une grande variabilité interindividuelle dans la distribution des différentes sous populations. Il n'y a pas de différence significative entre les deux groupes après 3 mois de traitement pour la majorité des populations, excepté une augmentation des Tc1* dans le groupe vitamine D dans l'analyse supervisée et une augmentation des Th2 dans le groupe vitamine D dans l'analyse non supervisée. Étonnamment, une baisse non significative des Th2 a été observée dans l'analyse supervisée (2-way ANOVA = 0,0960) contre une augmentation de cette population dans l'analyse non supervisée (2-way ANOVA = 0,0446). Ici encore, cette différence peut s'expliquer par les seuils définis pour l'identification des populations pouvant être trop ou pas assez stricts, ce qui ne permet pas une bonne définition des populations. Les effets immunomodulateurs de la vitamine D rapportés dans la littérature sont principalement liés à une réduction de la polarisation des cellules $CD4^+$ naïves vers les profils pro-inflammatoires (Th1 et Th17) au profit d'une augmentation du développement des cellules à profil anti-inflammatoires (Th2 et Treg)[115,116] en augmentant la production d'IL-4 et en inhibant

celle d'IFN-γ, de l'IL-6 et de l'IL-23. Les résultats de l'analyse non supervisée semblent donc plus en accord avec la littérature. Dans l'étape de validation en HT-qPCR, il aurait été intéressant d'analyser les effets de la vitamine D sur les Th2, qui sont des cellules plutôt à profil anti-inflammatoire. La vitamine D pourrait avoir un effet qualitatif en plus du quantitatif observé par l'analyse non supervisée.

Dans un second temps, j'ai pu évaluer les effets de la vitamine D sur l'expression des protéines de surfaces, marquées par les anticorps barcodés utilisés lors de l'analyse CITE-seq. Pour ce faire, j'ai calculé la moyenne d'expression de chaque protéine et utilisé la méthode du ratio global : ((M3/J0 Vitamin D) / (M3/J0 placebo)) avec RStudio pour les deux types d'analyse : supervisée et non supervisée.

L'analyse des marqueurs de surface ainsi que l'analyse du transcriptome n'a pas pu être effectuée sur l'ensemble des populations lymphocytaires préalablement décrites avec l'analyse supervisée puisque certaines sous-populations n'étaient pas identifiables à un des deux temps du traitement, rendant impossible l'utilisation de la méthode du ratio global. Les populations qui n'ont pas pu être étudiées dans l'analyse supervisée sont les suivantes : B transitionnels, B régulateurs (Breg), plasmablastes, Tr1, Tc1*, Tc2 et T-CD20.

Ainsi, dans l'analyse supervisée, on retrouve 16 protéines de surface différentiellement exprimées, réparties dans 11 sous-populations contre 15 dans 10 populations pour l'analyse non supervisée. Parmi les populations qui montrent une expression des protéines de surface modulée par la vitamine D, 5 sont communes dans les deux analyses (B naïfs, CD4 naïfs, Th17, Treg et les NKT). En revanche, peu de modulations identiques des protéines de surface au sein d'une même population sont retrouvées dans les deux analyses : CXCR4 est diminuée dans les CD4 naïfs et CD49d est augmentée dans les Th17. Dans des modèles de maladies inflammatoires (asthme, arthrite rhumatoïde) l'inhibition de CXCR4 entraîne une amélioration symptomatique, suggérant l'implication de CXCR4 dans la migration des leucocytes effecteurs vers le site

affecté[117,118]. Dans notre étude, cela pourrait suggérer que moins de lymphocytes CD4 naïfs peuvent être recrutées au site inflammatoire, pouvant ainsi entraîner une diminution de la prolifération de lymphocytes à profil pro- comme anti- inflammatoires. De façon inattendue, CD49d est significativement augmentée dans le groupe vitamine D dans les Th17. La chaîne α (CD49d), constitue l'une des chaînes des intégrines α4β1 (*very late activating antigen-4* - VLA-4). VLA-4 est essentiel pour la transmigration des cellules Th1 dans le SNC dans des conditions inflammatoires telles que la SEP[119]. Des traitements immunosuppresseurs comme le Natalizumab, un inhibiteur sélectif des molécules d'adhésion, sont déjà utilisés à ce jour dans le traitement de la SEP. Ce traitement se fixe sur la sous-unité α4 des intégrines humaines, plus spécifiquement, à l'intégrine α4ß1 en bloquant l'interaction de cette molécule avec son récepteur, la molécule VCAM-1 (*Vascular Cell Adhesion Molecule*-1). Le blocage des interactions moléculaires entre l'α4ß1 et ses cibles diminue l'activité inflammatoire cérébrale chez le sujet SEP et inhibe le recrutement ultérieur de cellules immunitaires dans les tissus inflammatoires, diminuant la formation ou l'extension des lésions de SEP[120]. Dans notre étude avec les deux types d'analyse, la vitamine D semble paradoxalement augmenter les capacités d'adhésion des Th17, une population pourtant pro-inflammatoire. Ce résultat peut s'expliquer par le phénomène de « off-Target » parfois observé pour certains traitements. De la même façon, une étude évaluant l'efficacité et la sécurité de l'Ocrélizumab, un anti-CD20, chez des patients SEP précoce n'ayant jamais été exposés à des traitements de fond a révélé des modifications inattendues dans d'autres populations lymphocytaires, notamment sur les cellules T effecteur mémoires, et en particulier une diminution des cellules T CD8 EM exprimant le CCR5[121].

