UNIVERSITÉ DE NANTES

FACULTÉ DE MÉDECINE

ÉTUDE DU ROLE EFFECTEUR ET RÉGULATEUR DES LYMPHOCYTES T DANS LA SCLÉROSE EN PLAQUES

THÈSE DE DOCTORAT

École Doctorale BIOLOGIE SANTÉ
Discipline : Biologie, Médecine et Santé
Spécialité : Immunologie

Présentée et soutenue publiquement par

Ségolène PETTRÉ

Le 30 octobre 2009, devant le jury ci-dessous

Président	Abdelhadi SAOUDI, Directeur de Recherche, Toulouse
Rapporteurs	Jean-François MOREAU, Professeur, Bordeaux
	Patrick VERMERSCH, Professeur, Lille
Examinateur	David-Axel LAPLAUD, Praticien Hospitalier, Nantes
Directeur de thèse	Jean-Paul SOULILLOU, Professeur, Nantes

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Abréviations

BHE : Barrière Hémato-Encéphalique

CMH : Complexe Majeur d'Histocompatibilité

CPA : Cellule Présentatrice d'Antigène

CTLA4 : *Cytotoxic T Lymphocyte Associated protein 4*

DCs : Cellules Dendritiques

EAE : Encéphalomyélite Auto-immune Expérimentale

EBV : Virus d'Epstein-Barr

GITR : *Glucocorticoid Induced TNF Receptor*

HLA : Antigènes Humains des Leucocytes

IFN : Interféron

IL : Interleukine

IRM : Imagerie par Résonnance Magnétique

LB : Lymphocytes B

LCR : Liquide Céphalorachidien

LFA1 : Antigène 1 associé à la Fonction des Lymphocytes

MAG : Glycoprotéine Associée à la Myéline

MBP : Protéine Basique de la Myéline

MMP : Métallo-Protéinase de la Matrice extracellulaire

MOG : Glycoprotéine des Oligodendrocytes et de la Myéline

PBMC: Cellules Mononuclées du Sang Périphérique

PSGL1: P-Sélectine Glycoprotéine Ligand-1

PLP: Protéine Protéo-Lipidique

SEP: Sclérose En Plaques

SNC: Système Nerveux Central

SNP : Single Nucleotide Polymorphism

TCR: Récepteur des Cellules T

TGFβ : *Transforming Growth Factor β*

TNFα: *Tumor Necrosis Factor α*

VCAM: Molécule d'Adhérence Cellulaire Vasculaire

VLA4: *Very Late Antigen-4* (nommée également α4β1 ou α4-intégrine)

INTRODUCTION

A. La Sclérose en Plaques

a. Historique et Épidémiologie de la Sclérose en Plaques

La Sclérose en Palques (SEP) fut décrite pour la première fois par Robert Carswell et Jean Cruveilhier dans les années 1830. La physiopathologie de cette maladie fut présentée par le neurologue Jean-Martin Charcot dès 1868 (Charcot 1868). La SEP est une maladie chronique inflammatoire diffuse du système nerveux central (SNC), caractérisée par une démyélinisation. Cette pathologie présente différentes formes, dont l'évolution clinique, l'infiltrat inflammatoire, le degré de démyélinisation, d'atteinte axonale et de remyélinisation varient d'un patient à l'autre. Les lésions sont généralement bien délimitées et sont concentrées le plus souvent dans la zone périventriculaire de la substance blanche, le nerf optique, le tronc cérébral mais aussi au niveau de la moelle épinière. Les symptômes, causés par la perturbation de l'influx nerveux, correspondent en général à des pertes de fonctions motrices, sensitives ou visuelles mais aussi cognitives. Lorsqu'il se concentre sur une courte période, ce phénomène est appelé une poussée. Environ une personne sur 1000 en France est touchée par cette pathologie, les symptômes débutant habituellement entre 20 et 40 ans et les femmes étant deux fois plus touchées que les hommes. Le diagnostic clinique peut être établi lorsque le patient présente des lésions (« plaques »), c'est-à-dire des zones démyélinisées au sein du SNC, détectées par imagerie à résonance magnétique (IRM). Ces plaques doivent être à la fois disséminées dans différents endroits du SNC et leur apparition doit être disséminée dans le temps (McDonald et al. 2001, Lublin et al. 2005, Polman et al. 2005). Au niveau du liquide cephalorachidien (LCR), un profil oligoclonal et/ou une sécrétion intrathécale des immunoglobulines peut être observé lors d'une ponction lombaire (Freedman et al. 2005). À ce jour aucun test biologique, clinique ou radiologique ne permet de s'assurer du diagnostic. Celui-ci repose donc sur un ensemble d'arguments.

b. Les différentes formes connues de la maladie

i. La Sclérose en Plaques de forme rémittente

Les personnes atteintes de Sclérose en Plaques de forme récurrente-rémittente représentent 85% des cas en début de maladie. Les symptômes alternent entre phase de poussée et phase de rémission (Figure 1). Au niveau anatomo-pathologique, les plaques sont multifocales, disséminées dans le temps, avec des plaques dites actives et des plaques chroniques. Les poussées sont le reflet de lésions focales aiguës.

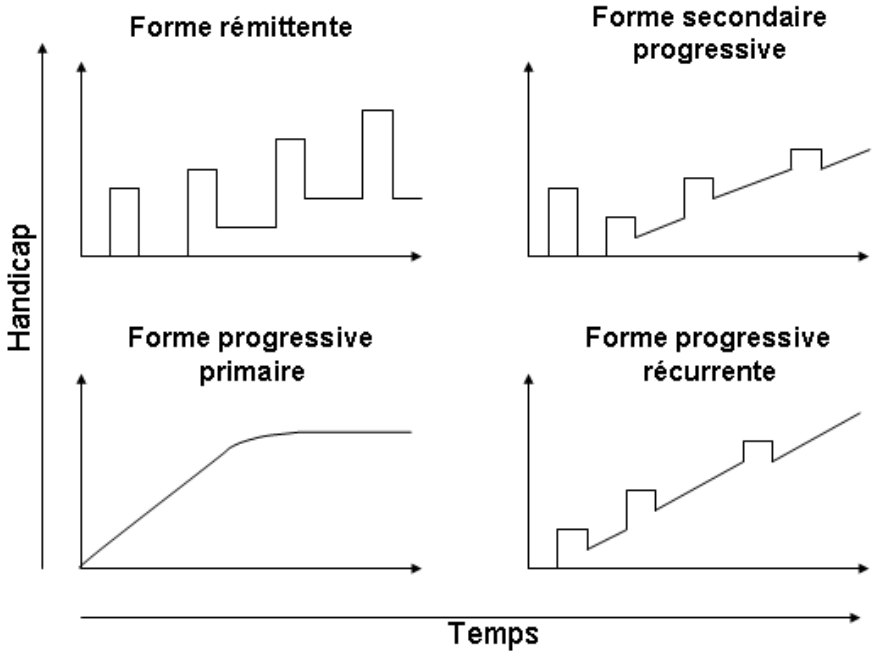

Figure 1: Évolutions du handicap pour les quatre formes de Sclérose en Plaques au cours du temps
La forme rémittente est caractérisée par l'alternance de poussées et de phases de rémission. Les formes secondaires progressives présentent une aggravation graduelle de l'état du patient et font suite aux formes rémittentes. Les formes progressives présentent une augmentation ininterrompue de séquelles.

ii. La Sclérose en Plaques de forme secondaire progressive

Les formes rémittentes de SEP évoluent en forme secondaire progressive pour 50% des malades en 15 ans et 90% des malades en 25 ans. Cette forme est caractérisée par une aggravation irréversible des anomalies neurologiques corrélée avec la perte axonale généralisée diffuse et le volume du parenchyme cérébral diminue (Khoury et al. 1994 ; Filippi

et al. 1995). La présence de poussée n'est pas systématique, mais elle est habituellement suivie de l'augmentation du nombre de séquelles.

iii. La Sclérose en Plaques de forme primaire progressive

La forme progressive primaire touche environ 10% des malades atteints de SEP, habituellement plus tardivement dans l'histoire du patient. La progression du handicap est permanente et le déclin neurologique est régulier (Figure 1). Les patients présentent une inflammation moindre, peu de destruction des axones mais une perte accrue des oligodendrocytes (Bruck et al. 2002).

iv. La Sclérose en Plaques de forme progressive récurrente

La forme progressive récurrente progressive représente 5% des cas de SEP. Elle associe une progression permanente et lente des symptômes sur laquelle se superposent des poussées aigues (Figure 1).

v. Autres formes associées à la Sclérose en Plaques

Plusieurs autres maladies partagent des caractéristiques communes à la SEP, en particulier deux variantes rares et fulgurantes : la maladie de type Marburg et la sclérose concentrique de Balo (Capello et al. 2004).

La maladie de type de Marburg présente une évolution fulminante avec une démyélinisation importante du tronc cérébral ou des hémisphères cérébraux. Si les malades survivent à la phase aigüe, ils présentent ensuite une forme de maladie rémittente. La destruction de la myéline survient de façon concomitante.

La sclérose concentrique de Balo est, quant à elle, une forme où les zones lésées sont organisées de façon concentrique, alternant des bandes démyélinisées et des bandes intactes ou remyélinisées.

Pendant longtemps la neuromyélite optique de Devic a été considérée comme une forme de SEP. Cette pathologie présente des lésions démyélinisantes concentrées au niveau du nerf optique et de la moelle épinière. La présence d'anticorps anti-aquaporine-4, une protéine impliquée dans le maintien de l'équilibre hydrique du SNC, permet de diagnostiquer cette pathologie et est un argument supplémentaire la distinguant de la SEP (Weinshenker et al. 2006).

c. Étiologie

Les causes de la Sclérose en Plaques ne sont actuellement pas connues. Cependant, l'étiologie de cette pathologie semble indiquer l'influence de facteurs environnementaux et de la prédisposition génétique sur la survenue de cette maladie.

i. Facteurs environnementaux

La SEP apparaît répartie géographiquement de façon inégale avec des zones de haute prévalence (en Europe, au Canada, aux Etats-Unis et en Australie) et des zones de prévalence basse comme l'Amérique latine (Marrie et al. 2004). L'hypothèse d'un facteur environnemental est étayée par les études sur les migrations de populations entre les zones de prévalence inégale : un individu migrant après l'âge de 15 ans présente le risque de la région de départ tandis qu'un individu migrant avant l'âge de 15 ans présentera le risque de la zone d'arrivée. Il semble donc qu'un événement décisif se passerait à l'adolescence (ie plusieurs années avant le début clinique de la maladie) (Alter et al. 1966 ; Hammond et al. 2000 ; Rosati et al. 2001).

Plusieurs facteurs de risque ont été étudiés comme les infections, les vaccinations, le climat et les expositions à des toxines mais les résultats sont contradictoires. Des arguments épidémiologiques et sérologiques ont suggéré que le virus d'Epstein-Barr (EBV) était fortement associé avec la SEP mais aussi avec d'autres maladies auto-immunes (Haahr et al. 2006). L'EBV est un herpèsvirus, également appelé HHV-4, responsable de la mononucléose infectieuse. Plus de 90% des adultes portent une trace de primo-infection et ce quelque soit la répartition géographique. Ce pourcentage est de l'ordre de 99% dans la population des patients atteints de SEP. Les études sur les cerveaux prélevés post-mortem de patients atteints de SEP ont montré un dérèglement des lymphocytes B infectés par l'EBV quelque soit la forme de la maladie. Les hypothèses suggérant l'implication de EBV dans la SEP incluent le mimétisme moléculaire, la production de clones B immortalisés et un dérèglement des cellules T cytotoxiques contre les cellules B infectées (pour revue Salvetti et al. 2009).

Aucun agent potentiel n'a pu à lui seul être mis en cause. La somme de plusieurs facteurs semblerait donc une cause possible du déclenchement de la maladie (pour revue Marrie et al. 2004).

ii. Facteurs génétiques

De nombreuses études épidémiologiques sur la SEP ont montré qu'il ne s'agissait pas d'une maladie héréditaire. Cependant, des facteurs génétiques influenceraient la survenue de cette pathologie. Seulement 15 à 20% des patients atteints de SEP ont un proche parent également atteint. Chez les jumeaux monozygotes, le risque de développer la maladie pour le deuxième jumeau lorsque le premier est atteint est seulement de 25 à 30% (Willer et al. 2003 ; Dyment et al. 2004). Les jumeaux dizygotes, quant à eux, ont un risque de 5% de développer la maladie (Sadovnick et al. 1993). Il semble donc que la SEP se déclenche plus fréquemment chez les individus prédisposés génétiquement.

1. Le système HLA et la susceptibilité génétique de la SEP

Ce constat a conduit à une recherche intense de gènes rendant les individus susceptibles à la survenue de la SEP. Les seuls liens clairement établis entre SEP et génétique concernent, entre autre, des gènes codant des molécules du système immunitaire (Tableau 1). De nombreuses études génétiques et épidémiologiques ont trouvé le locus du complexe majeur d'histocompatibilité (CMH) associé à la maladie SEP (Olerup et al. 1991, Sotgiu et al. 2002). L'allèle HLA-DRB1*1501 semble être un gène de susceptibilité (Haines et al. 1998 ; Fogdell-Hahn et al. 2000 ; Oksenberg et al. 2005). D'autres allèles codant des molécules du CMH de classe II comme les allèles DQA1*0102 et DQB1*0602 apparaissent également associés à la SEP (Fogdell et al. 1995) ainsi que les allèles HLA-A3 et -B7 pour les CMH de classe I (Jersild et al. 1972 ; Naito et al. 1972 ; Fogdell-Hahn et al. 2000 ; Harbo et al. 2004).

Concernant le gène HLA-C, l'allèle HLAC*05 semble être associé, non pas à un risque de développer la maladie, mais à une protection (Yeo et al. 2007).

2. Les gènes non HLA et la susceptibilité génétique de la SEP

D'autres études ont également associé à la SEP le polymorphisme de certains gènes impliqués dans le système immunitaire comme le récepteur des cellules T (TCR) et la molécule CTLA4 (Cytotoxic T lymphocyte-associated antigen-4), cependant selon les populations étudiées et les méthodes d'investigation, les résultats diffèrent (pour revue Dyment et al. 2004 ; Bagos et al. 2007).

Récemment, différentes collaborations internationales ont permis de mettre en évidence l'implication de marqueurs génétiques non lié au système HLA (Tableau 1). En effet, le polymorphisme de deux gènes codant des récepteurs de cytokines a été identifié comme

facteur de risque génétique dans la SEP. Il s'agit du gène codant la chaine α du récepteur à l'IL7 (IL7RA ou CD127) situé sur le chromosome 5p13 et du gène codant la chaine α du récepteur à l'IL2 (IL2RA ou CD25) situé sur le chromosome 10p15 (Hafler et al. 2007 ; Gregory et al. 2007 ; Lundmark et al. 2007 ; Weber et al. 2008). L'IL7 est une cytokine primordiale pour la survie, la prolifération et la différentiation des lymphocytes B et T (Fry et al. 2005). Il semble que la SNP identifiée au sein de l'exon 6 du gène codant l'IL7RA épissé alternativement aurait un effet sur les niveaux d'isoformes solubles et membranaires de la protéine allant dans le sens d'une diminution de la forme membranaire (Gregory et al. 2007). Dans le cas du récepteur à l'IL2, il semble que le variant génétique impliqué dans la SEP a pour conséquence de diminuer la forme soluble du récepteur mesurée dans le sérum. Cette forme soluble de l'IL2RA pourrait inhiber la voie de signalisation induite par l'IL2 mais aussi pourrait augmenter la prolifération des lymphocytes T ainsi que leur expansion (Maier et al. 2009a ; Maier et al. 2009b).

D'autres gènes possédant des fonctions immunologiques sont associés à un risque de développer la SEP dont LFA3, connu aussi sous la dénomination CD58 (molécule d'adhérence impliquée dans l'interaction des leucocytes ; Reich et al. 2005), TYK2 (membre de la famille JAK impliqué dans la signalisation de l'IFNα, IL6, IL10 et IL12 ; Ban et al. 2009), CD226 (impliqué dans l'adhérence cellulaire permettant la transduction de messages de co-stimulation ; Hafler et al. 2009) et PDE4B (phosphodiestérase spécifique de l'AMPc intervenant dans la transduction du signal ; Hafler et al. 2007).

Associated gene*	Proposed function	Chromosome location	SNP or allele	Position	Risk (odds ratio)‡	Replicated in independent study
Immunological genes						
HLA-DR	Antigen presentation	6p21.3	DRB1*1501	Entire allele	5.0	Yes
HLA-A	Antigen presentation	6p21.3	A*0301	Entire allele	1.9	No
			A*0201	Entire allele	0.6	No
HLA-C	Antigen presentation	6p21.3	C*05	Entire allele	ND	No
IL2RA (also known as *CD25*)	Cytokine receptor; apoptosis	10p15	rs2104286	Intron 1	0.8	Yes
IL7R (also known as *CD127*)	Cytokine receptor; cell survival	5p13	rs6897932	Exon 6	1.2	Yes
CD58 (also known as *LFA3*)	Cell–cell adhesion	1p13	rs2300747	Intron 1	0.8	Yes
TYK2	Cell signalling	19p13.2	rs34536443	Exon 23	1.3	Yes
CD226	Co-stimulation	18q22	rs763361	Exon 7	1.1	No
PDE4B	Signal transduction in inflammatory cells	1p31	rs1321172	5′ region	1.1	No
Neurological genes						
ACCN1	Neuronal pH-sensitive ion channel	17q11.2	rs28936	3′ UTR	2.0	No
KIF1B	Axonal transport	1p36.22	rs12044852	Intron 1	1.4	No
ALK	Protein tyrosine kinase receptor; brain development	2p23	rs7577363	Intron 3	1.4	No
ANKRD15	Specific function unknown; neuronal development	9p24	rs10975200	Intron 1	1.1	No
Genes of other or unknown function						
RPL5	Ribosomal protein; chaperone for the 5S rRNA	1p22	rs6604026	Intron 6	1.1	No
CLEC16A	Sugar-binding C-type lectin	16p13	rs6498169	Intron 22	1.2	Yes
			rs12708716	Intron 19	1.2	Yes
DBC1	Cell cycle arrest; apoptosis	9q33	rs10984447	Intron 5	1.17	No
FAM69A	Protein binding	1p22	rs11164838	Intron 1	1.11	No
			rs7536563	Intron 1	1.12	No
EVI5	Cell cycle control	1p22	rs10735781	Intron 11	1.11	No
			rs6680578	Intron 2	1.11	No

Tableau 1: Les gènes associés à un risque de développer la Sclérose en Plaques

Ces gènes ont été classés en trois sous-groupes fonctionnels : immunologique, neuronal et autres fonctions connues ou non-identifiées. Cette classification est discutable car certains gènes sont liés à plusieurs fonctions ou sont exprimés par différents types cellulaires. Le risque lié à la maladie est donné en Odds ratio.