Enfin, j'ai pu étudier les modifications transcriptomiques liés aux 3 mois de traitement à fortes doses de vitamine D grâce à l'analyse supervisée et non supervisée avec la méthode du ratio global ((M3/J0 Vitamin D)/(M3/J0 placebo)), et grâce à une analyse DESeq2 sur les clusters

issus de mon analyse non supervisée. De manière intéressante, on ne retrouve pas le même nombre de gènes modulés par la vitamine D en fonction du type d'analyse pour les mêmes populations lymphocytaires. Cela s'explique par l'application des divers seuils et par les cellules considérées dans ces clusters par l'analyse supervisée. En comparant les populations identiques aux deux types d'analyse (supervisée et non supervisée) et entre toutes les méthodes (ratio global et DESeq2), il a été possible d'identifier des gènes communs parmi 8 sous populations entre l'analyse supervisée et l'analyse non supervisée (Th17, Treg, CD4 naïf, CD8 naïf, B naïf, B mémoires, MAIT et NK). Entre l'analyse DESeq2 et l'analyse supervisée, on retrouve un gène en commun dans les Th17 (*TRA2B*), un gène avec une modulation opposée dans les Treg (*CD2*), 1 dans les NK (*HSH2D*). Entre le global ratio de l'analyse supervisée et celui de l'analyse non supervisée, 1 gène est en commun dans les Treg (*NDUFB8*), 1 dans les CD8 naïfs (*LAMTOR4*), 3 dans les MAIT (*STK17B*, *PAIP2*, *C9orf78*) et 1 dans les CD4 naïfs (*KMT2A*). Quatre populations ont des gènes en communs dans toutes les analyses : *SP100* dans les CD8 naïfs, *JUN* et *DUSP1* dans les B naïfs, *PSMB3* dans les B mémoires et *SUB1* dans les MAIT. Les gènes retrouvés dans toutes les analyses ont été choisis pour l'étape de validation en HT-qPCR ainsi que ceux présentant une forte modulation (> x 1.2 fold-change). Le seul gène à avoir été validé dans les deux analyses est *SUB1* (aussi nommé *PC4*) dans les MAIT. SUB1 est une protéine de réparation de l'ADN qui participe à la coordination des réponses cellulaires aux cassures de l'ADN double brin (*DNA double-strand breaks - DSB*). Il est intéressant de noter qu'il existe des parallèles fonctionnels potentiels entre l'alpha-synucléine, la protéine impliquée dans la maladie de Parkinson, une autre maladie neurodégénérative[122] et SUB1. En outre, PC4/SUB1 fonctionne comme un régulateur des réponses cellulaires au stress oxydatif[123], avec la capacité de protéger l'ADN des dommages oxydatifs et de coordonner des formes spécifiques de réparation des DSB[124]. Dans notre étude, SUB1 est augmenté dans toutes les

analyses statistiques dans les MAIT après 3 mois de traitement par vitamine D mais son rôle précis reste à déterminer par des études complémentaires.

Toutefois, mon travail de thèse comporte certaines limites. D'une part, le CITE-seq est une technique permettant d'effectuer le séquençage de l'ARN tout en obtenant des informations quantitatives et qualitatives sur les protéines de surface avec des anticorps à l'échelle de la cellule unique. Néanmoins, des études ont montré qu'il existait une faible corrélation entre l'expression de l'ARN et la présence de la protéine[125]. Ce que nous observons donc avec le CITE-seq n'est pas forcément le reflet de la présence ou non en surface des protéines correspondantes, excepté pour les protéines de surface dont nous avons utilisé des anticorps dirigés contre ces dernières pour le CITE-seq.

D'autre part, une seconde limite de mon projet est liée aux "*temps*" choisis pour mes échantillons. En effet, en utilisant J0 et M3, nous n'observons pas les mêmes gènes qu'après une stimulation ponctuelle. Hanel et Carlberg en 2022 ont mis en évidence un différentiel temporel des effets de la vitamine D sur le système immunitaire[126]. De ce fait, nous n'observons donc pas des modulations de gènes à réponse rapide dits "*early genes*" ou ciblés directement, mais plutôt des modulations de gènes à réponse lente, dits "*late genes*" ou ciblés indirectement, ce qui permet de distinguer les effets stables d'une stimulation par la vitamine D chez l'Homme. C'est pourquoi nous n'observons pas de modification significative de l'expression des gènes modulés par la vitamine D dans les études de pulse, *in vitro* ou dans des modèles EAE comme par exemple les gènes liés au métabolisme de la vitamine D. Ceci peut néanmoins constituer un avantage important lorsque l'on s'intéresse aux effets chroniques de la vitamine D sur le système immunitaire adaptatif dans le contexte d'un traitement au long cours dans la SEP comme dans d'autres pathologies inflammatoires.

Conclusion

Dans ce travail, j'ai mis au point deux méthodes d'analyse différentes (supervisée ou non supervisée) ainsi que deux types d'analyses statistiques (global ratio ou DESeq2) pour traiter les données de CITE-seq de PBMC de patients SEP traités avec de fortes doses de vitamine D. Au total, cela représente 1411 gènes identifiés comme cible par la vitamine D dans 21 populations lymphocytaires différentes. L'analyse supervisée n'est pas celle couramment utilisée dans la littérature, elle permet toutefois d'établir une liste de gènes à étudier dans des populations plus fines que l'analyse non supervisée. En revanche, l'analyse non supervisée fournit des résultats plus proches dans la globalité de ce qui a été montré dans la littérature et représente la méthode couramment utilisée dans le traitement de ce type de données. Pour conclure sur l'effet chronique de la vitamine D sur son effet et son mode d'action chez les patients atteints de sclérose en plaques, il semblerait que cette dernière contrebalance les dérèglements engendrés par EBV dans les lymphocytes B. Son action sur les lymphocytes T est plus nuancée notamment avec une augmentation de certains marqueurs d'adhésion dans certaines populations à profil pro-inflammatoire. Il serait intéressant d'étudier les effets combinés des traitements de fond de la sclérose en plaques avec un traitement à fortes doses de vitamine D pour un meilleur effet sur l'inflammation périphérique.

Références

1. Ramagopalan SV, Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. *The Lancet Neurology*. 2010;9(7):727-739. doi:10.1016/S1474-4422(10)70094-6

2. Compston A, Coles A. Multiple sclerosis. 2008;372:16.

3. Risk Alleles for Multiple Sclerosis Identified by a Genomewide Study. *N Engl J Med*. 2007;357(9):851-862. doi:10.1056/NEJMoa073493

4. The International Multiple Sclerosis Genetics Consortium & The Wellcome Trust Case Control Consortium 2. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature*. 2011;476(7359):214-219. doi:10.1038/nature10251

5. Ramagopalan SV, Dyment DA, Cader MZ, et al. Rare variants in the CYP27B1 gene are associated with multiple sclerosis. *Ann Neurol*. 2011;70(6):881-886. doi:10.1002/ana.22678

6. International Multiple Sclerosis Genetics Consortium (IMSGC). Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat Genet*. 2013;45(11):1353-1360. doi:10.1038/ng.2770

7. Vukusic S, Hutchinson M, Hours M, et al. Pregnancy and multiple sclerosis (the PRIMS study): clinical predictors of post-partum relapse. *Brain*. 2004;127(6):1353-1360. doi:10.1093/brain/awh152