Abréviations : ACCN1, amiloride-sensitive cation channel 1 neuronal ; ALK, anaplastic lymphoma kinase ; ANKRD15, ankyrin-repeat domain ; CLEC16A, C-type lectin domain family 16 member A ; DBC1, deleted in bladder cancer 1 ; EVI5, ecotropic viral integration site 5 ; FAM69A, family with sequense similarity 69 member A ; IL2RA, interleukin-2 receptor α chain ; KIF1B, kinesin family member 1B ; LFA3, lymphocyte function associated antigen 3 ; ND, non determiné ; PDE4B, phosphodiesterase 4B cAMP specific ; RPL5, ribosomal protein L5 ; SNP, single nucleotide polymorphism; TYK2, tyrosine kinase 2. *D'après Fugger et al. 2009*

d. Physiopathologie

i. Les différents types de lésions

Les lésions des patients atteints de SEP apparaissent très hétérogènes entre les individus. Certaines lésions sont dites « actives » et d'autres « chroniques », plus anciennes, illustrant la dynamique du processus pathologique (Frohman et al. 2006). Les études de tissus humains prélevés post-mortem et de biopsie ont permis de décrire quatre types de lésions, résultant de processus pathologiques différents (Lucchinetti et al. 1996 ; Lucchinetti et al. 1999 ; Lucchinetti et al. 2000). Les lésions de type I sont caractérisées par une démyélinisation et la présence de facteurs produits par les macrophages comme le TNFα, des protéinases et des espèces oxygénées réactives intermédiaires. Au niveau des lésions de type II, on retrouve des immunoglobulines ainsi que des molécules du complément. Les lésions de type III sont caractérisées par la perte précoce de la glycoprotéine associées à la myéline (MAG) et par l'absence de remyélinisation suggérant un dysfonctionnement des oligodendrocytes. La spécificité des lésions de type IV est l'apoptose des oligodendrocytes présentant une fragmentation de l'ADN.

Il est a noté que les lésions d'un même patient peuvent présenter les critères d'un ou de plusieurs types (Barnett et al. 2004) mais cela reste très discuté. Au niveau des lésions « actives », il a été observé la présence de transsections ainsi que de dommages substantiels axonaux (Trapp et al. 1998 ; Peterson et al. 2001). Cependant, certaines lésions présentent une dégénérescence des oligodendrocytes en l'absence d'infiltrat de cellules inflammatoires (Barnett et al. 2004). La question de l'inflammation ou de la neurodégénérescence à l'origine de la pathologie reste latente. Il semble d'après certaines études récentes que l'inflammation soit présente à toutes les phases et dans toutes les formes de SEP, mais différente selon les phases : en particulier avec une compartimentalisation variée des réponses immunes (Lassmann et al. 2007).

ii. L'atteinte diffuse de la substance blanche et grise

La substance blanche d'apparence normale présente également des lésions diffuses globales non visibles à l'IRM standard mais appréciées par la spectroIRM. Ces lésions sont caractérisées par de l'inflammation (Lindberg et al. 2004), une perte axonale (Evangelou et al. 2000 ; Kornek et al. 2000), un infiltrat de cellules T et une activation des cellules

microgliales. Malgré la prédominance des lésions au niveau de la substance blanche, les patients atteints de SEP présentent aussi des atteintes axonales au niveau de la substance grise comme par exemple au niveau des noyaux profonds cérébraux (Cifelli et al. 2002) et du cortex (Kidd et al. 1999 ; Peterson et al. 2001). Ces atteintes neuronales peuvent être dues en partie aux transsections axonales visibles dans la substance blanche (dégénérescence rétrogade) qui, cependant, n'explique pas tout. Ces lésions sont moins inflammatoires avec peu de destruction de la matrice extracellulaire et uniquement des cellules microgliales comme cellules infiltrantes. La démyélinisation corticale touche 90% des patients, pourtant le tissu apparaît généralement normal à l'IRM standard (Kutzelnigg et al. 2005 ; Albert et al. 2007). L'utilisation d'appareils plus performants (en particulier à hauts champs) permettrait de visualiser ces lésions.

iii. La rupture de la Barrière Hémato-Encéphalique

La barrière hémato-encéphalique (BHE) est composée de cellules endothéliales, liées par des jonctions serrées, tapissant les capillaires (Ballabh et al. 2004). La BHE limite les échanges moléculaires et le passage des cellules immunitaires entre le sang périphérique et le système nerveux central (SNC). Seules les cellules microgliales, cellules sentinelles de l'immunité sont présentes naturellement dans le SNC, à partir du stade embryonnaire, et peuvent assurer des fonctions de cellules présentatrices d'antigènes (Aloisi et al. 2001 ; Streit et al. 2002). Pour franchir la BHE, les cellules immunitaires présentes en périphérie doivent avoir été activées par des cellules présentatrices d'antigènes et ainsi exprimer à leur surface des molécules d'adhérence. Celles-ci leur permettent d'entrer en contact avec les cellules endothéliales. La production de chémokines facilite également l'attraction des cellules immunitaires et l'activation des cellules endothéliales. Enfin, les cellules immunitaires sécrètent des métallo-protéinases de la matrice extracellulaire (MMP) qui induisent une rupture de la membrane et permettent la transmigration au travers de la BHE (Figure 2).

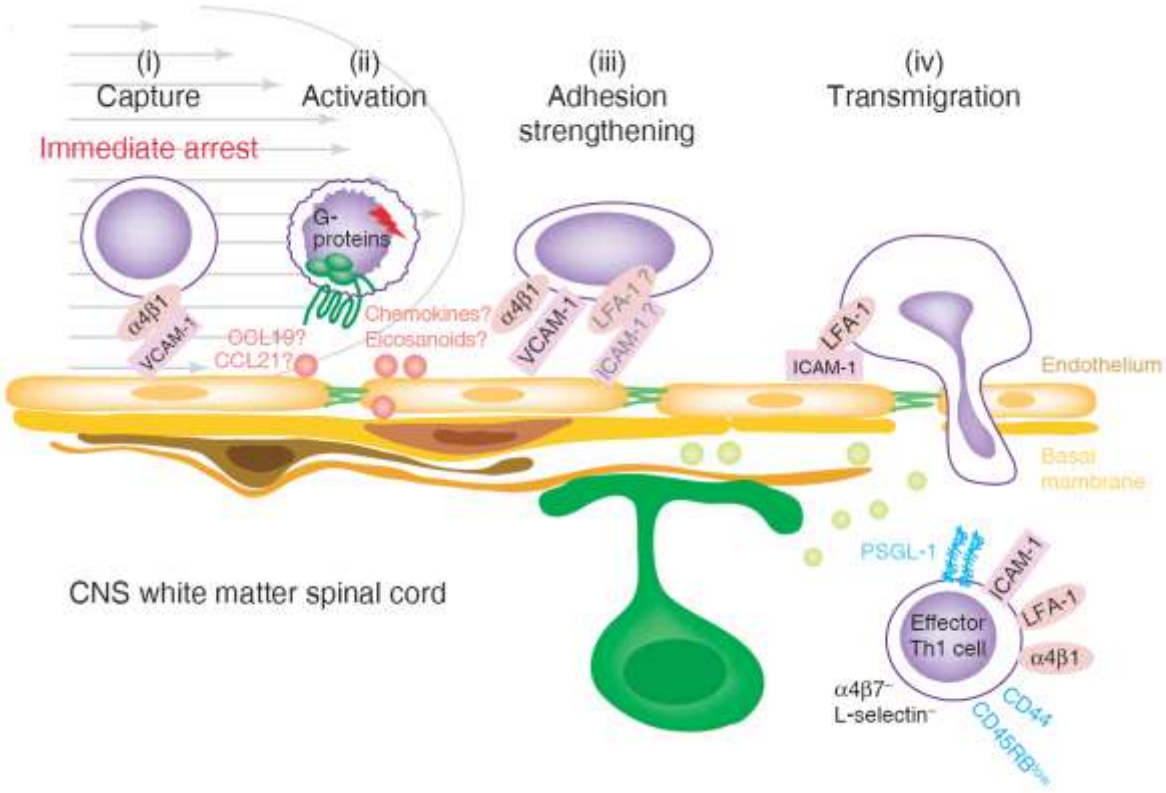

Figure 2 : Passage de la BHE

Lors de l'inflammation du SNC, les cellules immunitaires effectuent un roulement (rolling ou (i) capture). Les molécules E- et P-sélectinne, leur ligand PSGL1 et l'α4β1 (α4-intégrine ou VLA4) sont impliqués dans ce roulement des lymphocytes au niveau des vaisseaux superficiels. Les cellules sont activées via des protéines G (ii) et leur adhérence dépend de la liaison de la molécule LFA1 à ICAM1 (iii). Les cellules migrent à travers l'endothélium au niveau des jonctions serrées : c'est la diapédèse (iv). *D'après Engelhardt et al. 2005.*

Chez les patients atteints de SEP, l'augmentation de la prise de Gadolinium révélée par l'IRM reflète la rupture de la BHE (Katz et al. 1993). De plus, chez les malades, la molécule d'adhésion intercellulaire (ICAM1) et la molécule d'adhérence cellulaire vasculaire (VCAM1) sont présentes à des taux élevés sur les cellules endothéliales des lésions (Sobel et al. 1990 ; Washington et al. 1994 ; Cannella et al. 1995 ; Ransohoff et al. 1999). Les cellules infiltrant les lésions expriment quant à elles l'Antigène 1 associé à la Fonction des Lymphocytes (LFA1) et *Very Late Antigen-4* (VLA-4) (Bo et al. 1996). Les cellules circulant dans le sang des patients présentent également des taux élevés de LFA1 et VLA4 (Correale et al. 2003). Les lymphocytes T des patients atteints de SEP ont des capacités d'adhérence plus grandes par rapport aux individus sains (Lou et al. 1997) et les lymphocytes T CD8+ des patients atteints de SEP expriment la molécule P-Sélectine Glycoprotéine Ligand-1 (PGSL1) (Battistini et al 2003). La fréquence des cellules T CD4+PSGL1+ est significativement plus

importante chez les patients atteints de SEP. Celle des cellules T CD8+PSGL1+, quant à elle, est similaire entre les patients et les individus sains. De plus, les lymphocytes CD4+ et CD8+ de patients migrent mieux à travers un modèle de BHE que nous avons mis au point au laboratoire en collaboration avec l'équipe du Professeur P.O. Couraud (Bahbouhi et al. 2009).

Au niveau des métallo-protéinases, on note l'augmentation de l'expression de MMP-2, -7, -9, -12 dans les macrophages et lymphocytes autour des zones périvasculaires (Anthony et al.1997 ; Cossins et al. 1997 ; Lindberg et al. 2001 ; Vos et al. 2003) ainsi qu'une élévation du taux de MMP-9 dans le LCR et le sérum de patients SEP en particulier pendant les poussées cliniques et pendant l'augmentation de la prise de Gadolinium sur l'IRM (Lee et al. 1999 ; Lichtinghagen et al. 1999 ; Waubant et al. 1999). Les leucocytes des patients atteints de SEP présentent une augmentation des taux d'ARN messagers de MMP-1, -3, -7, -14 et -19 (Galboiz et al. 2001 ; Kouwenhoven et al. 2001a ; Van Horssen et al. 2006) ce qui peut faciliter la migration au travers de la BHE. Effectivement, les lymphocytes de type Th1 migrent plus efficacement que les lymphocytes Th2 et présentent des taux plus élevés de MMP-2 et -9 (Abraham et al. 2005).

e. Les traitements de la Sclérose en Plaques

Pour l'heure, il n'existe aucun traitement curatif de la SEP. Les traitements disponibles se divisent en deux grandes catégories : les immunosuppresseurs et les immunomodulateurs. Les immunosuppresseurs sont des agents ciblant la prolifération des cellules immunitaires et sont prescrits en général lors des poussées. Les immunomodulateurs, quant à eux, sont utilisés comme traitement de fond principalement pour la forme récurrente-rémittente de la maladie.

i. Les traitements immunosuppresseurs de la Sclérose en Plaques

Il existe plusieurs types de traitements immunosuppresseurs : l'azathioprine, le mycophénolate mofétil, la mitoxantrone, le cyclophosphamide, les corticoïdes et le natalizumab, qui est un immunosuppresseur particulier car sélectif.

1. AZATHIOPRINE

Historiquement, il s'agit du premier immunosuppresseur utilisé comme thérapie dans la SEP. À l'origine, prescrit chez les patients greffés, il a été testé dans la SEP. Ce traitement diminue probablement la survenue de poussées et peut-être la progression du handicap, mais aucune étude ayant une qualité méthodologique de niveau 1 n'a été réalisée jusqu'à présent (Yudkin et al. 1991 ; Palace et al. 1997). L'azathioprine n'est donc pas un traitement recommandé en première ligne chez les patients.

2. Mycophénolate mofétil

Cet immnosuppresseur mieux connu sous le nom de Cellcept, est lui aussi utilisé en transplantation pour prévenir le rejet de greffe. Pour l'instant aucun essai clinique n'a été réalisé dans la SEP mais il représente une alternative de traitement compte tenu de sa bonne tolérance. Un petit essai a montré son intérêt en association avec l'IFNβ (Vermersch et al. 2007).

3. Mitoxantrone (Elsep)

La mitoxantrone est un immunosuppresseur autorisé depuis 2003 dans le traitement des formes agressives de SEP à évolution rapide. Il diminue les poussées, la progression de la maladie et le nombre de lésions prenant le gadolinium (Edan et al. 1997 ; Goodin et al. 2003). Cet agent inhibe la prolifération des lymphocytes T et B ainsi que celle des macrophages en s'intercalant dans les molécules d'ADN et d'ARN. Il montrerait également des propriétés d'immunomodulation en diminuant les taux de certaines cytokines pro-inflammatoires comme le TNFα, l'IL2, et l'IFNγ. Ce traitement est limité par son potentiel de toxicité cardiaque et hématologique.

4. Cyclophosphamide

Cet immunosuppresseur aussi connu sous le nom d'Endoxan est habituellement prescrit dans les cas de formes secondaires progressives de SEP avec aggravation rapide du handicap. Son efficacité reste cependant à démontrer formellement sur des études prospectives et actuellement un PHRC est en cours à l'initiative du Pr Brochet (Bordeaux) pour régler cette question. Le cyclophosphamide a peu d'effets bénéfiques pendant les poussées (Drachman et al. 1975) mais diminue le taux de poussées.

5. Corticothérapie

La méthylprédnisolone est généralement injectée sur trois à cinq jours lors des hospitalisations pour une poussée des patients atteints de SEP. Les effets bénéfiques ont lieu à court terme chez les patients atteints de forme rémittente et de forme secondaire progressive (Milligan et al. 1987). Ce traitement permettrait d'accélérer la récupération des symptômes (Brochet et al. 2001).

6. Natalizumab (Tysabri)

Il s'agit du premier anticorps monoclonal développé pour le traitement de la SEP. Il a fait l'objet d'une autorisation de mise sur le marché européenne en juin 2006. C'est un anticorps humanisé dirigé contre la chaine α4 de l'intégrine α4β1 (VLA4) (Engelhardt et al. 2008). VLA4 est exprimée à la surface de tous les leucocytes excepté celle des neutrophiles. Le Natalizumab agit comme un antagoniste de VLA4 en empêchant la liaison des leucocytes à VCAM1 et à la fibronectine (O'Connor et al. 2004). VCAM1 appartient à la famille VCAM, molécules sur-exprimées lors du processus d'inflammation. Ainsi, en présence de Natalizumab, les leucocytes ne peuvent plus infiltrer les tissus cibles. Après traitement, le pourcentage de leucocytes activés produisant des cytokines pro-inflammatoires est augmenté dans le sang, suggérant une séquestration des cellules activées au niveau de la circulation périphérique (Kivisäkk et al. 2009).

Le Natalizumab est indiqué dans le traitement de fond des formes très actives de SEP rémittente-récurrente chez les patients adultes uniquement.

Les risques liés à ce traitement sont principalement un risque de développer une infection opportuniste, la LEMP mais qui reste exceptionnelle, voire d'autres infections non-opportunistes, la survenue de réactions allergiques (environ 5%), l'apparition d'anticorps anti-Natalizumab et des troubles hépatiques (rares).

ii. Les traitements immunomodulateurs de la Sclérose en Plaques

1. Les interférons β

Depuis les années 1990, trois protéines recombinantes interféron β (IFNβ) sont autorisées comme traitement de la SEP. Leurs effets semblent assez similaires malgré quelques différences dans leur structure (glycosilation) et dans leur posologie.