8. Harroud A, Mitchell RE, Richardson TG, et al. Childhood obesity and multiple sclerosis: A Mendelian randomization study. *Mult Scler*. 2021;27(14):2150-2158. doi:10.1177/13524585211001781

9. Marrodan M, Farez MF, Balbuena Aguirre ME, Correale J. Obesity and the risk of Multiple Sclerosis. The role of Leptin. *Ann Clin Transl Neurol*. 2021;8(2):406-424. doi:10.1002/acn3.51291

10. Rasul T, Frederiksen JL. Link between overweight/obese in children and youngsters and occurrence of multiple sclerosis. *J Neurol*. 2018;265(12):2755-2763. doi:10.1007/s00415-018-8869-9

11. Hedström AK, Olsson T, Alfredsson L. High body mass index before age 20 is associated with increased risk for multiple sclerosis in both men and women. *Mult Scler*. 2012;18(9):1334-1336. doi:10.1177/1352458512436596

12. D'hooghe MB, Haentjens P, Nagels G, De Keyser J. Alcohol, coffee, fish, smoking and disease progression in multiple sclerosis: Lifestyle associated with progression in MS. *European Journal of Neurology*. 2012;19(4):616-624. doi:10.1111/j.1468-1331.2011.03596.x

13. Hedström AK, Hillert J, Olsson T, Alfredsson L. Smoking and multiple sclerosis susceptibility. *Eur J Epidemiol*. 2013;28(11):867-874. doi:10.1007/s10654-013-9853-4

14. Hedström AK. Smoking and disability progression in multiple sclerosis. *Expert Review of Neurotherapeutics*. 2020;20(8):739-741. doi:10.1080/14737175.2020.1743176

15. Hedström AK, Hillert J, Olsson T, Alfredsson L. Alcohol as a Modifiable Lifestyle Factor Affecting Multiple Sclerosis Risk. *JAMA Neurol*. 2014;71(3):300. doi:10.1001/jamaneurol.2013.5858

16. Andersen C, Søndergaard HB, Bang Oturai D, et al. Alcohol consumption in adolescence is associated with a lower risk of multiple sclerosis in a Danish cohort. *Mult Scler*. 2019;25(12):1572-1579. doi:10.1177/1352458518795418

17. Zhu T, Ye X, Zhang T, et al. Association between alcohol consumption and multiple sclerosis: a meta-analysis of observational studies. *Neurol Sci*. 2015;36(9):1543-1550. doi:10.1007/s10072-015-2326-7

18. Massa J, O'Reilly E, Munger K, Ascherio A. Caffeine and alcohol intakes have no association with risk of multiple sclerosis. *Mult Scler*. 2013;19(1):53-58. doi:10.1177/1352458512448108

19. Pakpoor J, Goldacre R, Disanto G, Giovannoni G, Goldacre MJ. Alcohol Misuse Disorders and Multiple Sclerosis Risk. *JAMA Neurol*. 2014;71(9):1188. doi:10.1001/jamaneurol.2014.1795

20. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: The role of infection. *Ann Neurol*. 2007;61(4):288-299. doi:10.1002/ana.21117

21. Bjornevik K, Cortese M, Healy BC, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science*. Published online January 21, 2022:science.abj8222. doi:10.1126/science.abj8222

22. Cohen JI. Epstein–Barr Virus Infection. *N Engl J Med*. 2000;343(7):481-492. doi:10.1056/NEJM200008173430707

23. Thacker EL, Mirzaei F, Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: A meta-analysis. *Ann Neurol*. 2006;59(3):499-503. doi:10.1002/ana.20820

24. Sundstrom P, Juto P, Wadell G, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: A prospective study. *Neurology*. 2004;62(12):2277-2282. doi:10.1212/01.WNL.0000130496.51156.D7

25. Pender MP. The Essential Role of Epstein-Barr Virus in the Pathogenesis of Multiple Sclerosis. *Neuroscientist*. 2011;17(4):351-367. doi:10.1177/1073858410381531

26. Holick MF. Vitamin D Deficiency. *N Engl J Med*. 2007;357(3):266-281. doi:10.1056/NEJMra070553

27. Van Der Mei IAF. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. *BMJ*. 2003;327(7410):316-0. doi:10.1136/bmj.327.7410.316

28. Simpson S, Wang W, Otahal P, Blizzard L, Van Der Mei IAF, Taylor BV. Latitude continues to be significantly associated with the prevalence of multiple sclerosis: an updated meta-analysis. *J Neurol Neurosurg Psychiatry*. 2019;90(11):1193-1200. doi:10.1136/jnnp-2018-320189

29. Tao C, Simpson S, Van Der Mei I, et al. Higher latitude is significantly associated with an earlier age of disease onset in multiple sclerosis. *J Neurol Neurosurg Psychiatry*. 2016;87(12):1343-1349. doi:10.1136/jnnp-2016-314013

30. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA*. 2006;296(23):2832-2838. doi:10.1001/jama.296.23.2832

31. Ramagopalan SV, Maugeri NJ, Handunnetthi L, et al. Expression of the Multiple Sclerosis-Associated MHC Class II Allele HLA-DRB1*1501 Is Regulated by Vitamin D. Roopenian DC, ed. *PLoS Genet*. 2009;5(2):e1000369. doi:10.1371/journal.pgen.1000369

32. Miller D, Barkhof F, Montalban X, Thompson A, Filippi M. Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. *The Lancet Neurology*. 2005;4(5):281-288. doi:10.1016/S1474-4422(05)70071-5

33. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: Guidelines from the international panel on the diagnosis of multiple sclerosis. *Ann Neurol*. 2001;50(1):121-127. doi:10.1002/ana.1032

34. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria." *Ann Neurol*. 2005;58(6):840-846. doi:10.1002/ana.20703

35. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. *Ann Neurol*. 2011;69(2):292-302. doi:10.1002/ana.22366

36. Thompson AJ, Banwell BL, Barkhof F, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *The Lancet Neurology*. 2018;17(2):162-173. doi:10.1016/S1474-4422(17)30470-2

37. Calabrese M, Filippi M, Gallo P. Cortical lesions in multiple sclerosis. *Nat Rev Neurol*. 2010;6(8):438-444. doi:10.1038/nrneurol.2010.93