Les études cliniques de phase III ont montré une diminution d'environ 30% du taux annuel de poussée comparé au groupe placebo et 30% des patients après 2 ans de traitement étaient indemnes de poussées (Group et al. 1993). Plusieurs effets des IFNβ ont été décrits comme une suppression de la prolifération des cellules T (Rep et al. 1996 ; Pette et al. 1997) et une diminution de l'expression des molécules du CMH de classe II induite par l'IFNγ (Joseph et al. 1988 ; Lu et al. 1995). Après traitement aux IFNβ, la production de cytokines anti-inflammatoires est augmentée (Rudick et al. 1996 ; Rep et al. 1999). Les IFNβ semblent inhiber l'activation des monocytes (Van Weyenbergh et al. 1998) et agir également sur les cellules dendritiques via l'inhibition de la production d'IL12 (Bartholome et al. 1999). Ces traitements sont capables aussi de bloquer le trafic des cellules T en bloquant les protéines MMP (Stuve et al. 1997) et en diminuant l'expression de VLA4 au niveau des lymphocytes T des patients (Calabresi et al.1997).

2. L'acétate de glatiramère (Copaxone)

L'acétate de glatiramère est un copolymère randomisé de quatre acides aminés (Acide Glutamique, Lysine, Alanine et Tyrosine) dont la longueur moyenne et le poids peuvent varier (Teitelbaum et al.1971). Il est approuvé et utilisé dans les formes rémittentes de SEP. Les effets bénéfiques de ce traitement sont une diminution de 30% de la survenue de poussée (Johnson et al. 1995 ; Comi et al. 2001) et un ralentissement de la progression de la maladie (Johnson et al. 2000). Les études comparatives récentes ne montrent pas de différence d'efficacité entre les interférons β et la copaxone sur le risque de survenue de poussée. De plus, nos observations sur le répertoire T des patients atteints de SEP de forme rémittente, traités par l'acétate de glatiramère, ne montrent pas de différence dans les variations du répertoire ni dans celles de la production de cytokines entre malades et témoins (Berthelot et al. 2009, Annexe I).

iii. Les avancées thérapeutiques

Jusqu'à présent, l'ensemble des traitements proposés aux patients atteints de SEP ont pour voie d'admission des injections intraveineuses, intramusculaires ou sous-cutanée. Récemment, plusieurs études de phase III ont été lancées pour étudier l'effet de nouvelles thérapies dont la voie d'admission est la voie orale (Tableau 2). Si l'un ou plusieurs de ces traitements testés à l'heure actuelle recevaient l'accord de mise sur le marché, il s'agirait d'une réelle avancée permettant d'améliorer la qualité de vie des patients atteints de SEP.

1. Teriflunomide

Le teriflunomide est un inhibiteur oral de la dihydro-orate déshydrogénase. Il possède des propriétés immunomodulatrices, il diminue la prolifération des lymphocytes (Cherwinski et al. 1995). En phase II, ce traitement a eu pour effet de diminuer le nombre de lésions actives visible à l'IRM. Une phase III est en cours.

2. Cladribine

La Cladribine (2-chlorodeoxyadenosine) est un immunosuppresseur. Son mécanisme d'action n'est pas totalement connu. Cependant, cette molécule étant résistante à la dégradation par la deaminase, va s'accumuler dans les cellules. Cette accumulation est à l'origine d'un déséquilibre dans le métabolisme cellulaire, de dommages causés à l'ADN et de mort cellulaire (Beutler et al. 1992). Cette activité cytotoxique touche les lymphocytes en particulier mais aussi les monocytes. Kopadze et ses collaborateurs ont montré qu'à la suite d'un traitement des PBMCs par la Cladribine *in vitro*, les capacités de migration des monocytes ainsi que des lymphocytes CD4+ et CD8+ étaient diminuées (Kopadze et al. 2009). Les lymphocytes sont plus sensibles à cette action que les monocytes. L'étude de phase III en cours évaluant l'effet de la Cladribine par voie orale chez des patients atteints de SEP rémittente-récurrente montre une diminution significative du taux de poussée annuel pour les deux doses testées.

3. FTY720 (fingolimod)

Le FTY720, connu aussi sous le nom de fingolimod, est une molécule testée par voie orale. Ces études sont réalisées chez des patients atteints de SEP rémittente-récurrente mais aussi de forme primaire progressive. Cette molécule se lie au récepteur de la sphingosine-1-

phosphate, celle-ci est exprimée de façon prédominante dans les lymphocytes et régule leur migration des organes lymphoïdes secondaires vers le site de l'infection. FTY720 a donc une action de rétention au niveau des organes lymphatiques, aboutissant à l'absence des lymphocytes au niveau de la circulation, les éloignant de leur cible. Les résultats de phase II semblent indiquer que le traitement par voie orale de fingolimod est associé à une diminution de l'apparition de nouvelles lésions en T2 ou des lésions actives sur l'IRM ainsi que la diminution du taux annuel de poussée comparé au traitement par IFNβ.

4. Laquinimod

Le laquinimod est un analogue de la linomide. Son mécanisme d'action n'est pas totalement élucidé mais il semble altérer l'équilibre entre le profil cytokinique Th1 et Th2 (Zou et al. 2002). En phase II, ce traitement, comparé à un placebo, diminue le nombre de nouvelles lésions focales inflammatoires chez les patients atteints de SEP rémittente-récurrente (Comi et al. 2008). Une phase III est en cours.

	Phase III (indication, comparator)	**Phase II (Primary end point)**	**Adverse effects**
Teriflunomide	(1) CIS, placebo (2) RR-MS, placebo (3) RR-MS, IFN-β	MRI[41] (mean number of CU active lesions per scan): 7 or 14 mg/day: reduction by 61%	GI symptoms, hepatotoxicity, low risk of pancytopenia, low risk of endogenous infections, teratogenicity
Cladribine	(1) CIS, placebo (2) RR-MS, placebo (3) RR-MS, add-on to IFN-β	Not performed for oral formulation	Not published for oral formulation
Fingolimod	(1) RR-MS, placebo (2) RR-MS, placebo (3) RR-MS, IFN-β (4) PP-MS	MRI[42] (median total number of gadolinium-enhanced lesions on MRI): 1.25 mg or 5 mg or placebo: 1 ($P < 0.001$) or 3 lesions ($P = 0.006$) or 5 lesions	Nasopharyngitis, dyspnea, headache, GI-symptoms, hepatotoxicity, case of the posterior reversible encephalopathy syndrome, cardiovascular side effects
Laquinimod	(1) RR-MS, placebo (2) RR-MS, IFN-β	MRI[43] (cumulative number of active lesions over 24 weeks): reduction by 44%	Iritis and burning sensation; during follow-up acute tonsillitis, one case of breast cancer

Tableau 2 : Les traitements oraux en phase III pour les formes rémittente-récurrente de SEP

Abréviations : CIS, syndrome clinique isolé ; CU, combined unique ; IFN, interféron ; GI, gastrointestinal ; MRI, image par résonance magnétique ; MS, Sclérose en Plaques ; RR, forme rémittente-récurrente ; PP, forme primaire progressive. *D'après Warnke et al. 2009*.

5. Abatacept (CTLA4-Ig)

Il s'agit d'une protéine chimérique de fusion bloquant la voie de costimulation CD28-CD80/CD86 (Viglietta et al. 2008). Ce traitement est utilisé dans la polyarthrite rhumatoïde. La phase I a montré qu'il était bien toléré dans la SEP. Ces résultats préliminaires sont encourageants et suggèrent que le blocage de la voie de costimulation peut être un axe thérapeutique prometteur dans la SEP.

6. Rituximab

Le rituximab est un anticorps monoclonal chimérique homme/souris ciblant le marqueur CD20 exprimé à la surface des lymphocytes B. Il a été approuvé en 1997 comme traitement des lymphomes B. Il a pour effet de dépléter les cellules B circulantes positives pour le CD20. Cette thérapie a déjà montré des effets bénéfiques chez les patients atteints de neuromyélite optique (Cree et al. 2005).

7. Ocrelizumab

L'ocrelizumab est un anticorps monoclonal totalement humanisé proposé à la fois dans le traitement des formes de SEP rémittent-récurrente mais aussi progressive. Comme le rituximab, il cible les lymphocytes B en bloquant le CD20. Une phase II est en cours.

8. Daclizumab

Cet anticorps monoclonal humanisé est un traitement dans le rejet d'allogreffe rénale approuvé depuis 1997. Il se lie au CD25 (chaine α du récepteur à l'IL2) exprimé sur les cellules T activées. Cet anticorps va bloquer l'expansion des lymphocytes T. Le marqueur CD25 est fortement exprimé à la surface des cellules régulatrices. Ce traitement aboutit entre autre à une diminution des cellules régulatrices (Oh et al. 2009). Le daclizumab n'exerce pas seulement une action anti-inflammatoire en diminuant l'activation des cellules T mais il induit aussi une population de cellules NK exprimant fortement le CD56 (Bielekova et al. 2006). Chez les patients atteints de SEP, ce traitement est à l'origine d'une diminution de l'inflammation au sein du SNC.

9. Alemtuzumab (Campath-1h)

Cet anticorps monoclonal humanisé neutralise la protéine membranaire CD52 exprimée à la surface de plus de 95% des lymphocytes T et B, des monocytes et des macrophages. Cette déplétion montre des effets bénéfiques sur le nombre de lésions visibles sur l'IRM, le taux de poussées chez des patients atteints de SEP de forme rémittente mais aussi de forme secondaire progressive (Coles et al. 2006). Il a un effet remarquable sur l'évolution du handicap (Coles et al. 2008). Malheureusement, certains effets indésirables graves ont également été observés : disthyroïdies, purpura thrombopénique en particulier.

B. L'immunologie de la Sclérose en Plaques

a. Les cellules effectrices

i. Les monocytes et les macrophages

Les monocytes sont des cellules mononuclées circulantes qui migrent dans les tissus, devenant des macrophages. L'IFNγ permet l'activation des macrophages. Ceux-ci sécrètent des cytokines inflammatoires telles que l'IL1, l'IL6, l'IL12, l'IL18, le TNFα, l'IFNγ, des radicaux oxygénés et des facteurs pro-coagulants. Ces cellules phagocytaires jouent de nombreux rôles dans l'immunité adaptative et innée.

Dans le sang des patients atteints de SEP, les monocytes sécrètent plus de cytokines pro-inflammatoires IL6 et IL12, et expriment à niveau élevé les molécules des voies de costimulation CD80 et CD86 (Kouwenhoven et al. 2001b). Ils expriment également des taux importants de LFA1 et VLA4 (Correale et al. 2003), facilitant leur passage dans le SNC. En effet, chez les patients, les infiltrats cellulaires sont composés entre autre de monocytes (Prineas et al. 1978). Les macrophages, contenant des débris de myéline, sont retrouvés au centre des lésions (Lucchinetti et al. 1996) et dans les ganglions cervicaux (Fabriek et al. 2005). *In vitro*, les macrophages, contenant les protéines αβ-cristalline et MOG après incubation avec des membranes de myéline, sont capables d'activer des lymphocytes T (Barjramovic et al. 2000).

ii. La microglie

La microglie possède des propriétés de cellules présentatrices d'antigène et de phagocytes. Lorsqu'elle est activée, elle sécrète des cytokines pro-inflammatoires telles que l'IL1, l'IL16, l'IL12, l'IL23, le TNFα et du monoxyde d'azote (NO) (John et al. 2003).

Le TNFα et le NO peuvent endommager les oligodendrocytes. La microglie se trouve fréquemment dans un état activé au niveau des lésions actives des patients atteints de SEP (Lassmann et al. 2001). Chez les malades, ces cellules produisent de grandes quantités d'IL23, une cytokine pro-inflammatoire, au niveau des lésions du SNC (Li et al. 2007).

iii. Les cellules dendritiques

Les cellules dendritiques (DCs) représentent une population rare de cellules présentatrices d'antigène (CPA) dites professionnelles possédant des prolongements cytoplasmiques caractéristiques et dont la fonction principale est la capture, l'apprêtage et la présentation des antigènes aux lymphocytes T. Deux grandes populations de DCs peuvent être identifiées chez l'homme : les DCs myéloïdes (mDCs, $CD4^+CD11c^+HLA\text{-}DR^+$) qui comme leur nom l'indique, ont une origine myéloïde et les DCs plasmacytoïdes (pDCs, $CD4^+CD11c^-HLA\text{-}DR^+$) qui ont, quant à elles, une origine lymphoïde (Shortman et al. 2002). Ces deux types de cellules diffèrent par leur profil de sécrétion de cytokines mais aussi par leur répertoire de pattern de reconnaissance. Elles interviennent aussi bien dans les réactions de l'immunité innée ou spécifique et les réponses immunes engagées sont très différentes car dépendantes des facteurs environnementaux.

Malheureusement, peu d'études se sont penchées sur l'implication des DCs dans le contexte de la SEP. Huang et ses collaborateurs rapportent un nombre élevé de DCs dans le sang périphérique des patients atteints de SEP (Huang et al.1999). Il a aussi été observé que les mDCs mais aussi les pDCs sont présentes dans le LCR de ces patients (Pashenkov et al. 2001). Serafini et ses collaborateurs ont montré que les DCs présentes au niveau du SNC des patients atteints de SEP sont préférentiellement localisées dans les zones périvasculaires, aussi bien dans les lésions actives que chroniques et elles contiennent dans leur cytoplasme des composants de la myéline (Serafini et al. 2000 ; Serafini et al. 2006). Des DCs avec un phénotype altéré ainsi que des interactions DCs/Treg (cellules T régulatrices) non fonctionnelles ont été décrites chez les malades (Navarro et al. 2006 ; Stasiolek et al. 2006). La fréquence et le phénotype des mDCs et des pDCs circulantes dans le sang des patients atteints de forme primaire progressive de SEP suggèrent que les DCs ont un état de maturation moindre dans ce cas (Lopez et al. 2006). Lorsque les patients sont traités par une forte dose de methylprednisolone en intraveineuse lors d'une poussée, une corrélation entre l'augmentation du nombre des Treg et la diminution du nombre des mDCs et des pDCs a été observée (Navarro et al. 2006). Les DCs des patients atteints de SEP produisent plus d'IL23 ce qui affecte la production d'IL10 (Vaknin-Dembinsky et al. 2008).

iv. Les mastocytes

Les mastocytes sont des cellules présentes au niveau des muqueuses. Elles sont surtout impliquées dans des phénomènes d'allergie mais aussi dans l'inflammation. Elles expriment à leur surface le récepteur de haute affinité pour les immunoglobulines de type IgE (FcεRI).

Au sein du SNC des patients atteints de SEP, les mastocytes sont retrouvés plus fréquemment dans des lésions chroniques et moins fréquemment au niveau des lésions actives (Ibrahim et al. 1996). Dans le LCR des patients, les mastocytes sécrètent des taux élevés de facteurs dirigés contre l'histamine (Tuomisto et al. 1983), la trypase et la protéase spécifique des mastocytes (Rozniecki et al. 1995). En réponse à la protéine basique de la myéline (MBP), les mastocytes peuvent dégranuler et induire une démyélinisation *in vitro* (Jonhson et al. 1988 ; Theoharides et al. 1993). De plus, les mastocytes peuvent réguler la rupture de la BHE (Zhuang et al.1996).

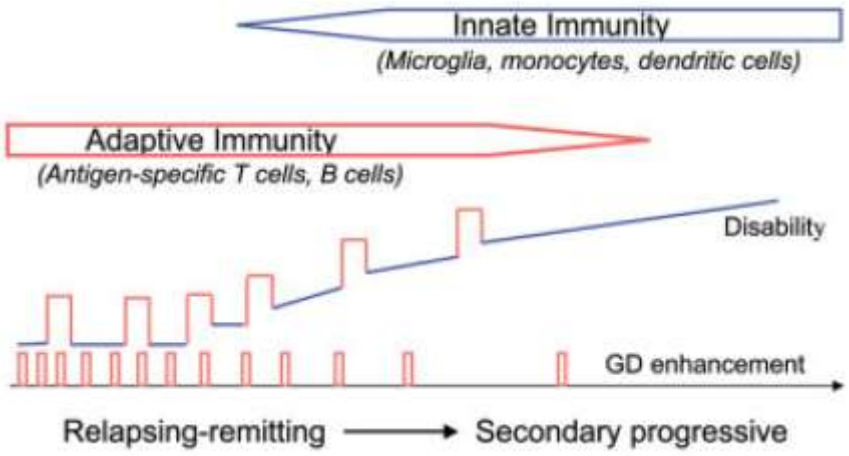

Figure 3 : Réactions immunitaires et évolution de la SEP au cours du temps
Schématiquement, le patient atteint de SEP débute la maladie par une forme rémittente-récurrente évoluant au bout d'un certain temps vers une forme secondaire progressive. La première phase de la maladie correspond à la forme rémittente-récurrente. Elle est caractérisée par des poussées, des prises de Gadolinium visibles à l'IRM et un handicap réduit. Ces troubles seraient le résultat de la réaction immunitaire dite adaptative. La seconde phase de la maladie caractérisée par la forme secondaire progressive est constituée d'une accumulation progressive du handicap en l'absence de poussées. Ces phénomènes seraient le résultat de la réaction immunitaire dite innée. *D'après Weiner et al. 2009*

b. Les différentes populations de lymphocytes

i. Les lymphocytes B

Les lymphocytes B interviennent dans la réponse immunitaire adaptative de type humorale. Les cellules B naïves matures caractérisées par l'expression du CD19 et l'absence du CD27 et du CD38 à leur surface sont libérées dans la circulation sanguine périphérique après leur développement et maturation dans la moelle osseuse et les organes lymphoïdes secondaires. Une fois activées, les cellules B vont proliférer et se transformer en plasmocytes (CD19-CD27highCD138+CD38+) sécrétant des anticorps. Chez les individus sains, la majorité des cellules B retrouvée dans le sang est composée de cellules naïves et quasiment aucun lymphocyte B n'est retrouvé dans le LCR (Tableau 3).

Plusieurs observations suggèrent que le système humoral est fortement impliqué dans la SEP. La première étude à mettre en évidence un rôle des lymphocytes B et des anticorps dans la physiopathologie de la SEP a été réalisée au niveau du LCR des patients. En effet, les immunoglobulines y étaient augmentées mais pas dans le sérum (Kabat et al. 1948 ; Cross et al. 2001). Cette augmentation est observée chez 90% des patients et elle est caractérisée par un profil oligoclonal (Correale et al. 2002). Bien que les lymphocytes B retrouvés dans le sang des patients aient le même phénotype que ceux des individus sains (CD19+CD27-) (Cepok et al. 2005 ; Corcione et al. 2004), des différences importantes existent. Le LCR des patients atteints de SEP contient environ 4 à 5% de cellules B CD19+, ces cellules présentent en majorité le phénotype mémoire (CD19+CD27+CD38-CD138-). Les plasmocytes ont aussi été décrits comme une population importante du LCR des patients atteints de SEP (Kuenz et al. 2008).

	Naïve B cell	Memory B cell	Plasma blast	Plasma cell	Centroblast
Surface markers	$CD19^+$ $CD27^-$ $CD38^-$	$CD19^+$ $CD27^+$ $CD138^-$ $CD38^-$	$CD19^+$ $CD27^{high}$ $CD138^+$ $CD38^+$	$CD19^-$ $CD27^{high}$ $CD138^+$ $CD38^+$	$CD19^+$ $CD77^+$ $Ki67^+$ $Bcl\text{-}2^-$ $CD38^{high}$
Healthy persons					
Periphery	+	(+)	−	−	−
CSF	−	−	−	−	−
Multiple sclerosis					
Periphery	+	(+)	−	−	−
CSF	−	+	+	+/(+)	+

Tableau 3: Distribution des sous-populations de lymphocytes B dans la SEP

+ présent ;- absent ; (+) présent mais à un faible niveau. *D'après Fraussen et al. 2009*.

Les lymphocytes B ne passent pas la BHE lorsque celle-ci est intacte. Cependant, une fois l'inflammation démarrée, les cellules B, les anticorps et le complément peuvent entrer dans le SNC. L'augmentation du niveau des immunoglobulines observée dans le LCR des patients mais non pas dans leur sérum semble indiquer une production locale.

Les lymphocytes B peuvent exercer différentes fonctions dans la SEP comme la fixation du complément, la cytotoxicité médiée de façon cellulaire, la présentation d'antigènes, la costimulation des lymphocytes T et la production de cytokines et de facteurs neurotrophiques (Cross et al. 2001 ; Ziemssen et al. 2005 ; Youinou et al. 2006 ; Meinl et al. 2006) cependant les recherches se sont surtout concentrées sur leur capacité à produire des auto-anticorps. Ces auto-anticorps peuvent détruire les tissus en recrutant des macrophages exprimant le récepteur au fragment Fc et en activant le complément. Ces auto-anticorps réagissent contre diverses protéines de la myéline comme la MBP (Gerriste et al. 1994), la glycoprotéine des oligodendrocytes et de la myéline (MOG) (Genain et al. 1999), la protéine protéolipidique (PLP) (Laman et al. 2001), la glycoprotéine associée à la myéline (MAG) (Baig et al. 1991), la protéine spécifique des oligodendrocytes (OSP) (Bronstein et al. 1999), la transaldolase (TAL) (Banki et al. 1994), l'αβ-crystalline (Agius et al. 1999) mais aussi contre des protéines ubiquitaires comme celles appartenant au protéasome (Mayo et al. 2002) ou des protéines chaperonnes neurales (protéines de choc thermique) (Cid et al. 2004). Des auto-anticorps réagissant contre des protéines neurales ont aussi été mis en évidence (Ehling et al. 2004) et Mathey et ses collaborateurs ont observé une augmentation de la concentration d'anticorps dirigés contre la neurofascine dans le sérum de patients atteints de SEP de forme secondairement progressive (Mathey et al. 2007). Les anticorps les plus décrits sont ceux anti-MOG (pour revue Reindl et al. 2006). Les cellules B jouent aussi un rôle dans les formes progressives de SEP (Magliozzi et al. 2007).

Les lymphocytes B, ainsi que les plasmocytes, ont été détectés au sein des lésions et des infiltrats lymphocytaires chez les patients atteints de SEP (Prineas et al. 1978). L'augmentation du nombre de lymphocytes B dans le LCR des malades est associée à une progression plus rapide de la pathologie (Cepok et al. 2001). Il est à noter qu'au niveau du LCR, on retrouve tous les types de différentiation de cellules B : allant de la cellule B naïve au plasmocyte (Corcione et al. 2004). En outre, il y a plus de lymphocytes B mémoires dans le LCR que dans le sang de patients atteints de SEP (Cepock et al. 2005). Le traitement par Rituximab, un anticorps monoclonal déplétant les cellules B, réduit fortement l'activité inflammatoire de la maladie comme le montrent les IRM, ceci sans affecter le niveau des

immunoglobulines. Ces résultats suggèrent un rôle des lymphocytes B dans la forme rémittente de SEP (Hauser et al. 2008).

Niino et son équipe ont montré que les cellules B mémoires pouvaient exprimer un important niveau de VLA4, une molécule d'adhérence se liant à VCAM1 exprimée par les cellules endothéliales. La partie α4-intégrine de VLA4 est une cible du Natalizumab, un autre anticorps capable de réduire l'activité de la maladie (Polman et al. 2006). Le Natalizumab réduit l'expression de VLA4 sur les cellules immunitaires circulantes et inhibe l'entrée des lymphocytes B et des lymphocytes T dans le SNC (Niino et al. 2006).

L'identification des follicules B ectopiques permettant la production des lymphocytes B et des cellules plasmacytoïdes dans le cerveau de patients atteints de SEP de forme secondaire progressive renforce l'idée que l'immunité humorale joue un rôle important dans l'évolution de cette pathologie (Serrafini et al. 2004).

ii. Les lymphocytes T γδ

Les lymphocytes T γδ représentent environ 5% des cellules T et sont essentiellement présents au niveau des muqueuses digestives. Ces cellules expriment CD1 et reconnaissent des antigènes non protéiques. Ce type de lymphocyte est présent au niveau des lésions du SNC des malades (Wucherpfenning et al. 1992) et sont plutôt du type δ2 alors qu'au niveau du sang des patients atteints de SEP on retrouve le type δ1 (Battisitni et al. 1997). Les lymphopcytes T γδ sont capables de lyser des oligodendrocytes *in vitro* (Zeine et al. 1998). Des expansions clonales des lymphocytes T γδ ont été retrouvées chez les malades en tout début de maladie (Shimonkevitz et al. 1993) et au niveau des lésions du SNC (Hvas et al. 1993).

iii. Les lymphocytes αβ auto-réactifs

Une fréquenc élevée de lymphocytes T réactifs contre la myéline est montrée dans le LCR de patients atteints de SEP (Lovett-Racke et al. 1998, Scholz et al. 1998). La présence de lymphocytes T auto-réactifs contre divers antigènes dérivés de la myéline dans le sang des patients a été détectée *in vitro* par de nombreuses équipes en établissant des lignées cellulaires spécifiques, en partculier contre :

- la MBP (Burns et al. 1983 ; Martin et al. 1990 ; Ota et al. 1990 ; Pette et al. 1990 ; Jingwu et al. 1992)

- la PLP (Sun et al. 1991 ; Markovic-Plese et al. 1995)
- la MOG (Kerlero de Rosbo et al. 1993 ; Lindert et al. 1999)
- l'αβ-crystalline (Chou et al. 2004)

Cependant, les lymphocytes T auto-réactifs contre la myéline font également partie du répertoire immun d'individus sains adultes (Tsuchida et al. 1994 ; Burns et al. 2002) malgré la sélection thymique. La présence de ce type de lymphocytes n'est pas à l'origine du déclenchement de la maladie mais peut participer par la suite au processus auto-immun.

D'autre part, plusieurs études ont montré un phénomène d'« epitope spreading » chez les patients, les épitopes reconnus par les patients varient au cours du temps (Davies et al. 2005 ; Goebels et al. 2000 ; Muraro et al. 2003).

iv. Les lymphocytes T CD8+

1. Les lymphocytes CD8+ cytotoxiques

Les lymphocytes T CD8+ sont des cellules cytotoxiques, capables de détruire des cibles cellulaires (exprimant le CMH de classe I). Leur activation s'effectue grâce à l'interaction entre le TCR et le complexe CMH de classe I / antigène porté par la CPA. Ils peuvent alors agir de plusieurs façons sur les cellules cibles :

- voie FasLigand/Fas : l'interaction entre FasLigand présent à la membrane du lymphocyte T CD8+ et Fas à la membrane de la cellule cible entraine l'activation d'une cascade de caspases, menant la cellule à l'apoptose.

- voie perforine/granzyme : l'exocytose de vésicules contenant les molécules perforine et granzyme permet à la perforine de former des pores au niveau de la membrane de la cellule cible et ainsi permettre l'entrée de granzymes entraînant également l'activation des caspases et l'apopstose (Trapani et al. 2002).

- voie du TNFα : la sécrétion de cytokines comme le TNFα participe à la lyse de la cellule cible.

Le rôle des lymphocytes T CD8+ a longtemps été méconnu tant l'importance des lymphocytes T CD4+ de type Th1 semblait prépondérante dans la pathologie de SEP.

Les lymphocytes T CD8+ cytotoxiques, présents en nombre au niveau du SNC chez les patients atteints de SEP, sont donc capables de causer des dommages aux oligodendrocytes mais aussi potentiellement aux axones au moins *in vitro* (Neumann et al. 2002 ; Pouly et al. 1999). Ces cellules sont effectivement capables d'endommager les axones, les neurones étant particulièrement vulnérables à la présence des lymphocytes. Dans un modèle *in vitro*

d'infection virale, les lymphocytes T cytotoxiques ont montré clairement leur capacité à détruire les axones. Chez les patients atteints de SEP, les dommages au niveau des axones corrèlent avec la présence de lymphocytes T CD8+ au niveau des lésions (Bitsch et al. 2000 ; Kuhlmann et al. 2002).

2. Les lymphocytes CD8+ effecteurs

En 2001, deux nouveaux modèles d'EAE ont été induits par l'injection de lymphocytes T CD8+ réactifs contre la MBP (Huseby et al. 2001) et la MOG (Sun et al. 2001). Chez les patients atteints de SEP, les infiltrats cellulaires au niveau des lésions sont majoritairement constitués de lymphocytes T CD8+ (Booss et al. 1983, Gay et al. 1997, Babbe et al. 2000). Les clones infiltrants peuvent persister dans le SNC, le LCR et le sang de ces patients pendant plusieurs années et des clones identiques peuvent exister dans différentes régions du cerveau d'un même malade (Jacobsen et al. 2002 ; Skulina et al. 2004 ; Junker et al. 2007). Dans le compartiment sanguin, la production de cytokines par les lymphocytes T CD8+ corrèle avec la formation de lésions détectées par IRM (Killestein et al. 2003) et avec les scores de handicap (Moldovan et al. 2003 ; Sepulcre et al. 2005). Notre équipe a montré que les altérations du répertoire des cellules T présentes dans le sang des patients atteints de SEP concernaient principalement le compartiment T CD8+ (Laplaud et al. 2004). Il a été également montré la présence de lymphocytes T CD8+ auto-réactifs contre des peptides de la myéline chez les patients atteints de SEP mais aussi chez des individus sains (Tsuchida et al. 1994 ; Honma et al. 1997 ; Crawford et al ; 2004 ; Zang et al. 2004). De plus, des lymphocytes T CD8+ spécifiques d'un peptide de la MBP (MBP110-118) ont montré des capacités de lyse d'oligodendrocytes *in vitro* (Jurewicz et al. 1998). Neumann et ses collaborateurs ont également montré que les lymphocytes CD8+ au niveau de lésions du SNC de patients SEP présentent une polarisation des vésicules contenant la molécule granzyme B dirigée vers un axone démyélinisé (Neumann et al. 2002).

v. Les lymphocytes T CD4+

Selon plusieurs paramètres comme l'environnement cytokinique ou la reconnaissance TCR/CMH, les précurseurs alloréactifs CD4 (Th0) ont la possibilité de se différencier selon quatre voies : Th1, Th2, Th17 ou iTreg (lymphocytes T régulateurs induits incluant les cellules Th3, Tr1,…) (Zhu et al. 2008) (Figure 4). Cette dernière voie de différenciation sera développée un peu plus tard dans le chapitre « Tolérance périphérique ». Les cellules T

CD4+, une fois activées par les cellules présentatrices d'antigènes, jouent un rôle primordial en activant de nombreuses cellules effectrices par l'intermédiaire de leur production de cytokines.

1. Les lymphocytes Th1

Dans l'initiation de la réponse Th1, la sécrétion d'IL12 joue un rôle primordial en permettant la différentiation de type Th1 en jouant directement sur les cellules NK et les lymphocytes CD4+. Les cellules NK activées vont produire l'interféron-γ (IFNγ) ce qui va engendrer, de concert avec l'IL12, la différentiation des lymphocytes CD4+ vers la voie Th1 (Murphy et al. 2002). Ces cellules de type Th1 activées vont alors sécréter d'importante quantité d'IFNγ, d'IL2 et de lymphotoxine-α (LTα). Les cellules CD4+ de type Th1 ont été largement décrites comme médiateurs de la réponse immune dirigée contre les pathogènes intracellulaires (Mosmann et al. 1989, Paul et al. 1994). Le facteur de transcription T-bet a été identifié comme un élément clef pour la différentiation en cellules de type Th1 et pour l'induction de la production d'IFNγ (Szabo et al. 2000).

Le gène de susceptibilité reconnu de la SEP codant une molécule de CMH de clase II et les modèles animaux d'EAE étant uniquement induits par l'injection de ce type de lymphocytes, un postulat général plaçait les lymphocytes CD4+ de type Th1 comme effecteurs principaux dans la pathologie SEP. De nombreuses études ont en effet montré une augmentation des taux de cytokines pro-inflammatoires TNFα et IFNγ sécrétées par ces cellules, aussi bien au niveau du LCR (Benvenuto et al. 1991) que dans le cerveau (Hofman et al. 1989) ou dans le sang des patients atteints de SEP. De plus, la production de TNFα, d'IFNγ et d'IL2 par les cellules circulantes corrèle avec les lésions actives visibles à l'IRM (Chofflon et al. 1997, Calabresi et al. 1998 ; Killestein et al. 2001a ; Killestein et al. 2001b). Le taux de cellules CD4+ mémoires, contenant des marqueurs de type Th1 montre également une corrélation avec l'activité et la sévérité de la maladie (Krakauer et al. 2006). Des infiltrats de cellules Th1 ont été décrits au niveau de cerveau de patients atteints de SEP (Kivisakk et al. 2003). Les lymphocytes T CD4+ de type Th1 sont non seulement capables d'activer des effecteurs cellulaires mais aussi de causer eux-mêmes des dommages aux oligodendrocytes. *In vitro*, l'IFNγ rend les oligodendrocytes susceptibles à l'apoptose déclenchée par Fas (Pouly et al. 2000). Enfin, un essai thérapeutique utilisant un IFNγ recombinant a aggravé la maladie chez les patients testés (Panitch et al. 1987). Une forte augmentation du facteur de

transcription T-bet a été observée dans les PBMC, en particulier dans les CD4+, des patients atteints de SEP en phase de rémission (Frisullo et al. 2006).

2. Les lymphocytes Th2

La différentiation de type Th2 est induite principalement par deux cytokines : l'IL2 et l'IL4 (Le Gros et al. 1990 ; Swain et al. 1990). Les cellules CD4+ de type Th2 activées vont alors sécréter de nombreuses cytokines dont l'IL4, l'IL5, l'IL9, l'IL10 et l'IL13. Ces cellules jouent un rôle majeur dans la réponse immune vis-à-vis de pathogènes extracellulaires, en agissant sur le « switch » isotypique des immunoglobulines produites par les lymphocytes B, notamment par l'interaction CD40-CD40L (Lumsden et al. 2003), mais aussi en induisant l'activation des éosinophiles.