38. Dutta R, Chang A, Doud MK, et al. Demyelination causes synaptic alterations in hippocampi from multiple sclerosis patients. *Ann Neurol*. 2011;69(3):445-454. doi:10.1002/ana.22337

39. Lucchinetti CF, Popescu BFG, Bunyan RF, et al. Inflammatory Cortical Demyelination in Early Multiple Sclerosis. *N Engl J Med*. 2011;365(23):2188-2197. doi:10.1056/NEJMoa1100648

40. Fitzner B, Hecker M, Zettl UK. Molecular biomarkers in cerebrospinal fluid of multiple sclerosis patients. *Autoimmunity Reviews*. 2015;14(10):903-913. doi:10.1016/j.autrev.2015.06.001

41. Stangel M, Fredrikson S, Meinl E, Petzold A, Stüve O, Tumani H. The utility of cerebrospinal fluid analysis in patients with multiple sclerosis. *Nat Rev Neurol*. 2013;9(5):267-276. doi:10.1038/nrneurol.2013.41

42. Chitnis T, Qureshi F, Gehman VM, et al. *Inflammatory and Neurodegenerative Serum Protein Biomarkers Increase Sensitivity to Detect Disease Activity in Multiple Sclerosis*. Neurology; 2023. doi:10.1101/2023.06.28.23291157

43. Awad A, Hemmer B, Hartung HP, Kieseier B, Bennett JL, Stuve O. Analyses of cerebrospinal fluid in the diagnosis and monitoring of multiple sclerosis. *Journal of Neuroimmunology*. 2010;219(1-2):1-7. doi:10.1016/j.jneuroim.2009.09.002

44. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KHG. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clinical and Experimental Immunology*. 2010;162(1):1-11. doi:10.1111/j.1365-2249.2010.04143.x

45. Huseby ES, Huseby PG, Shah S, Smith R, Stadinski BD. Pathogenic CD8 T Cells in Multiple Sclerosis and Its Experimental Models. *Front Immun*. 2012;3. doi:10.3389/fimmu.2012.00064

46. Bettelli E, Sullivan B, Szabo SJ, Sobel RA, Glimcher LH, Kuchroo VK. Loss of T-bet, But Not STAT1, Prevents the Development of Experimental Autoimmune Encephalomyelitis. *The Journal of Experimental Medicine*. 2004;200(1):79-87. doi:10.1084/jem.20031819

47. Dettke M, Scheidt P, Prange H, Kirchner H. [No title found]. *Journal of Clinical Immunology*. 1997;17(4):293-300. doi:10.1023/A:1027374615106

48. Petereit HF, Richter N, Pukrop R, Bamborschke S. Interferon gamma production in blood lymphocytes correlates with disability score in multiple sclerosis patients. *Mult Scler*. 2000;6(1):19-23. doi:10.1177/135245850000600105

49. Drulović J, Mostarica-Stojković M, Lević Z, Stojsavljević N, Pravica V, Mesaroš Š. Interleukin-12 and tumor necrosis factor-α levels in cerebrospinal fluid of multiple sclerosis patients. *Journal of the Neurological Sciences*. 1997;147(2):145-150. doi:10.1016/S0022-510X(96)05320-8

50. Hofstetter HH, Grau C, Buttmann M, et al. The PLPp-specific T-cell population promoted by pertussis toxin is characterized by high frequencies of IL-17-producing cells. *Cytokine*. 2007;40(1):35-43. doi:10.1016/j.cyto.2007.07.192

51. Kebir H, Kreymborg K, Ifergan I, et al. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med*. 2007;13(10):1173-1175. doi:10.1038/nm1651

52. Tzartos JS, Friese MA, Craner MJ, et al. Interleukin-17 Production in Central Nervous System-Infiltrating T Cells and Glial Cells Is Associated with Active Disease in Multiple Sclerosis. *The American Journal of Pathology*. 2008;172(1):146-155. doi:10.2353/ajpath.2008.070690

53. Komiyama Y, Nakae S, Matsuki T, et al. IL-17 Plays an Important Role in the Development of Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology*. 2006;177(1):566-573. doi:10.4049/jimmunol.177.1.566

54. Hall JA, Pokrovskii M, Kroehling L, et al. Transcription factor RORα enforces stability of the Th17 cell effector program by binding to a Rorc cis-regulatory element. *Immunity*. 2022;55(11):2027-2043.e9. doi:10.1016/j.immuni.2022.09.013

55. Brucklacher-Waldert V, Stuerner K, Kolster M, Wolthausen J, Tolosa E. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. *Brain*. 2009;132(12):3329-3341. doi:10.1093/brain/awp289

56. Matusevicius D, Kivisäkk P, He B, et al. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler*. 1999;5(2):101-104. doi:10.1177/135245859900500206

57. Arellano G, Acuña E, Reyes LI, et al. Th1 and Th17 Cells and Associated Cytokines Discriminate among Clinically Isolated Syndrome and Multiple Sclerosis Phenotypes. *Front Immunol*. 2017;8:753. doi:10.3389/fimmu.2017.00753

58. Kunkl M, Frascolla S, Amormino C, Volpe E, Tuosto L. T Helper Cells: The Modulators of Inflammation in Multiple Sclerosis. *Cells*. 2020;9(2):482. doi:10.3390/cells9020482

59. Loos J, Schmaul S, Noll TM, et al. Functional characteristics of Th1, Th17, and ex-Th17 cells in EAE revealed by intravital two-photon microscopy. *J Neuroinflammation*. 2020;17(1):357. doi:10.1186/s12974-020-02021-x

60. Shevach EM. Mechanisms of Foxp3+ T Regulatory Cell-Mediated Suppression. *Immunity*. 2009;30(5):636-645. doi:10.1016/j.immuni.2009.04.010

61. Haas J, Hug A, Viehöver A, et al. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol*. 2005;35(11):3343-3352. doi:10.1002/eji.200526065

62. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *The Journal of experimental medicine*. 2004;199(7):971-979. doi:10.1084/jem.20031579

63. Venken K, Hellings N, Thewissen M, et al. Compromised CD4 $^{+}$ CD25 ^{high} regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology*. 2008;123(1):79-89. doi:10.1111/j.1365-2567.2007.02690.x

64. Venken K, Hellings N, Broekmans T, Hensen K, Rummens JL, Stinissen P. Natural Naive CD4+CD25+CD127low Regulatory T Cell (Treg) Development and Function Are Disturbed in Multiple Sclerosis Patients: Recovery of Memory Treg Homeostasis during Disease Progression. *The Journal of Immunology*. 2008;180(9):6411-6420. doi:10.4049/jimmunol.180.9.6411