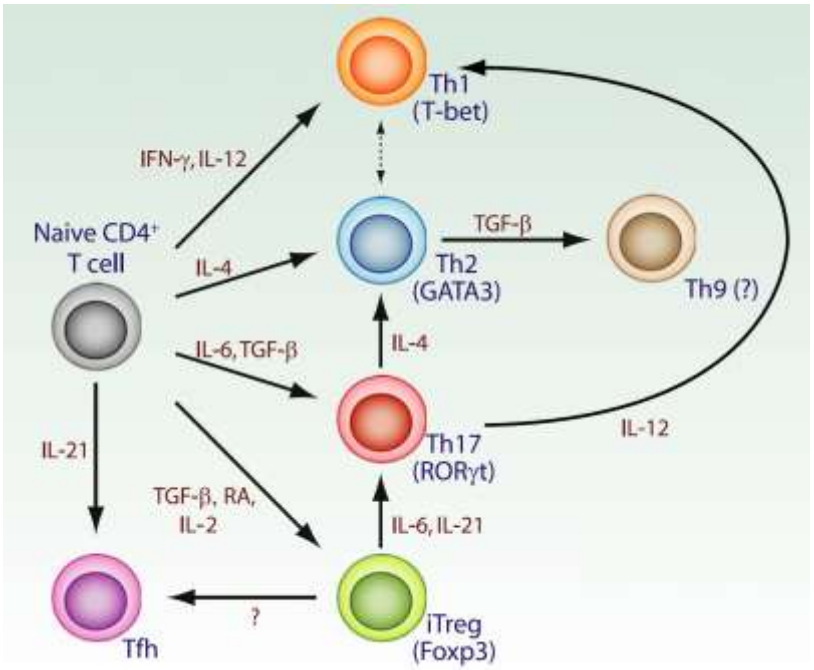

Figure 4 : La différentiation des lymphocytes T CD4+ naïfs dépend de l'environnement cytokinique
Les cellules naïves T CD4+ peuvent se différencier en cellules de type Th1, Th2, Th17, iTreg et Tfh. Ces programmes de différentiation sont contrôlés par les cytokines produites par les cellules de l'immunité innée, comme l'IL12 ou l'IFNγ, deux cytokines importantes pour le profil Th1, ou l'IL4, cytokine primordiale pour la différentiation en cellules de type Th2. La présence de TGFβ associée à celle d'IL6 conduit les cellules vers la voie Th17 tandis que la différentiation en iTreg est induite par le TGFβ, l'acide rétinoique et l'IL2. La différentiation en cellules Tfh requiert, quant à elle, la sécrétion d'IL21. *D'après Zhou et al. Immunity 2009*

3. Les lymphocytes Th17

Le TGFβ joue un rôle critique dans la différentiation du type Th17. En présence d'IL6 produite par les cellules de l'immunité innée, le TGFβ initie la différentiation du type Th17 et induit la production d'IL21 et l'expression du récepteur à l'IL23 et de RORγt (Bettelli et al. 2006 ; Mangan et al. 2006 ; Veldhoen et al. 2006). L'IL21 est responsable de l'amplification de la différentiation Th17 mais peut aussi remplacer l'IL6 dans l'initiation de cette différenciation (Korn et al. 2007 ; Nurieva et al. 2007). Finalement, l'IL23 qui avait été proposé initialement comme facteur de différentiation pour les cellules Th17, joue en réalité le rôle dans le maintien de la fonction de différentiation pour les cellules Th17 différenciées (Veldhoen et al. 2006). Ces cellules CD4+ de type Th17 produisent de l'IL17A, de l'IL17F, de l'IL21 et de l'IL22. Il a été démontré que ces cellules jouent un rôle dans la réponse vis-à-vis de bactéries extracellulaires et de champignons (Weaver et al. 2006). Les cellules Th17 ont été identifiées comme une population cellulaire indépendante de la population cellulaire «T helper » grâce à l'identification de facteurs de différenciation et de transcription spécifiques de ces cellules. En 2006, trois études indépendantes ont montré qu'une combinaison entre le TGFβ et l'IL6, cytokine pro-inflammatoire, était nécessaire pour induire la production d'IL17 par des cellules T naïves (Veldhoen et al. 2006 ; Betteli et al. 2006 ; Mangan et al. 2006). Les cellules Th17 ont des fonctions effectrices bien distinctes de celles des cellules Th1 et Th2. Ces cellules jouent aussi un rôle dans le rejet aigu d'allogreffes (Afzali et al. 2007) mais aussi dans le développement de maladies auto-immunes dont la SEP ou la polyarthrite rhumatoïde (Betteli et al. 2007 ; Bettelli et al. 2008).

Les lymphocytes de type Th17 ont un rôle dans l'induction de l'EAE (Steinman et al ; 2007). Les souris KO pour l'IL23 ou déficientes en IL17 sont en effet résistantes à l'induction d'une EAE (Cua et al. 2003 ; Komiyama et al. 2006). Chez les patients atteints de SEP, une augmentation des taux d'IL17 et d'IL23 dans les cellules mononuclées du sang et du LCR ainsi qu'au sein des lésions du SNC a été notée (Matusevicius et al. 1999 ; Lock et al. 2002 ; Tzartos et al. 2008). Kebir et ses collaborateurs ont montré l'expression des récepteurs à l'IL17 et l'IL22 sur les cellules endothéliales constituant la BHE. Ces cytokines endommagent les jonctions serrées de la BHE *in vitro* et *in vivo*. De plus, dans ce modèle *in vitro* les cellules de type Th17 transmigrent efficacement en exprimant du granzyme B (Kebir et al. 2007). Une étude immunohistochimique a mis en évidence que l'infiltrat de cellules T était enrichi en cellules T du type Th17 (Montes et al. 2009). De plus, l'expansion des cellules de type Th17 est associée à l'activité de la maladie. L'IFNβ inhibe les cellules de type Th17 à

la fois chez les patients et chez les témoins, cet effet n'est pas observé pour les cellules de type Th1 (Durelli et al. 2009).

4. Les lymphocytes Tfh

Une nouvelle population cellulaire au sein des lymphocytes T CD4+ a été mise en évidence. Il s'agit des cellules « follicular helper T » (Tfh). Ces cellules régulent le développement de la réponse immune passant par les lymphocytes B et ce de façon spécifique d'un antigène spécialisé (Figure 5) (Fazilleau et al. 2007 ; King et al. 2008 ; Vinuesa et al. 2005). Les lymphocytes de type Tfh sont caractérisés par l'expression d'un récepteur au chemiokine : CXCR5 mais aussi par l'expression de ICOS. La différentiation des cellules de type Tfh à partir de cellules naïves Th0 requiert un processus faisant intervenir l'IL21 (Vogelzang et al. 2008 ; Nurieva et al. 2008) et dépendant du facteur de transcription Bcl-6 (Fazilleau et al. 2009) (Figure 4).

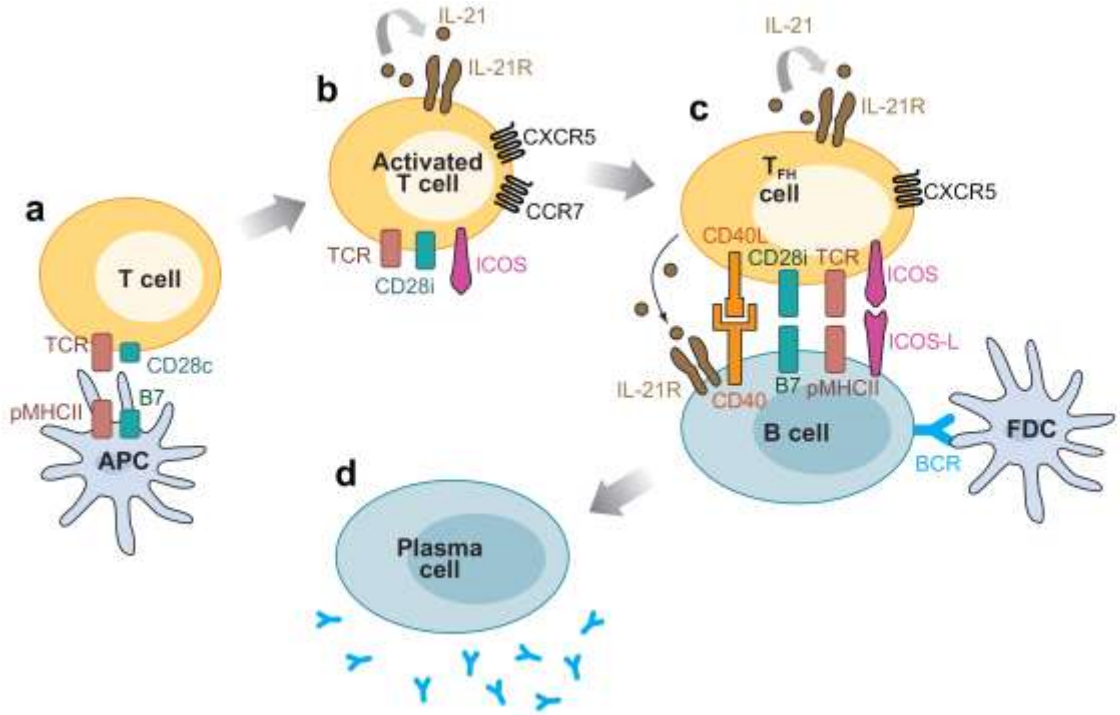

Figure 5 : La différentiation des lymphocytes T CD4+ de type Tfh et leur rôle dans l'immunité humorale CD28, ICOS et l'IL21 costimulent les cellules T CD4+ activées par leur TCR. L'environnement cytokinique, les molécules de costimulation ainsi que les récepteurs interviennent de façon ordonnée dans les étapes de différentiation des cellules de type Tfh. **a**. Au sein de la zone T des tissus lymphoïdes, les cellules naïves T CD4+ sont activées via leur TCR. **b.** Les cellules activées sécrètent de l'IL21 et induisent l'expression de CD28 et d'ICOS. **c.** L'ensemble des signaux conduit à la modulation de l'expression de molécules importantes pour la migration et de molécules de costimulation. **d.** la migration de lymphocytes de type Tfh fonctionnels dans les follicules va entraîner la sélection de cellules B activées secrétant des immunoglobulines *D'après King et al. 2008*

L'IL21 est un membre de la famille des cytokines IL2 jouant un rôle important dans le développement des cellules Tfh (Parrish-Novak et al. 2000 ; Spolski et al. 2008). Les cellules de type Th17, Th2 et Tfh sécrètent de l'IL21. L'absence d'IL21 et de son récepteur a un impact sur le développement de l'immunité humorale avec une diminution du « switch » isotypique et un défaut du développement des centres germinatifs (Ozaki et al. 2002, Spolski et al. 2008). Les lymphocytes de type Tfh produisent des quantités importantes d'IL21 (Nurieva et al 2008 ; Volgelzang et al. 2008) avec une action autocrine du fait de l'expression concomitante du récepteur à l'IL21 (Chtanova et al. 2004 ; Rasheed et al. 2006 ; Vinuesa et al. 2005).

L'IL6 peut aussi induire la sécrétion d'IL21 par les cellules de type Tfh. Cette production d'IL21 fait intervenir une voie de signalisation passant par Stat3 (Nurieva et al. 2008).

Le TGFβ, quant à lui, n'est pas requis pour l'induction des cellules effectrices de type Tfh et RORγt n'est pas exprimé par ces cellules (Nurieva et al. 2008). Il semble donc que les étapes menant à la différentiation des cellules en Th17 ou Tfh soient bien indépendantes.

À l'heure actuelle, l'implication des lymphocytes de type Tfh n'a pas été étudiée chez les patients atteints de SEP (ou d'autres pathologies inflammatoires du SNC). Il pourrait être intéressant de regarder particulièrement ce sous-type cellulaire dans les pathologies auto-immunes impliquant des anticorps pathologiques telles que la maladie de Devic ou l'Encéphalite de Rasmussen.

5. Les lymphocytes Th9

Très récemment, une nouvelle population de lymphocytes T CD4+ a été mise en évidence. Les groupes de V.Kuchroo et B. Stockinger ont décrit une population de cellules T CD4+ de type Th9 caractérisées par leur forte production d'IL10 et d'IL9 (Dardalhon et al. 2008. Veldhoen et al. 2008). Ces cellules sont générées en présence de TGFβ et d'IL4, cette dernière cytokine bloquant la différenciation en cellules T régulatrices. Ces cellules ne présentent pas de capacité de régulation *in vitro* ni *in vivo*, malgré leur forte production d'IL10. En effet, l'injection de ces cellules à des souris RAG déficientes induit l'apparition de colite et de neurite montrant leur capacité à promouvoir l'inflammation.

C. La tolérance

a. La tolérance centrale

La tolérance immunitaire spécifique se définie comme une absence de réaction vis-à-vis de certains antigènes, en l'absence d'immunosuppression, tout en maintenant la réponse immunitaire contre d'autres antigènes.

Les lymphocytes se développent dans le thymus à partir de précurseurs provenant de la moelle osseuse. Le réarrangement des gènes codant leur TCR se produit lors de ce développement, puis le répertoire lymphocytaire est défini par l'intermédiaire de la sélection positive puis négative. Lors de ces étapes, plus de 95% des thymocytes sont éliminés.

La sélection positive permet de conserver les thymocytes capables de reconnaître via leur TCR un peptide associé à une molécule du CMH du soi (Fink et al. 1978). Au cours de ce processus, les cellules épithéliales du cortex thymique présentent aux cellules T des complexes CMH/peptides et fournissent un signal de survie aux cellules capables d'interagir avec les molécules du CMH du soi (Benoist et al. 1989). Les cellules T qui reconnaissent trop faiblement les molécules du CMH du soi meurent par apoptose dans le cortex.

La sélection négative joue un rôle primordial dans l'établissement de la tolérance aux antigènes du soi en éliminant la plupart des lymphocytes auto-réactifs. Cette sélection peut être effectuée par les cellules épithéliales médullaires mais ce sont les cellules dendritiques présentes dans le thymus qui apparaissent comme les principaux acteurs de cette selection (Anderson et al. 1998). Récemment, il a été montré que le répertoire des peptides du soi présentés dans le thymus est étonnamment large. En effet, des antigènes exclusivement exprimés dans certains tissus peuvent être inclus dans ce répertoire. C'est le cas de l'insuline, un antigène spécifique des îlots pancréatiques (Kyewski et al. 2002 ; Gotter et al. 2004). La tolérance centrale est un phénomène important dans le contexte de la transplantation car elle peut avoir lieu lorsqu'un chimérisme s'est créé au sein du thymus du receveur. Un chimérisme thymique se définit par la présence de CPA du donneur dans le thymus du receveur. Ainsi les CPA du donneur induisent par sélection négative l'élimination des clones T allogéniques reconnaissant les molécules du CMH du donneur.

Les thymocytes réactifs au soi ne sont cependant pas tous éliminés après la déplétion négative dans le thymus (Bouneaud et al. 2000). Les mécanismes périphériques du maintien de la tolérance sont donc également essentiels et peuvent constituer des approches pour l'induction de tolérance aux allo-antigènes. L'expression au sein du thymus d'antigènes

restreints à un tissu est probablement sous le contrôle de plusieurs facteurs de transcription dont AIRE (autoimmune regulator). Les mutations touchant ce facteur de transcription ont pour conséquence une dérégulation transcriptionnelle menant à l'expression de nombreux antigènes du soi par les cellules épithéliales de la medulla thymique ainsi qu'à différentes maladies auto-immunes spécifiques d'organe (Anderson et al. 2002 ; Liston et al. 2003 ; Liston et al. 2004).

Différents auto-antigènes du SNC pertinents dans le cadre de la SEP sont exprimés dans le thymus. En effet, de l'ARNm de la MBP ainsi que la protéine sont synthétisés par plusieurs types cellulaires dans le thymus dont les macrophages (Liu et al. 2001).

En dépit des mécanismes de tolérance thymique, des cellules T spécifiques de la MBP et potentiellement auto-réactives peuvent circuler dans l'organisme (Kuchroo et al. 2002). Leur fréquence et leur avidité peuvent cependant varier ceci étant lié l'étendue de l'expression de la MBP au sein du thymus.

b. La tolérance périphérique

Quatre mécanismes principaux de tolérance périphérique, mutuellement non exclusifs, ont été décrits : l'anergie, la délétion clonale, l'ignorance et la suppression (Figure 6).

i. L'anergie

L'anergie est un état d'inactivation fonctionnelle de la cellule T devenant réfractaire à toute restimulation par l'antigène (Lecher et al. 2001, Schwartz et al. 2003). Elle peut être induite in vitro par une forte stimulation des cellules T via leur TCR en l'absence de second signal de costimulation. L'état anergique se caractérise par une incapacité des cellules T à proliférer et à produire de l'IL2. Celui-ci peut être levé par l'ajout d'IL2 (Essery et al. 1988). Plus récemment, il a été décrit que de faibles stimulations en présence de costimulation pouvaient aussi engendrer un état anergique mais uniquement sur des cellules T mémoires (Mirshahidi et al. 2001). Ces cellules anergiques peuvent aussi jouer un rôle immunosuppresseur en inhibant la prolifération des cellules T naïves (Chai et al. 1999) ou en inhibant la capacité des DCs (nécessité de contact) à stimuler les lymphocytes T naïfs (Vendetti et al. 2000 ; Salcido-Ochoa et al. 2002).

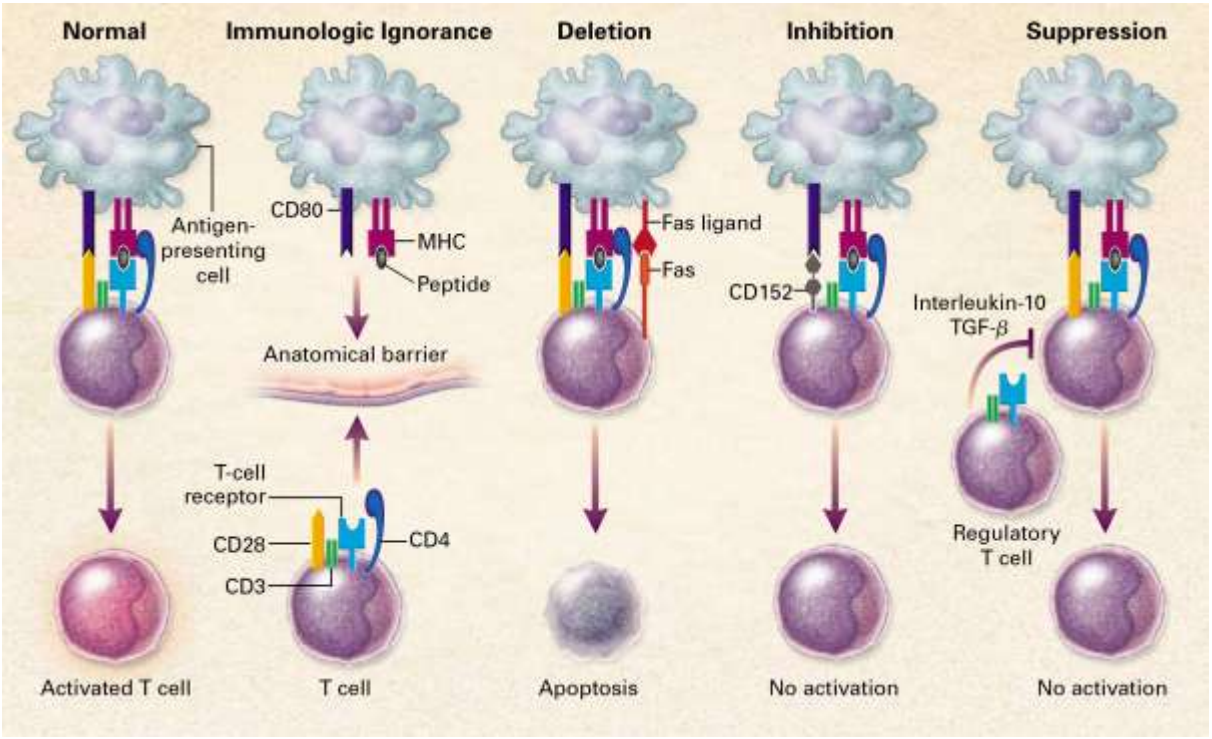

Figure 6: Les mécanismes de tolérance périphérique
D'après Kamradt et al. 2001

ii. La délétion clonale

Nous avons vu que les mécanismes de tolérance centrale permettent d'éliminer les cellules autoréactives dans le thymus, avant leur maturation. La délétion des cellules T peut également avoir lieu à la périphérie, lorsque celles-ci rencontrent l'antigène dans des conditions particulières (Kabelitz et al. 1993). Deux situations peuvent aboutir à la mort des cellules T : soit un défaut de signaux de survie, par exemple en l'absence de facteurs de croissance comme l'IL2 (Steller et al. 1995), soit une activation trop importante. Ce deuxième mécanisme est nommé « mort cellulaire induite par l'activation » (AICD) et fait intervenir principalement le système Fas/FasL (Brunner et al.1995).

iii. L'ignorance

Le terme d'ignorance s'applique à une stimulation dans laquelle des cellules T potentiellement réactives ne répondent pas à une stimulation antigénique. Ces cellules ne sont pas inactivées, les différenciant des cellules anergiques, mais elles ignorent l'antigène (Miller et al. 1990). Plusieurs mécanismes peuvent être responsables de ce phénomène d'ignorance.

Dans un modèle d'allogreffe cardiaque chez la souris en absence d'organes lymphoïdes secondaires, la rencontre entre lymphocytes T et les cellules dendritiques ne se fait pas, conduisant à l'établissement d'une ignorance immunologique et favorisant la tolérance à l'allogreffe (Lakkis et al. 2000). De plus, l'ignorance peut résulter de la séquestration de l'antigène dans des sites dits « privilégiés », non accessibles aux cellules T réactives, tels que l'œil ou le SNC (Barker et al. 1977) ou d'une absence de présentation par les CPA. Elle peut aussi être due à une faible concentration d'antigènes, les rendant invisibles aux lymphocytes T (Kurts et al. 1999).

iv. La suppression

Contrairement aux autres mécanismes de tolérance centrale et périphérique, la suppression est un phénomène actif dans lequel une population cellulaire contrôle ou régule l'activité d'une autre population. En 1985, le groupe de Hall a montré que l'état de tolérance allogénique pouvait être transféré à des receveurs naïfs par l'injection de cellules T CD4+ dérivant d'animaux tolérants (Hall et al. 1985), fournissant ainsi la première démonstration du caractère actif de la suppression.

La suppression est réalisée par des cellules régulatrices. De nombreux types de cellules régulatrices ont été décrits, certains présents de façon naturelle dans l'organisme et d'autres pouvant être induits dans des conditions de stimulations particulières. Les principales cellules régulatrices décrites sont des cellules T CD4+ mais des cellules régulatrices ont aussi été décrites dans la population T CD8+ et dans d'autres types cellulaires comme les NKT et les cellules T γδ (Jameson et al. 2003 ; Van Kaer et al. 2004). De plus, les cellules dendritiques sont fortement impliquées dans la tolérance et dans l'induction des cellules régulatrices.

c. Les cellules régulatrices

Le concept de lymphocytes T régulateurs (Treg) capables d'inhiber une réponse immunitaire, est apparu dans les années 1970. Le groupe de Gershon et Kondo a montré que certains lymphocytes T, différents des lymphocytes T « helpers », pouvaient diminuer l'intensité de la réponse immune (Gershon et al. 1970 ; Gershon et al. 1971). De plus, ce concept a été renforcé par des études montrant que le transfert de cellules T était capable de prévenir le développement de maladies auto-immunes, telles que la thyroïdite (Penhale et al. 1976), l'oophorite (Sakaguchi et al. 1982) ou le diabète (Boitard et al. 1989). Cependant,

l'incapacité à identifier un marqueur de surface spécifique de ces cellules ou des médiateurs solubles associés à leur fonction a conduit à l'essoufflement des recherches dans ce domaine et à la contestation de l'existence des lymphocytes T suppressifs (Green et al. 1993).

Dans le milieu des années 1990, les travaux de Sakaguchi et de ses collaborateurs ont apporté un regain d'intérêt à ce concept en décrivant pour la première fois le phénotype d'une population de cellules T CD4+ suppressives définies par l'expression de la chaine α du récepteur à l'IL2 (CD25), qui étaient cruciales pour le contrôle des cellules T *in vivo* (Sakaguchi et al. 1995 ; Asano et al. 1996) (Figure 7).

i. Les lymphocytes T régulateurs naturels

1. Origine et caractéristiques phénotypiques

Les cellules T régulatrices naturelles ou « innées » (nTreg) sont générées dans le thymus (Stephens et al. 2001 ; Annunziato et al. 2002 ; Fehervari et al. 2004) et représentent 5 à 10% des thymocytes matures CD4+ et 2 à 5% des PBMC (Bacchetta et al. 2005). Un des problèmes majeurs a été et continue d'être l'indentification d'un marqueur spécifique de ces cellules. Cependant, il est bien établi qu'elles expriment de manière constitutive la chaine α du récepteur à l'IL2 (CD25). Chez l'homme, seules les cellules exprimant fortement l'IL2RA (CD25high) possèdent des propriétés suppressives (Baecher-Allan et al. 2001), alors qu'un niveau d'expression intermédiaire correspond aux cellules activées, mais cette distinction n'est pas absolue puisque des cellules régulatrices CD25low ont également été décrites (Tsaknaridis et al. 2003).

En plus du CD25, de nombreux marqueurs ont été proposés pour identifier plus précisément cette population cellulaire sans qu'aucun d'eux ne soit réellement spécifique du phénotype régulateur : le marqueur CD45RO (marqueur des cellules mémoires), CD122 (chaine β du récepteur à l'IL2), CD62L (L-sélectine), CD103 (intégrine $\alpha_E\beta_7$). Ils se caractérisent également par l'expression constitutive du CD152 (CTLA4) et de GITR (Wood et al. 2003).

Plus récemment, un facteur de transcription, Foxp3 (Forkhead box P3), un nouveau membre de la famille des régulateurs transcriptionnels forkhead/winged helix, a été identifié comme un facteur de transcription majeur pour le contrôle du développement et de la fonction des cellules T régulatrices CD4+CD25+ (Fontenot et al. 2003 ; Hori et al. 2003 ; Kim et al. 2006). En effet, l'expression ectopique de Foxp3 dans les cellules CD4+CD25+ leur confère un phénotype et une activité suppressive. Différentes études ont ainsi montré que les souris ou

les individus n'exprimant pas ou exprimant une protéine mutée de Foxp3 développent de graves syndromes lymphoprolifératifs (syndrome IPEX chez l'homme) (Bennet et al. 2001 ; Brunkow et al. 2001 ; Wildin et al. 2001). Même s'il a été montré que l'expression de Foxp3 seule n'était pas suffisante pour conférer une activité régulatrice (Baecher-Allan et al. 2005) et qu'elle était aussi retrouvée chez des lymphocytes activés chez l'homme (Morgan et al. 2005 ; Ziegler et al. 2007), à l'heure actuelle, il reste tout de même un marqueur très spécifique de ces cellules T régulatrices. Cependant, sa localisation intracellulaire ne permet pas son utilisation pour trier ces cellules et de réaliser des études fonctionnelles.

Récemment, Seddiki et ses collaborateurs ont montré que la faible expression du marqueur CD127 (chaine α du récepteur à l'IL7) combinée à la forte expression du CD25 permettait de mieux différencier les cellules T régulatrices des lymphocytes T activés, que ce soit dans le sang périphérique, le cordon ombilical ou les organes lymphoïdes (Seddiki et al. 2006). Ainsi les cellules T CD4+ exprimant fortement CD25 et faiblement CD127 ($CD25^{high}CD127^{low}$) sont hautement suppressives dans les tests fonctionnels réalisés. Ce nouveau marqueur CD127 est, de plus, corrélé négativement avec l'expression de Foxp3 (alors qu'il n'existe pas de corrélation entre CD25 et Foxp3). Cette corrélation négative entre les deux molécules s'explique par le fait que Foxp3 interagit avec le promoteur de CD127 et contribue ainsi à la faible expression de cette molécule au niveau des cellules régulatrices (Liu et al. 2006).

Très récemment, il a été proposé de combiner le CD49d au CD127 pour obtenir une population nTreg encore plus pure exempte de cellules effectrices CD25+ (Kleinewietfeld et al. 2009). Miyara et ses collaborateurs, quand à eux, proposent de combiner uniquement le CD45RA ou CD45RO au CD25 pour discriminer au mieux les nTreg. En effet, ils ont montré que les lymphocytes T humains Foxp3+CD4+ sont composés de trois sous-populations distinctes aussi bien sur le plan phénotypique que fonctionnel. Ils ont identifié les cellules CD45RA+Foxp3low « resting Treg » (rTreg), les cellules CD45RA-Foxp3high « activated Treg » (aTreg) et les cellules CD45RA-Foxp3low sécrétrices de cytokines. Les rTreg et aTreg possèdent des capacités suppressives tandis que la dernière sous population CD45RA-Foxp3low n'est pas une population régulatrice. De plus, cette dernière population comprend des cellules ayant un potentiel Th17 (Miyara et al. 2009).

2. Mécanismes d'action

Les mécanismes d'action d'immunosuppression des cellules nTreg et des molécules impliquées dans cette suppression bien que largement étudiés sont toutefois encore mal connus. Cependant, il est clairement établi maintenant que les cellules nTreg sont activées par leur TCR (Sanchez et al. 2006), sont anergiques et ne produisent pas d'IL2 (Thornton et al. 1998 ; Shevach et al. 2002 ; Thornton et al. 2004). Cette anergie peut être levée par ajout d'IL2 (Dieckmann et al. 2001) ou par une forte stimulation de la molécule CD28 (Baecher-Allan et al. 2001). Les cellules régulatrices sont bloquées dans leur cycle cellulaire, elles présentent une diminution des cyclines E et A (Li et al. 2005). Le niveau d'activation des protéines kinases MEK1/2 et Erk1/2 est diminué au sein de ces cellules. La présence exogène d'IL2 augmente le niveau de cyclines E et A ainsi que les protéines kinases engendrant la survie et la prolifération des nTreg (Li et al. 2005). Une fois activées par un antigène, leur action suppressive n'est plus dépendante de l'antigène les ayant stimulées. Leur fonction régulatrice est donc non spécifique (Thornton et al. 2000).

Elles sont alors capables d'inhiber la prolifération et la production de cytokines des lymphocytes T CD4+CD25-, des lymphocytes T CD8+ (Suvas et al. 2003 ; Camara et al. 2003) et elles sont capables d'inhiber la différentiation Th1/Th2 (Xu et al. 2003 ; Toda et al. 2006). L'action régulatrice des cellules nTreg sur les CD4+CD25- s'exerce en inhibant la transcription d'IL2 et nécessite *in vitro* un contact cellulaire (Thornton et al. 1998 ; Dieckmann et al. 2001). Cette activité régulatrice semble indépendante des CPA puisque leur fonction suppressive s'exerce dans des cultures en absence de CPA (Shevach et al. 2002).

Les cellules régulatrices T naturelles ont depuis longtemps été décrites comme exerçant leur fonction suppressive par contact cellulaire et non par l'intermédiaire d'IL10 (cellules Tr1) et du TGFβ (cellules Th3) (Takahashi et al. 1998 ; Thornton et al. 1998). Cependant, un rôle de cytokines ne peut être totalement exclu (Vignali et al. 2008). En effet, récemment l'IL35 a été décrite comme une cytokine aux propriétés immunosuppressives. Elle est nécessaire aux cellules T régulatrices pour que celles-ci puissent exercer une suppression maximale. De plus, l'IL35 inhibe la différentiation des cellules CD4+ de type Th17 et atténue la sévérité de l'arthrite dans un modèle murin en diminuant la production d'IL17 (Niedbala et al. 2007). Contrairement à ces résultats obtenus chez la souris, une récente étude chez l'homme a montré que les cellules régulatrices n'exprimaient pas l'IL35 de façon constitutive (Bardel et al. 2008).

3. Rôle dans la Sclérose en Plaques

Plusieurs auteurs ont pu mettre en évidence des fonctions suppressives altérées de la population cellulaire T CD4+CD25+ chez les patients atteints de SEP (Viglietta et al. 2004). Cependant, le taux de cellules T régulatrices CD4+CD25+ chez les patients atteints de SEP est similaire à celui des individus sains (Feger et al. 2007 ; Haas et al. 2005 ; Putheti et al. 2004 ; Viglietta et al. 2004). Deux équipes ont rapporté ce défaut de suppression envers des réponses dirigées contre des protéines de la myéline (MOG et MBP) (Kumar et al. 2006 ; Haas et al. 2005). Frisullo et ses collaborateurs ont observé une diminution à la fois de l'expression de Foxp3 et de la fréquence de cellules T CD4+CD25+Foxp3+ circulantes lors des phases de poussées comparées aux phases de rémission chez les patients atteints de SEP de forme récurrente-rémittente. Chez ces patients, les cellules régulatrices ont une capacité régulatrice diminuée sur la prolifération des cellules CD4+ exprimant un niveau élevé du facteur T-bet (Frisullo et al. 2008). Il faut noter que toutes ces études ont été réalisées en utilisant des cellules régulatrices sélectionnées uniquement grâce au marqueur CD25, peu discriminant et peu reproductible.

Les études réalisées sur les cellules régulatrices T CD4+CD25+ chez des patients atteints de SEP de forme secondaire progressive n'ont pas mis en évidence de défaut de régulation à la différence des résultats obtenus chez les patients atteints de SEP de forme rémittente-récurrente (Venken et al. 2006). Les mécanismes expliquant ce défaut de régulation sont mal connus à ce jour. Cependant, Haas et ses collaborateurs nous apportent des éléments de réponse en suggérant que chez les patients atteints de SEP il y aurait une production altérée de Treg au niveau du thymus (Haas et al. 2007).

Après une corticothérapie au methylprednisolone, la fréquence des cellules T CD4+CD25high ainsi que l'expression de Foxp3 sont augmentées chez les patients atteints de SEP de forme récurrente-rémittente (Braitch et al. 2009). Les cellules régulatrices T expriment un niveau faible de VLA4 et se lient moins bien au Natalizumab. Le défaut de régulation est toujours présent après traitement (Stenner et al. 2009). Haas et ses collaborateurs n'ont pas mis en évidence de différence de fréquence des cellules T CD4+CD25+ dans le sang ni dans le LCR (Haas et al. 2005). Il existe une augmentation des Treg au sein du LCR comparé au sang chez les patients atteints de SEP de forme récurrente-rémittente. Pour les patients atteints de SEP de forme secondaire progressive, les Treg sont moins nombreux dans le LCR que dans le sang (Feger et al. 2007 ; Venken et al. 2007). L'expression de CD49d et de CD103 à la surface des Treg retrouvés dans les PBMCs suggère que ces cellules peuvent migrer vers le SNC chez les patients atteints de SEP.

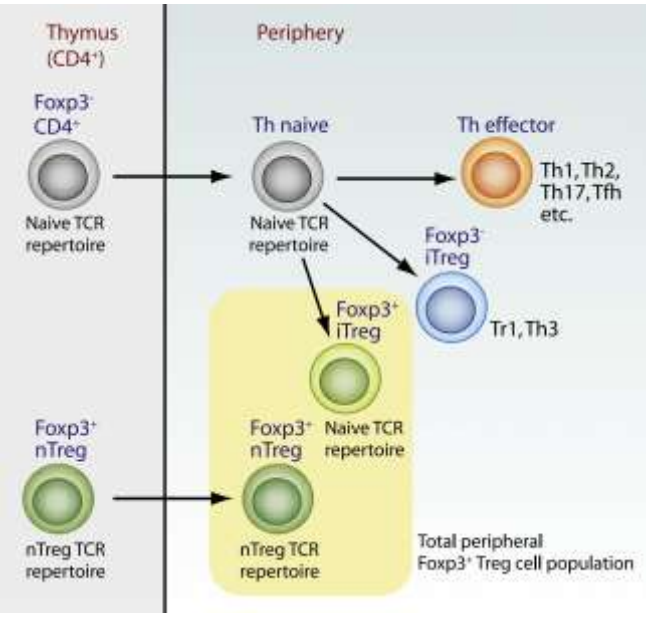

Figure 7: Les cellules régulatrices T CD4+
D'après Curotto de Lafaille et al. 2009

ii. Les lymphocytes T régulateurs induits

Par opposition aux cellules régulatrices T naturelles, des lymphocytes T dont l'activité régulatrice est induite, ou acquise, ont été identifiées. Ces cellules appelées cellules régulatrices T induites sont générées *in vitro* comme *in vivo*, à partir de précurseurs périphériques naïfs CD4+. Elles sont induites par un antigène et leur capacité suppressive est spécifique de cet antigène. Parmi les lymphocytes CD4+ nous pouvons distinguer trois populations de Treg induits : les cellules CD4+CD25-Foxp3+, les cellules Tr1 productrices d'IL10 (Groux et al. 1997) et les cellules Th3 productrice de TGFβ (Chen et al. 1994 ; Weiner et al. 2001). Parmi les cellules CD8+, les principales cellules, décrites à l'heure actuelle, présentent le phénotype CD8+CD28-. Cette dernière population sera développée dans le paragraphe « les lymphocytes régulateurs CD8+ ».