65. Michel L, Berthelot L, Pettré S, et al. Patients with relapsing-remitting multiple sclerosis have normal Treg function when cells expressing IL-7 receptor α-chain are excluded from the analysis. *J Clin Invest*. Published online September 1, 2008:JCI35365. doi:10.1172/JCI35365

66. Roy A. Myelin Basic Protein-primed T Helper 2 Cells Suppress Microglial Activation via AlphaVBeta3 Integrin: Implications for Multiple Sclerosis. *J Clin Cell Immunol*. 2013;04(04). doi:10.4172/2155-9899.1000158

67. Planas R, Metz I, Ortiz Y, et al. Central role of Th2/Tc2 lymphocytes in pattern II multiple sclerosis lesions. *Ann Clin Transl Neurol*. 2015;2(9):875-893. doi:10.1002/acn3.218

68. Fernando V, Omura S, Sato F, et al. Regulation of an Autoimmune Model for Multiple Sclerosis in Th2-Biased GATA3 Transgenic Mice. *IJMS*. 2014;15(2):1700-1718. doi:10.3390/ijms15021700

69. Booss J, Esiri MM, Tourtellotte WW, Mason DY. Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. *Journal of the Neurological Sciences*. 1983;62(1-3):219-232. doi:10.1016/0022-510X(83)90201-0

70. Hauser SL, Bhan AK, Gilles F, Kemp M, Kerr C, Weiner HL. Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. *Ann Neurol*. 1986;19(6):578-587. doi:10.1002/ana.410190610

71. Salou M, Garcia A, Michel L, et al. Expanded CD 8 T-cell sharing between periphery and CNS in multiple sclerosis. *Ann Clin Transl Neurol*. 2015;2(6):609-622. doi:10.1002/acn3.199

72. Lolli F, Martini H, Citro A, et al. Increased CD8+ T cell responses to apoptotic T cell-associated antigens in multiple sclerosis. *J Neuroinflammation*. 2013;10(1):862. doi:10.1186/1742-2094-10-94

73. Crotty S. Follicular Helper CD4 T Cells (T_{FH}). *Annu Rev Immunol*. 2011;29(1):621-663. doi:10.1146/annurev-immunol-031210-101400

74. Dhaeze T, Peelen E, Hombrouck A, et al. Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis. *The Journal of Immunology*. 2015;195(3):832-840. doi:10.4049/jimmunol.1500759

75. Crotty S. T Follicular Helper Cell Differentiation, Function, and Roles in Disease. *Immunity*. 2014;41(4):529-542. doi:10.1016/j.immuni.2014.10.004

76. Kroenke MA, Eto D, Locci M, et al. Bcl6 and Maf Cooperate To Instruct Human Follicular Helper CD4 T Cell Differentiation. *The Journal of Immunology*. 2012;188(8):3734-3744. doi:10.4049/jimmunol.1103246

77. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells: Tr1 cells. *Immunological Reviews*. 2001;182(1):68-79. doi:10.1034/j.1600-065X.2001.1820105.x

78. Gregori S, Amodio G, Passerini L, Santoni De Sio FR. Alteration of interleukin-10-producing Type 1 regulatory cells in autoimmune diseases. *Current Opinion in Hematology*. 2022;29(4):218-224. doi:10.1097/MOH.0000000000000720

79. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol*. 2007;7(8):585-598. doi:10.1038/nri2138

80. Montalban X, Arnold DL, Weber MS, et al. Placebo-Controlled Trial of an Oral BTK Inhibitor in Multiple Sclerosis. *N Engl J Med*. 2019;380(25):2406-2417. doi:10.1056/NEJMoa1901981

81. Bar-Or A, Fawaz L, Fan B, et al. Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol*. 2010;67(4):452-461. doi:10.1002/ana.21939

82. Duddy M, Niino M, Adatia F, et al. Distinct Effector Cytokine Profiles of Memory and Naive Human B Cell Subsets and Implication in Multiple Sclerosis. *The Journal of Immunology*. 2007;178(10):6092-6099. doi:10.4049/jimmunol.178.10.6092

83. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest*. Published online September 18, 2008:JCI36030. doi:10.1172/JCI36030

84. Couloume L, Ferrant J, Le Gallou S, et al. Mass Cytometry Identifies Expansion of T-bet+ B Cells and CD206+ Monocytes in Early Multiple Sclerosis. *Front Immunol*. 2021;12:653577. doi:10.3389/fimmu.2021.653577

85. Gandhi R, Laroni A, Weiner HL. Role of the innate immune system in the pathogenesis of multiple sclerosis. *Journal of Neuroimmunology*. 2010;221(1-2):7-14. doi:10.1016/j.jneuroim.2009.10.015

86. Carnero Contentti E, Farez MF, Correale J. Mucosal-Associated Invariant T Cell Features and TCR Repertoire Characteristics During the Course of Multiple Sclerosis. *Front Immunol*. 2019;10:2690. doi:10.3389/fimmu.2019.02690

87. Le Bourhis L, Martin E, Péguillet I, et al. Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol*. 2010;11(8):701-708. doi:10.1038/ni.1890

88. Filippi M, Bar-Or A, Piehl F, et al. Multiple sclerosis. *Nat Rev Dis Primers*. 2018;4(1):43. doi:10.1038/s41572-018-0041-4

89. Larochelle C, Alvarez JI, Prat A. How do immune cells overcome the blood-brain barrier in multiple sclerosis? *FEBS Letters*. 2011;585(23):3770-3780. doi:10.1016/j.febslet.2011.04.066

90. Ortiz GG, Pacheco-Moisés FP, Macías-Islas MÁ, et al. Role of the Blood–Brain Barrier in Multiple Sclerosis. *Archives of Medical Research*. 2014;45(8):687-697. doi:10.1016/j.arcmed.2014.11.013

91. Tonietto M, Poirion E, Lazzarotto A, et al. Periventricular remyelination failure in multiple sclerosis: a substrate for neurodegeneration. *Brain*. 2023;146(1):182-194. doi:10.1093/brain/awac334

92. Papadopoulos D, Dukes S, Patel R, Nicholas R, Vora A, Reynolds R. Substantial Archaeocortical Atrophy and Neuronal Loss in Multiple Sclerosis. *Brain Pathology*. 2009;19(2):238-253. doi:10.1111/j.1750-3639.2008.00177.x