1. Les cellules T CD4+CD25+Foxp3+

Il a été démontré que des cellules ayant le même phénotype et les mêmes propriétés que les lymphocytes régulateurs T naturels pouvaient être générés en périphérie par différents mécanismes (Shevach et al. 2006). D'une part, de nombreuses études ont montré que le TGFβ pouvait induire la conversion de cellules T CD4+CD25- en cellules régulatrices

CD4+CD25+Foxp3+ *in vitro* et *in vivo* (Yamagiwa et al. 2001 ; Chen et al. 2003 ; Fantini et al. 2004).

2. Les cellules Tr1

Les lymphocytes T CD4+ régulateurs de type 1 (Tr1) ont été identifiés à l'origine uniquement par leur production de cytokines (Groux et al. 1997). Roncarolo et ses collaborateurs ont observé pour la première fois que l'activation *in vitro* des lymphocytes T CD4+ humains ou murins avec de fortes doses d'IL10 permettait la génération de clones T produisant des cytokines distinctes de celles produites par les lymphocytes T de type Th1 et de type Th2 (Battaglia et al. 2006 ; Grazia et al. 2006). De plus, ces clones sont comparables à ceux isolés à partir de patients atteints d'immunodéficience sévère combinée (SCID) qui produisent de fortes quantités d'IL10. Les cellules Tr1, après activation via leur TCR, produisent de l'IL10, du TGFβ, de l'IL5 mais peu d'IFNγ et peu d'IL2 et pas d'IL4 (Groux et al. 1997). Il est également possible de générer ces cellules à partir de lymphocytes T CD4+ humains en utilisant de l'IFNα en combinaison avec de l'IL10 (Levings et al. 2001). L'IL10 est une cytokine cruciale pour les cellules régulatrices Tr1, à la fois pour leur génération et pour leur fonction. L'ajout d'anticorps anti-IL10 bloque l'activité suppressive des cellules Tr1 (Groux et al. 1997). L'addition de vitamine D et de dexamethasone permet de générer des cellules Tr1 *in vitro* (Barrat et al. 2002). Le groupe d'Atkinson a montré que le CD46 était impliqué dans la génération des cellules Tr1 (Kemper et al.2003). L'activation de la voie de signalisation d'OX40L inhibe la différentiation des cellules Tr1 (Ito et al. 2006). Ces cellules ne possèdent pas à ce jour de marqueurs spécifiques. Elles expriment des molécules telles que CXCR3, GATA3, CCR3, CCR4, CCR5, CCR8, mais ces molécules sont aussi exprimées par les cellules de type Th1 et/ou de type Th2 (Sebastiani et al. 2001 ; Cobbold et al. 2003). Ces cellules sont anergiques *in vitro* (Roncarolo et al. 2001).

Chez les patients atteints de SEP, l'augmentation de la production d'IL10 est associée aux phases de rémission de la maladie (Navikas et al. 1995 ; Correale et al. 1995 ; Clerrici et al. 2001) mais aussi au traitement par IFNβ (Chabot et al. 2000). De faibles taux d'IL10 sont associés à la charge lésionnelle visible à l'IRM chez les patients atteints de SEP de forme secondaire progressive (Petereit et al. 2003). De plus, lorsque l'induction des cellules Tr1 via l'activation de CD46 est observée chez les patients atteints de SEP, celle-ci est significativement diminuée comparé aux individus sains (Astier et al. 2006). Récemment, Martinez-Forero et ses collaborateurs ont montré que les lymphocytes CD4+ issus de patients

atteints de SEP se différenciaient moins bien en cellules régulatrices Tr1 *ex vivo* et que ces cellules produisaient moins d'IL10. De plus, les cellules T CD4+ des patients sont plus résistantes à la suppression induite par l'IL10 car la cascade de signalisation passant par le récepteur de l'IL10 est défectueuse (Martinez-Forero et al. 2008).

3. Les cellules Th3

Les cellules régulatrices induites Th3 ont été identifiées chez des souris protégées du développement de l'EAE (Encéphalomyélite Allergique Expérimentale) par administration orale de MBP et de PLP (Chen et al. 1994). Lorsque la MBP est administrée par voie orale, des cellules T capables d'inhiber les réactions inflammatoires sont générées (Faria et al. 2005). La suppression des symptômes cliniques de l'EAE est bloquée par l'injection d'anticorps anti-TGFβ (Chen et al. 1994). En effet, les cellules Th3 sont caractérisées par leur forte production de TGFβ, leur faible production d'IL10 et d'IL4 et l'absence de sécretion d'IL2 et d'IFNβ suite à l'engagement de leur TCR (Weiner et al. 1997 ; Kitani et al. 2000 ; Fukaura et al. 1996).

In vitro, les cellules régulatrices Th3 peuvent être générées à partir de précurseurs Th0 en présence de TGFβ, d'IL10, d'IL4 et cette différentiation est inhibée par l'IL12 (Weiner et al. 2001).

Les cellules Th3 sont capables de réguler les cellules de type Th1 (Weiner et al. 2001). Plus récemment, il a été montré que ces cellules avaient la capacité d'induire la différentiation des lymphocytes T en cellules régulatrices CD4+CD25+Foxp3+ (Carrier et al. 2007).

Les cellules Th3 agissent, contrairement aux cellules régulatrices T naturelles, par l'intermédiaire de cytokines immunosuppressives : le TGFβ et l'IL10 (Weiner et al. 2001). Ainsi, elles sont capables d'inhiber le développement du phénomène autoimmun dans plusieurs modèles animaux (Weiner et al. 1997) et inhibent en particulier la prolifération cellulaire et la synthèse de cytokines des clones Th1 et Th2 (Weiner et al. 1997 ; Beissert et al. 2006). Elles agissent de manière non-spécifique de l'antigène et par effet « bystander » via surtout la sécrétion de TGFβ. La fixation de cette cytokine sur son récepteur active le complexe intracellulaire Smad qui est alors transloqué vers le noyau entraînant l'inhibition de la prolifération et de la différentiation des cellules T ainsi que le blocage de la maturation des cellules dendritiques (Vigouroux et al. 2004).

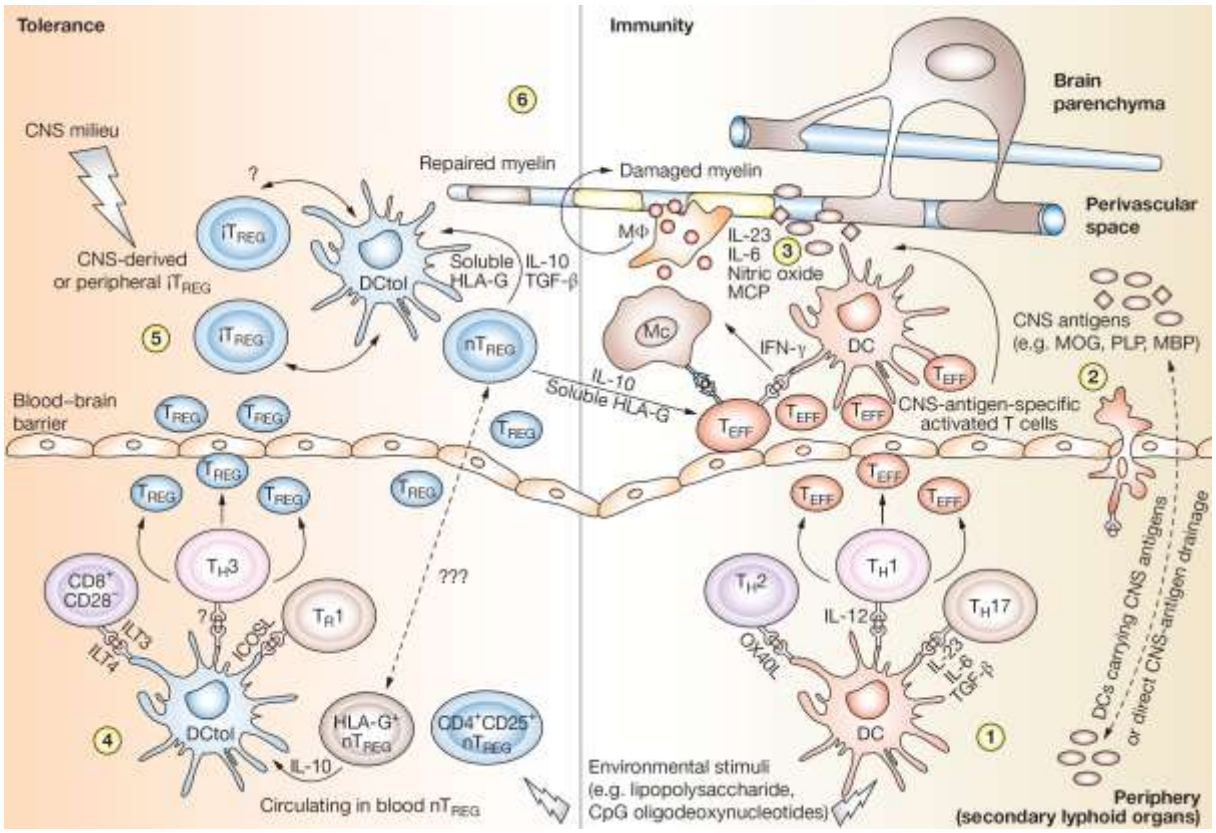

Figure 8 : L'équilibre rompu entre les mécanismes immunogène et tolérogène dans la Sclérose en Plaques
L'inflammation semble résulter d'une rupture de l'équilibre entre les cellules de l'immunité causnat les dommages (cellules T effectrices) et les cellules ayant des fonctions de régulation (cellules Treg et DCs tolérogènes). **1.** En réponse aux stimuli environnementaux, les DCs (en rouge) peuvent activer les cellules T CD4+ naïves pour produire de l'IFNγ (cellules Th1), de l'IL4 (cellules Th2) ou de l'IL17 (cellules Th17). **2.** Les cellules T activées, spécifiques de la myéline, peuvent transmigrer au travers de la BHE pour rejoindre les zones périvasculaires du SNC où elles seront réactivées par des DCs présentant des antigènes de la myéline. **3.** Ces cellules T CD4+ exerceront des fonctions effectrices par sécrétion de cytokines proinflammatoires comme l' IFNγ, l'IL6 et l'IL23…Ceci activera la microglie et les macrophages contribuant à une cascade d'évènements ayant pour effet la démyélinisation et les dommages neuronaux. **4.** Les DCs tolérogènes (en bleu) interviennent de sorte que les cellules T CD4+ naïves se différencient en cellules régulatrices (Th3, Tr1 ou CD8+CD28-). Les iTreg comme les nTreg semblent pouvoir migrer du sang périphérique vers le SNC en franchissant la BHE. **5.** Au sein du SNC, les cellules régulatrices peuvent directement réguler les cellules T effectrices activées par leur secretion de cytokines (IL10, TNFα, HLA-G soluble) et par l'interaction cellules/cellules.
D'après Zozulya et al. 2008

iii. Les cellules régulatrices CD8+

1. Les cellules régulatrices CD8+ naturelles

Les cellules régulatrices naturelles CD8+ proviennent du thymus. Elles contrôlent directement la production d'IFNγ et la prolifération des cellules CD8+. Leur mécanisme d'action ne nécessite pas l'intervention de la présentation de l'antigène mais agirait par une voie dépendante de l'IL10 (Endharti et al. 2005). Les cellules régulatrices CD8+CD122+

reconnaissent les lymphocytes T activés grâce à l'interaction des molécules du CMH de classe I avec le TCR de type αβ (Rifa'i et al. 2008). Une nouvelle population de cellules régulatrices CD8+ naturelles a été identifiée chez l'homme. Ces cellules sont caractérisées par l'expression constitutive de HLA-G. Leur fonction suppressive affecte les cellules autologues CD4+ et CD8+ via un mécanisme d'action indépendant du contact (Feger et al. 2007).

2. Les cellules régulatrices CD8+ induites

Les cellules régulatrices induites CD8+ se développent en périphérie. Elles ont la capacité de supprimer la réponse immune en interagissant directement avec les CPA et les rendant ainsi tolérogènes (Najafian et al. 2003). Elles sont caractérisées par l'expression de CD103 à leur surface (Uss et al. 2006), par la sécrétion d'IL10 (Noble et al. 2006) et de TGFβ (Balashov et al. 1995 ; Fukaura et al. 1996) cependant leur mécanisme d'action est dépendant du contact et spécifique de l'antigène.

3. Rôle dans la Sclérose en Plaques

Antel et ses collaborateurs furent les premiers à suggérer que la fonction suppressive des cellules CD8+ puisse être altérée dans la SEP (Antel et al. 1986 ; Bania et al. 1986). Chez des patients atteints de SEP, les lymphocytes T CD8+ activés ont des propriétés suppressives altérées (Balashov et al. 1995). De plus, les taux d'IFNγ plus élevés dans les formes secondaires progressives que dans les formes rémittentes, semblent être inversement corrélés à des fonctions suppressives (Becher et al. 1999). Correale et ses collaborateurs ont étudié le rôle des cellules régulatrices CD8+ lors des phases de poussée et de rémission chez des sujets atteints de SEP. Ils ont observé des cellules régulatrices CD8+ spécifiques reconnaissant et lysant les cellules T activées réactives à la myéline. Au sein du LCR en particulier, ces clones sont diminués lors des poussées (Correale et al. 2008).

L'acétate de glatiramère induit une réponse CD8+ chez les patients atteints de SEP dans des études menées *in vitro*. L'action bénéfique de ce traitement semble s'exercer, au moins en partie, via les cellules régulatrices CD8+ (Tennakoon et al. 2006). Cependant, dans un travail récent, nous avons montré que ce traitement n'entraînait pas de prolifération clonale CD8+ *in vivo* (Berthelot et al. 2009, Annexe I).

iv. Les cellules NKT/CD1d

Les cellules NKT constituent une population présentant des caractéristiques des cellules NK et des lymphocytes T (Van der Vliet et al. 2002 ; Cava et al. 2006). Elles expriment un TCR invariant constitué de segments Vα24 et Vβ11 et des marqueurs NK tels que CD161 ou NKR-P1. Bien que la majorité des NKT soient CD4+, la plupart des cellules restantes sont CD4-CD8-. Une petite partie de NKT est CD8+ chez l'homme. Elles reconnaissent les molécules du CMH de classe I non classiques CD1d qui présente des glycolipides (Goddfrey et al. 2000). Les NKT possèdent des caractéristiques communes avec les lymphocytes T régulateurs. En effet, elles peuvent inhiber la prolifération cellulaire et la production de cytokines (Terabe et al. 2004). Elles peuvent également inhiber la génération des lymphocytes Th1 (Miyamoto et al. 2001 ; Moodycliffe et al. 2000 ; Beaudouin et al. 2002) et CD8+ (Terabe et al. 2004 ; Hammond et al. 2003).

Chez les patients atteints de SEP, une forte diminution du taux de NKT a été observé (Illes et al. 2000 ; Demoulins et al. 2003). Pendant les phases de rémission des patients, une sous-population de cellules NKT CD4+ aurait un rôle immunomodulateur en sécrétant de grandes quantités d'IL4 et peu d'IFNγ (Akari et al. 2003). Chez un patient, une diminution de la diversité de Vα24 a été observée (Demoulins et al. 2003). De plus, une nouvelle population de NKT (Vα7.2-Jα33) est accumulée au niveau des lésions du SNC de patient SEP (Illes et al. 2004).

v. Les cellules NK

Les cellules NK sont des cellules dites « tueuses naturelles ». Elles constituent une population hétérogène contribuant à la défense de l'organisme contre les infections et les cancers. Ces cellules vont être activées par l'IL2 produit par les lymphocytes T CD4+. Leur mécanisme d'action passe par une lyse non spécifique de l'antigène. Elles expriment au niveau de leur surface membranaire les marqueurs CD56 et CD16 (récepteur FcγRIII) et des récepteurs invariants qui reconnaissent des molécules de CMH non classiques (Lanier et al. 1998). Les cellules NK représentent environ 10% des PBMC humains environ. (Steinman et al. 2001). La majorité des cellules NK, environ 95%, présente le phénotype suivant : CD56dimCD16+. Ces cellules ont une action de lyse via la production de perforine. Une petite proportion des cellules NK, environ 5%, sont CD56brightCD16-. Elles ne produisent pas de perforine mais secrètent de l'IFNγ et du TNFα (Moretta et al. 2001).

Les cellules NK peuvent lyser les neurones (Backstrom et al. 2003) mais aussi les oligodendrocytes, les astrocytes et la microglie (Morse et al. 2001 ; Antel et al. 1998).

Cependant, plusieurs études ont suggéré que les cellules NK jouaient un rôle dans l'immunorégulation (Zimmer et al. 2001 ; Kerschensteiner et al. 2003 ; Hammarberg et al. 2000) et possédaient un rôle protecteur dans la SEP (Takahashi et al. 2004). La déplétion des cellules NK a pour conséquence d'accentuer l'EAE, ceci suggère que cette population cellulaire présente des capacités régulatrices (Takahashi et al. 2001). Une augmentation de la proportion des NK exprimant le CD95 (Fas, récepteur pouvant induire l'apoptose) a été mise en évidence dans le sang périphérique des patients SEP de forme rémittente en phase de rémission. Ces cellules NK sécrètent des cytokines de type Th2 comme l'IL5 et l'IL13 (Takahashi et al. 2004 ; Takahashi et al. 2001 ; Loza et al. 2002). Des défauts dans la fonction et au niveau du nombre de cellules NK chez les patients atteints de SEP ont été décrits (Merrill et al. 1982 ; Vranes et al. 1989 ; Munschauer et al. 1995).