93. La Mantia L, Munari LM, Lovati R. Glatiramer acetate for multiple sclerosis. Cochrane Multiple Sclerosis and Rare Diseases of the CNS Group, ed. *Cochrane Database of Systematic Reviews*. Published online May 12, 2010. doi:10.1002/14651858.CD004678.pub2

94. Jakimovski D, Kolb C, Ramanathan M, Zivadinov R, Weinstock-Guttman B. Interferon β for Multiple Sclerosis. *Cold Spring Harb Perspect Med*. 2018;8(11):a032003. doi:10.1101/cshperspect.a032003

95. Hauser SL, Bar-Or A, Comi G, et al. Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *N Engl J Med*. 2017;376(3):221-234. doi:10.1056/NEJMoa1601277

96. Polman CH, O'Connor PW, Havrdova E, et al. A Randomized, Placebo-Controlled Trial of Natalizumab for Relapsing Multiple Sclerosis. *N Engl J Med*. 2006;354(9):899-910. doi:10.1056/NEJMoa044397

97. Chitnis T, Arnold DL, Banwell B, et al. Trial of Fingolimod versus Interferon Beta-1a in Pediatric Multiple Sclerosis. *N Engl J Med*. 2018;379(11):1017-1027. doi:10.1056/NEJMoa1800149

98. Hartung HP, Gonsette R, Konig N, et al. Mitoxantrone in progressive multiple sclerosis: a placebo-controlled, double-blind, randomised, multicentre trial. *The Lancet*. 2002;360(9350):2018-2025. doi:10.1016/S0140-6736(02)12023-X

99. Coles AJ, Twyman CL, Arnold DL, et al. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *The Lancet*. 2012;380(9856):1829-1839. doi:10.1016/S0140-6736(12)61768-1

100. Giovannoni G, Comi G, Cook S, et al. A Placebo-Controlled Trial of Oral Cladribine for Relapsing Multiple Sclerosis. *N Engl J Med*. 2010;362(5):416-426. doi:10.1056/NEJMoa0902533

101. Reich DS, Arnold DL, Vermersch P, et al. Safety and efficacy of tolebrutinib, an oral brain-penetrant BTK inhibitor, in relapsing multiple sclerosis: a phase 2b, randomised, double-blind, placebo-controlled trial. *The Lancet Neurology*. 2021;20(9):729-738. doi:10.1016/S1474-4422(21)00237-4

102. Cadavid D, Mellion M, Hupperts R, et al. Safety and efficacy of opicinumab in patients with relapsing multiple sclerosis (SYNERGY): a randomised, placebo-controlled, phase 2 trial. *The Lancet Neurology*. 2019;18(9):845-856. doi:10.1016/S1474-4422(19)30137-1

103. Abdalla MA, Zakhary CM, Rushdi H, et al. The Effectiveness of Statins as Potential Therapy for Multiple Sclerosis: A Systematic Review of Randomized Controlled trials. *Cureus*. Published online September 19, 2021. doi:10.7759/cureus.18092

104. Cree BAC, Cutter G, Wolinsky JS, et al. Safety and efficacy of MD1003 (high-dose biotin) in patients with progressive multiple sclerosis (SPI2): a randomised, double-blind,

placebo-controlled, phase 3 trial. *The Lancet Neurology*. 2020;19(12):988-997. doi:10.1016/S1474-4422(20)30347-1

105. Alfredsson L, Olsson T. Lifestyle and Environmental Factors in Multiple Sclerosis. *Cold Spring Harb Perspect Med*. 2019;9(4):a028944. doi:10.1101/cshperspect.a028944

106. Westerlind H, Ramanujam R, Uvehag D, et al. Modest familial risks for multiple sclerosis: a registry-based study of the population of Sweden. *Brain*. 2014;137(Pt 3):770-778. doi:10.1093/brain/awt356

107. Simpson S, Taylor B, Blizzard L, et al. Higher 25-hydroxyvitamin D is associated with lower relapse risk in MS. *Ann Neurol*. Published online April 6, 2010:n/a-n/a. doi:10.1002/ana.22043

108. Mowry EM, Waubant E, McCulloch CE, et al. Vitamin D status predicts new brain magnetic resonance imaging activity in multiple sclerosis. *Ann Neurol*. 2012;72(2):234-240. doi:10.1002/ana.23591

109. Ostkamp P, Salmen A, Pignolet B, et al. Sunlight exposure exerts immunomodulatory effects to reduce multiple sclerosis severity. *Proc Natl Acad Sci USA*. 2021;118(1):e2018457118. doi:10.1073/pnas.2018457118

110. Thouvenot E, Orsini M, Daures JP, Camu W. Vitamin D is associated with degree of disability in patients with fully ambulatory relapsing-remitting multiple sclerosis. *Eur J Neurol*. 2015;22(3):564-569. doi:10.1111/ene.12617

111. Muscogiuri G, Barrea L, Somma CD, et al. Sex Differences of Vitamin D Status across BMI Classes: An Observational Prospective Cohort Study. *Nutrients*. 2019;11(12):3034. doi:10.3390/nu11123034

112. Orton SM, Wald L, Confavreux C, et al. Association of UV radiation with multiple sclerosis prevalence and sex ratio in France. *Neurology*. 2011;76(5):425-431. doi:10.1212/WNL.0b013e31820a0a9f

113. Chen M, He Y, Hu X, et al. Vitamin D3 attenuates SARS-CoV-2 nucleocapsid protein-caused hyperinflammation by inactivating the NLRP3 inflammasome through the VDR-BRCC3 signaling pathway in vitro and in vivo. *MedComm*. 2023;4(4):e318. doi:10.1002/mco2.318

114. Mahjoub L, Youssef R, Yaakoubi H, et al. Melatonin, vitamins and minerals supplements for the treatment of Covid-19 and Covid-like illness: A prospective, randomized, double-blind multicenter study. *EXPLORE*. Published online June 2023:S1550830723001295. doi:10.1016/j.explore.2023.06.009

115. Wei R, Christakos S. Mechanisms Underlying the Regulation of Innate and Adaptive Immunity by Vitamin D. *Nutrients*. 2015;7(10):8251-8260. doi:10.3390/nu7105392

116. Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. *Current Opinion in Pharmacology*. 2010;10(4):482-496. doi:10.1016/j.coph.2010.04.001

117. Lukacs NW. Role of chemokines in the pathogenesis of asthma. *Nat Rev Immunol*. 2001;1(2):108-116. doi:10.1038/35100503

118. Matthys P, Hatse S, Vermeire K, et al. AMD3100, a Potent and Specific Antagonist of the Stromal Cell-Derived Factor-1 Chemokine Receptor CXCR4, Inhibits Autoimmune Joint Inflammation in IFN-γ Receptor-Deficient Mice. *The Journal of Immunology*. 2001;167(8):4686-4692. doi:10.4049/jimmunol.167.8.4686

119. Breuer J, Korpos E, Hannocks MJ, et al. Blockade of MCAM/CD146 impedes CNS infiltration of T cells over the choroid plexus. *J Neuroinflammation*. 2018;15(1):236. doi:10.1186/s12974-018-1276-4

120. Rice GPA, Hartung HP, Calabresi PA. Anti- 4 integrin therapy for multiple sclerosis: Mechanisms and rationale. *Neurology*. 2005;64(8):1336-1342. doi:10.1212/01.WNL.0000158329.30470.D0

121. Garcia A, Dugast E, Shah S, et al. Immune Profiling Reveals the T-Cell Effect of Ocrelizumab in Early Relapsing-Remitting Multiple Sclerosis. *Neurol Neuroimmunol Neuroinflamm*. 2023;10(3):e200091. doi:10.1212/NXI.0000000000200091

122. Schaser AJ, Osterberg VR, Dent SE, et al. Alpha-synuclein is a DNA binding protein that modulates DNA repair with implications for Lewy body disorders. *Sci Rep*. 2019;9(1):10919. doi:10.1038/s41598-019-47227-z

123. Yu L, Ma H, Ji X, Volkert MR. The Sub1 nuclear protein protects DNA from oxidative damage. *Mol Cell Biochem*. 2016;412(1-2):165-171. doi:10.1007/s11010-015-2621-x

124. Yu L, Volkert MR. Differential Requirement for SUB1 in Chromosomal and Plasmid Double-Strand DNA Break Repair. Lichten M, ed. *PLoS ONE*. 2013;8(3):e58015. doi:10.1371/journal.pone.0058015

125. Haider S, Pal R. Integrated Analysis of Transcriptomic and Proteomic Data. *CG*. 2013;14(2):91-110. doi:10.2174/1389202911314020003

126. Hanel A, Carlberg C. Time-Resolved Gene Expression Analysis Monitors the Regulation of Inflammatory Mediators and Attenuation of Adaptive Immune Response by Vitamin D. *IJMS*. 2022;23(2):911. doi:10.3390/ijms23020911

Annexes

Annexe 1 : **DEGs identifiés dans l'analyse supervisée dans les sous populations lymphocytaires.**

	B memory Non Switched	B memory Switched	B naive	CD4 CM	CD4 EM	CD4 EMRA+	CD4 naive	CD8 CM	CD8 EM	CD8 EMRA+	CD8 naive	gdTcells	MAIT	NK	NKT	Tc1	Tc17	Th1	Th1*	Th2	Th17	Treg
DAZAP2	0,79																	1,42				
RSRP1	0,71																					
JUN	0,49		0,56																			
N4BP2L2	0,78																					
CAST	0,69											1,47										
EIF5	0,68																					
SRSF10	0,76																					
FAM49B	0,78																					
DDX5	0,78																					
ITSN2	0,68																					
DDX21	0,68																					
PSMB3		2,43																				
COMMD6		1,53																				
TBCA		3,42																				
ENO1		1,63																				
PRELID1		1,64																				
KMT2A		0,36					0,80											1,96				
NME2		1,51																				
EPS15		2,14																				
SMG1		0,57																2,15				
MEF2C		1,73																				
HNRNPH3		1,39																				
PSMB9		1,61																				
CUTA		2,08																				
TP53INP1		0,48																				
APRT		1,62																				
RPL27A		1,38																				
ATP5MC3		2,10																				3,21
PAPOLA		1,64																				
EMP3		2,11																				
SEPTIN7		1,40																				
PPP1CC		2,18											1,29									
RPL26		1,31																				
PHKB		1,81																				
SUB1		1,60																				
UQCRB		1,37																				
RPL29		1,36																				
COX5B		1,39																				

	B memory Non Switched	B memory Switched	B naive	CD4 CM	CD4 EM	CD4 EMRA+	CD4 naive	CD8 CM	CD8 EM	CD8 EMRA+	CD8 naive	gdTcells	MAIT	NK	NKT	Tc1	Tc17	Th1	Th1*	Th2	Th17	Treg
EIF3K		1,51																				
RPL36A		1,29																				
MT.ND1			1,12	1,17						1,18			1,16				1,11					
AKAP13			0,81																			
IRF8			0,74																			
DUSP1			0,45																			
EIF4A1				1,52																		
SPOCK2				1,44																		
MT.ND2				1,17																		
TNRC6B				0,79																		
EMB				1,69																		
ODF2L				1,88																		
SCAF11				0,73																		
UQCRH				0,70																		
ARGLU1					0,89													1,40				
ASH1L					0,82																	
RCAN3					1,22																	
SRSF2					1,17																1,29	
LDHB						0,67																
ANKRD44						1,68																
MBNL1						0,87																
CD44						1,31																
PSMA3.AS1							0,82															
LCK							0,80															
SAMHD1							1,18															
RIPOR2								0,59														
HNRNPA2B1								0,59										1,64				
NDUFA13								0,51														
ANKRD12									1,31	1,22								1,39				
PRKCH									1,35													
TCF25									1,13						0,58							
KLF13									0,77				0,73									
UTRN										1,56												
ABLIM1											1,29											
ATP5F1D											1,29											
C12orf57											0,82											
CBX3											1,19											
CYBA											0,86											

	B memory Non Switched	B memory Switched	B naive	CD4 CM	CD4 EM	CD4 EMRA+	CD4 naive	CD8 CM	CD8 EM	CD8 EMRA+	CD8 naive	gdTcells	MAIT	NK	NKT	Tc1	Tc17	Th1	Th1*	Th2	Th17	Treg
SP100											1,23											
SLC38A1											0,84							2,15				
LAMTOR4											1,19											
FOXP1											1,16											
CD8A											1,26											
TAF1D											1,16							2,41				
SATB1											1,26											
OXNAD1											1,44											
PDE7A											1,40											
MT.ND4												1,17										
HLA.F												1,40	1,46									
CORO1A												1,30										
IKZF1												1,42						1,87			1,14	
ARL4C												1,27						1,72				
LCP1												1,43						1,65				
STK17B												1,38	1,41					1,80				
MT.ATP6												1,15										
RBM25												1,49										
GCC2												1,43										
BPTF												1,54										
C9orf78													1,47									
DDX18													0,70									
PAIP2													1,82									
ISG20													1,42									
AKAP9													1,27									
PRF1													2,23									
HSH2D														1,52								
RUNX3														1,29								
SYTL3														1,38								
ADD3														0,82								
HELZ															0,49							
AKNA															0,51							
ATRX																1,49						
PIP4K2A																1,44						
EIF4G2																1,52		1,39				
PCBP2																0,71						
LRRFIP1																1,40						
SEMA4D																1,74						

	B memory Non Switched	B memory Switched	B naive	CD4 CM	CD4 EM	CD4 EMRA+	CD4 naive	CD8 CM	CD8 EM	CD8 EMRA+	CD8 naive	gdTcells	MAIT	NK	NKT	Tc1	Tc17	Th1	Th1*	Th2	Th17	Treg
EIF4B																1,36						
SEC62																	0,70					
GPBP1																	0,77					
SRSF9																	0,65					
SELENOK																	1,70					
ZRANB2																	0,72					
SYNE2																		1,80				
ARF1																		1,91				
MGA																		2,80				
PRKAG2																		2,50				
ACAP1																		1,35				
C12orf75																		2,25				
PPIA																		2,29				
STUB1																		2,37				
UBE2D3																		1,95				
VPS13C																		1,89				
COX8A																		2,01				
BCLAF1																		1,59				
UBE2E3																		0,44				
BTG1																		1,27				
PTP4A2																		1,98				
KCNAB2																		2,78				
GPRIN3																		2,45				
FOXJ3																		0,36				
TGFB1																		2,03				
STT3B																		1,51				
TACC3																		0,43				
HSP90AA1																		1,69				
CDC42																		1,91				
CCT7																		2,00				
KLF12																		1,57				
ORMDL1																		1,77				
MZT2B																		2,00				
CSNK2B																		2,34				
ACTR3																		2,09				
PREX1																		2,43				
PCED1B.AS1																		1,60				
DYNC1H1																		2,13				

	B memory Non Switched	B memory Switched	B naive	CD4 CM	CD4 EM	CD4 EMRA+	CD4 naive	CD8 CM	CD8 EM	CD8 EMRA+	CD8 naive	gdTcells	MAIT	NK	NKT	Tc1	Tc17	Th1	Th1*	Th2	Th17	Treg
PTPRC																		1,56				
EIF3I																		2,37				
DICER1																		1,52				
BAZ1A																		2,62				
AKAP8L																		2,27				
CEP85L																		2,56				
EIF1AX																		1,65				
MAML2																		0,52				
EIF4A2																		1,55				
VAPA																		3,01				
CIRBP																		1,76				
HERPUD2																		2,90				
RALGAPA1																		1,64				
IL6ST																		2,00				
CHD3																		2,37				
HLA.C																		1,78				
CCNI																		1,54				
ZC3H7A																		1,78				
CUL5																		2,22				
GNB1																		1,84				
RCSD1																		2,24				
PIK3R1																		3,36				
EIF3G																		1,68				
H2AFZ																		2,20				
TPR																		1,68				
ZFP36L2																		1,44				
DYNLT1																		2,02				
OSBPL8																		1,65				
ARPC2																		2,28				
RPS20																		1,83				
CAPZB																		1,66				1,73
LARP1																		0,41				
COX7A2																		2,58				
TOMM20																		1,98				
UBTF																		1,97				
CYTIP																		1,97				
ABHD17A																		1,65				
POLR2B																		1,53				

	B memory Non Switched	B memory Switched	B naive	CD4 CM	CD4 EM	CD4 EMRA+	CD4 naive	CD8 CM	CD8 EM	CD8 EMRA+	CD8 naive	gdTcells	MAIT	NK	NKT	Tc1	Tc17	Th1	Th1*	Th2	Th17	Treg
KRT10																		2,25				
NEDD8																		2,35				
PSMA2																		2,57				
ANAPC16																		1,69				
SF1																		1,40				
CAND1																		1,96				
HNRNPM																		1,67				
SNX3																		1,98				
USP7																		2,51				
HNRNPD																		2,10				
PSMB1																		2,46				
HNRNPH1																		1,51				
IPCEF1																		1,74				
BRD2																		1,38				
GRK6																		3,55				
PRKX																		1,41				
TMC8																			0,59			
DDX17																			0,78			1,93
TAX1BP1																			2,39			
GGNBP2																			0,61			
DGKZ																			0,55			
JPX																			0,50			
SRGN																				2,61		
IL10RA																				2,35		
SEPTIN9																				1,83		
NKTR																				1,62		
ATP5F1E																				2,13		
MYO1F																					0,80	
ARHGEF1																					0,78	
TRA2B																					0,70	
CFLAR																					0,80	
SYNE1																						3,33
SEPTIN6																						1,53
NSD3																						1,83
TAPBP																						2,96
CLIC1																						2,14
PRDX1																						1,96
NDUFB8																						3,35

	B memory Non Switched	B memory Switched	B naive	CD4 CM	CD4 EM	CD4 EMRA+	CD4 naive	CD8 CM	CD8 EM	CD8 EMRA+	CD8 naive	gdTcells	MAIT	NK	NKT	Tc1	Tc17	Th1	Th1*	Th2	Th17	Treg
CALM2																						1,68
CDC42SE1																						1,72
NECAP2																						1,64
CD74																						3,40
RNASEK																						1,85
SARAF																						1,33
EPB41																						2,03
STK10																						4,94
ANP32A																						3,94
CDC37																						2,42
SRRM2																						1,38
MACF1																						2,11
RNF125																						2,74
CD2																						1,67
ARPC1B																						2,17
DAD1																						2,55
YWHAB																						1,41
RNF19A																						3,17
HMGB2																						1,83
ANP32E																						2,59
CLDND1																						3,51
NDUFA4																						1,77
FYB1																						1,88
RBX1																						1,52
LAPTM5																						1,76
OGA																						1,92