Il a été observé une expansion des cellules NK CD56bright chez les patients atteints de SEP traités avec du Daclizumab (Bielekova et al. 2006). Une expansion similaire de ce sous-type de cellules NK est observée au niveau du sang périphérique mais aussi au sein des organes lymphoïdes secondaires des patients atteints de SEP traités à l'IFNβ (Saraste et al. 2007) et pendant le dernier trimestre de grossesse, période associée à une baisse du taux de poussées (Airas et al. 2008). Ce même sous-type cellulaire aux propriétés régulatrices est diminué chez les patients atteints de SEP non-traités comparé aux sujets sains (De Jager et al. 2008).

OBJECTIFS

La SEP est une maladie à laquelle sont associés des lymphocytes T reconnaissant les protéines de la myéline. Ces lymphocytes T auto-réactifs sont aussi bien des CD4+ que des CD8+. Cependant, peu d'épitopes de la myéline restreints aux molécules de CMH de classe I ont été caractérisés. L'étude du répertoire Vβ dans le sang de patients atteints de SEP réalisée dans notre laboratoire a montré que les patients SEP présentent dès le début de leur maladie significativement plus d'altérations de la répartition des différentes longueurs de CDR3 que le groupe d'individus sains (Laplaud et al. 2004). Ce phénomène est retrouvé de façon prédominante dans le compartiment lymphocytaire T CD8+. De plus, en isolant les lymphocytes T exprimant à leur surface des Vβ appartenant aux familles Vβ altérées, nous avons montré que ces lymphocytes sont auto-réactifs. Ils répondent en effet à une stimulation par la MBP et produisent de l'IFNγ.

Étant donné la prépondérance des lymphocytes CD8+ dans les altérations du répertoire T chez les patients atteints de SEP, nous nous sommes intéressés plus particulièrement à ces cellules et avons cherché à déterminer les épitopes issus de la myéline et reconnus par les lymphocytes T CD8+ dans ce contexte (Article I dans la partie résultats).

À partir de ces observations, nous avons réalisés une synthèse sur la question de la fréquence des cellules T auto-réactives détectée dans la SEP (Revue I en préparation dans la partie résultats).

Plusieurs équipes ont rapporté un défaut de régulation au sein du compartiment T CD4+CD25high chez les patients atteints de SEP. Un autre objectif de ma thèse a été d'étudier la fonction régulatrice de ces cellules T en incluant le nouveau marqueur CD127 (chaine α du récepteur à l'IL7) permettant une meilleure sélection de cette population (Article II dans la partie résultats). Cette étude a été réalisée chez des patients atteints de SEP de forme rémittente, non-traités.

Enfin, suite aux différentes observations obtenues, nous nous sommes à nouveau penchés sur les cellules T auto-réactives chez les patients atteints de SEP de forme rémittente. Pour cette étude, une nouvelle technique a été utilisée intitulée TRAP (T Recognition of APC by Protein Transfer). La nouveauté de ce travail est d'utiliser une technique jamais appliquée dans le contexte de la SEP mais aussi d'utiliser la myéline totale comme antigène.

La méthode TRAP décrite chez la souris, consiste à marquer la membrane (par la biotine et un fluorochrome) des CPA préalablement incubées avec la protéine d'intérêt (Beadling et al. 2006). Les CPA sont ensuite mises en présence de lymphocytes. Les lymphocytes reconnaissant les peptides issus de l'apprêtage des protéines par les CPA et dont le TCR a interagi avec les complexes CMH/peptides auront capté des morceaux de membrane provenant des CPA et sont identifiables en cytométrie en flux. Cette méthode présente l'avantage de s'affranchir de la restriction par différents CMH et de l'utilisation peptides non restreints et d'étudier tous les lymphocytes potentiellement auto-réactifs. Cette technique chez l'Homme a été mise au point par notre équipe en utilisant la réponse anti-CMV de sujets CMV séropositifs et des patients atteints de SEP connus pour leur réactivité à des peptides de la myéline en ELISPOT. La myéline totale a, elle aussi, été aussi utilisée. Ce travail est en cours de soumission (Article III partie résultats).

RÉSULTATS

ARTICLE I : Réponses des cellules T CD8+ du sang périphérique contre des antigènes de la myéline dans la Sclérose en Plaques et chez les individus sains.

Par : Berthelot L, Laplaud DA, Pettré S, Ballet C, Michel L, Hillion S, Braudeau C, Connan F, Lefrère F, Wiertlewski S, Guillet JG, Brouard S, Choppin J, Soulillou JP

Publié dans : European Journal of Immunology. 2008. 38(7) : 1889-1899

Eur. J. Immunol. 2008. 38: 1889–1899 DOI 10.1002/eji.200838023 **Cellular immune response**

European Journal of Immunology

Blood CD8$^+$ T cell responses against myelin determinants in multiple sclerosis and healthy individuals

Laureline Berthelot[1], David-Axel Laplaud[1,2], Ségolène Pettré[1], Caroline Ballet[1], Laure Michel[1], Sophie Hillion[1], Cécile Braudeau[1], Francine Connan[3,4], Fabienne Lefrère[5], Sandrine Wiertlewski[2], Jean-Gérard Guillet[*3,4], Sophie Brouard[1], Jeannine Choppin[3,4] and Jean-Paul Soulillou[1]*

[1] INSERM, U643, et ITERT, Nantes, France
[2] CHU de Nantes, Service de Neurologie, Nantes, France
[3] Institut Cochin, Université Paris Descartes, CNRS (UMR 8104), Paris, France
[4] INSERM, U567, Paris, France
[5] INSERM, CIC004, CHU de Nantes, France

Patients with multiple sclerosis (MS) display significant peripheral blood CD8$^+$ T cell receptor biases, suggesting clonal selection. Our objective was to identify relevant myelin-derived peptides capable of eliciting responses of fresh blood CD8$^+$ T cells in MS patients. We focused our analysis on the HLA supertypes (HLA-A3, -A2, -B7, -B27, -B44) predominant in a patient cohort. Three myelin protein (MBP, PLP and MOG) sequences were screened for HLA binding motifs and peptides were tested for their binding to HLA molecules. The cellular responses of 27 MS patients and 19 age- and sex-matched healthy controls (HC) were tested in IFN-γ ELISPOT assays only detecting pre-committed CD8$^+$ T cells. Sixty-nine new epitopes elicited positive responses, with MOG-derived peptides being the most immunogenic and peptides binding to HLA-A3 being the most frequent. However, MS patients and HC displayed the same frequency of autoreactive cells. The epitopes inducing the strongest responses were not those with the highest HLA binding, suggesting an effective thymic selection in MS patients. Our data extend the concept that the frequency of myelin-reactive T cells in MS patient blood is not increased compared to HC. The description of this set of myelin-derived peptides (MHC class I restricted, recognized by CD8$^+$ T cells) offers new tools to explore the CD8$^+$ cell role in MS.

Key words: CD8$^+$ T cells · Multiple sclerosis · Myelin epitopes

Introduction

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system associated with the presence of autoantibodies and T lymphocytes reactive against myelin determinants, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) [1–3]. Individuals with the class II HLA-DR2 are more susceptible to the disease [4] and most studies have focused on the CD4$^+$ T cell response. However, a potential role for CD8$^+$ T cells in MS pathology has recently been emphasized. CD8$^+$ T cells predominantly infiltrate the white matter lesions and CD8$^+$ clones can persist in the cerebrospinal fluid and blood of MS patients [5–7]. The production of various cytokines by CD8$^+$ T cells also correlates with magnetic resonance imaging (MRI) features of tissue destruction [8] and with disability scores [9]. In agreement

Correspondence: Prof. Jean-Paul Soulillou
e-mail: Jean-Paul.Soulillou@univ-nantes.fr

[*] Deceased

with these observations, we have shown that the peripheral lymphocytes of MS patients present greater biases in their TCR Vβ chain usage than those of healthy individuals [10] and that these biases correlate with the number of Gadolinium-positive lesions at MRI [11]. T cell repertoire alterations mostly concerned $CD8^+$ T cells, and sorted T cells with altered Vβ TCR usage contained a high frequency of MBP-reactive cells [10]. In addition, oligodendrocytes can be lysed by $CD8^+$ lymphocytes *in vitro* [12]. In rodents, passive transfer of MBP-specific or MOG-specific $CD8^+$ lymphocytes from immunized animals can induce encephalomyelitis [13, 14]. Collectively, these results suggest that circulating $CD8^+$ effectors can contribute to tissue destruction by direct cytotoxicity on oligodendrocytes or by indirect release of pro-inflammatory factors in the local environment (see [15] for review). There is thus a need to better understand the myelin-reactive $CD8^+$ T cells and their actual role in MS. Only few studies have focused on these cells in MS [16, 17]. HLA-A3 is the most common HLA class I molecule associated with MS [18, 19] and a single HLA-A3-restricted peptide (PLP_{43-53}) has been characterized [20]. Other myelin epitopes have also been described for the HLA-A2 allele [17, 21] and HLA-A24 [21]. However, no study has compared the presence of committed myelin-reactive $CD8^+$ T cells in the blood of MS patients and healthy controls (HC).

The main objective of this work was to extensively characterize the myelin epitopes restricted to the most representative HLA class I molecules present in MS patients and able to stimulate blood $CD8^+$ T cells. We focused our analysis on the five HLA supertypes (HLA-A3, -A2, -B27, -B44 and -B7) predominant in MS patients. The search for HLA-binding motifs was first directed by the sequence analysis of three myelin proteins (MBP, PLP and MOG). Next, the selected peptides were tested for their binding to the five HLA molecules and for their capacity to stimulate the PBMC of 27 patients with MS and 19 HC in an IFN-γ ELISPOT assay. To avoid any *in vitro* artifacts, T cells were analyzed directly following their isolation from fresh blood and the test used only detected pre-committed $CD8^+$ T cells. Altogether, 71 HLA class I-restricted peptides eliciting $CD8^+$ T cell responses were characterized. However, we show that MS patients do not exhibit a higher frequency of autoreactive $CD8^+$ T cells in blood than HC.

Results

Binding of myelin peptides to HLA class I molecules

The most frequent HLA class I molecules in our patient cohort were HLA-A3, -A2, -B7, -B27 and -B44. Peptides potentially able to bind to these HLA molecules were first selected by sequence analysis and detection of HLA anchor residues in myelin proteins. Peptides were then tested for their binding to the corresponding HLA molecules. The binding test was performed using purified HLA molecules incubated with the peptides and human β2-microglobulin. When a HLA/peptide complex is formed, it can be recognized by a conformation-dependent anti-HLA antibody [22]. The number of peptides eliciting binding above the threshold was: 42 for HLA-A2 (79%), 33 for HLA-A3 (75%), 16 for HLA-B7/35/51 (73%), 26 for HLA-B27 (52%) and 15 for HLA-B44 (78%). Thus, a total of 132 of the 188 tested peptides significantly interacted with their HLA class I molecules. The method of peptide selection based on anchor binding sites [23] appeared effective for the identification of HLA binders.

Detection of $CD8^+$ memory T cell responses by the IFN-γ ELISPOT assay

Because cell lines may not reflect the actual profile of blood autoreactive cells, our study was restricted to the analysis of fresh blood $CD8^+$ T cell reactivity against myelin-derived peptides. Monocytes, B cells, $CD4^+$ T cells and $CD8^+$ T cells were thus sorted from fresh PBMC and their reactivity against peptide stimulation in the ELISPOT assay was studied. A viral pool of peptides (from influenza virus, CMV and EBV) was systematically used as a positive control [24]. Figure 1A shows a representative experiment indicating that purified monocytes, B lymphocytes, $CD4^+$ T cells and naive $CD8^+$ T cells ($CD45RO^-$) alone or $CD8^+$ T cell-depleted PBMC did not produce IFN-γ in the presence of the autoreactive peptides tested in our experimental setting. Only memory $CD8^+$ T cells ($CD45RO^+$) were able to produce IFN-γ in response to the stimulation with the class I-restricted peptides derived from virus and from myelin. Adding antigen-presenting cells (B cells, monocytes or irradiated PBMC) to naive $CD8^+$ cells did not induce a response (data not shown). In contrast, the addition of B cells to $CD8^+$ $CD45RO^+$ T cells induced an increase in IFN-γ production ($p=0.06$) (Fig. 1B), which may enhance the sensitivity of the test when PBMC are used. These results confirmed that the 15-h ELISPOT assay only detected committed effectors from blood and did not generate detectable *in vitro* stimulation of naive cells.

MS patient and HC IFN-γ-positive $CD8^+$ T cell responses to myelin peptides

Fresh PBMC from 27 patients with the relapsing-remitting form of multiple sclerosis (RR-MS) and from 19 HC were isolated from blood every 3 months over a period of 1 year. MS patients displayed the same number of blood $CD8^+$ T cells as HC (data not shown). Without peptide stimulation in the ELISPOT assay, IFN-γ-producing $CD8^+$ T cell frequencies in MS patient and HC approached 0 (Fig. 2A). The frequency of responding cells against the pool of viral epitopes was roughly comparable between the two groups (mean: 54.9 ± 7.6 (SD) spots for HC and 63.0 ± 9.72 spots for MS patients (not significant)) (Fig. 2D). Because activated $CD8^+$ T cells were found at sites of major accumulations of EBV-infected B cells in the brains of MS patients [25], we performed separate stimulation with EBV peptides in the ELISPOT assay. MS patients and HC displayed a comparable frequency of EBV-specific $CD8^+$ T cells in their blood. These results are in agreement with previous studies [26, 27]. MS patients and HC also showed roughly similar autoreactive responses against the myelin peptides (Fig. 2B) as well as to each

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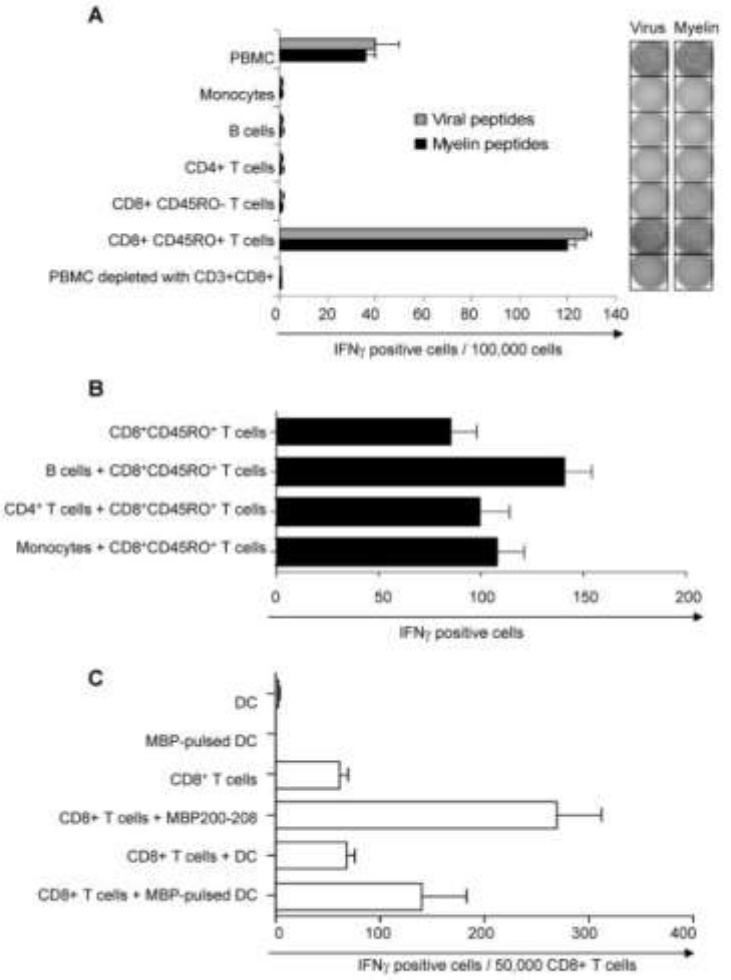

Figure 1. Stimulation of purified PBMC subpopulations with myelin peptides. Subpopulations were sorted with an ARIA sorter with antibodies against CD19-PE-Cy7, CD14-FITC, CD3-PE, CD4- or CD8-APC and CD45RO-ECD. The sorted cells (4×10^4 cells/well) and PBMC (4×10^5 cells/well) were incubated for 15 h in complete RPMI (containing 10% AB serum), with a viral peptide pool (CMV, EBV, influenza) and a myelin peptide pool (MOG_{74-81}, MOG_{97-105}, $MOG_{225-232}$, $PLP_{202-210}$, $MBP_{244-252}$, $MBP_{138-147}$) at 10 μg/mL in ELISPOT plates. (A) Membranes of ELISPOT shown in this figure correspond to the patient MS-9, a representative sample of the four MS patients and two HC tested. (B) Cells were incubated with a myelin peptide pool; ratio of $CD8^+CD45RO^+$ T cells to other cells 1:2 (results are representative of five MS patients and three HC; x axis shows the number of IFN-γ positive cells per 1×10^5 cells). (C) DC from one MS patient and two HC were derived from monocytes and cultured with GM-CSF and IL-4. $CD8^+$ T cells were stimulated for 7 days with the peptide $MBP_{200-208}$. On day 7, cells were incubated in ELISPOT plates at a $CD8^+$ T cell to DC ratio of 50:1. Data represent mean + SEM.

tested peptide (an example for peptide MOG243–51 is given in Fig. 2C). Collectively, the frequency of IFN-γ-producing cells appeared lower with myelin peptide stimulation than with viral peptide stimulation, both in MS patients and HC ($p<0.01$). There was also no difference between MS patients with or without immunomodulatory treatment (glatiramer acetate or IFN-β). Thus, our data, based on a large set of autoantigen-derived peptides and a large group of MS patients and normal individuals, show that the frequency of peripheral blood $CD8^+$ T cells reactive to myelin autoantigens is not significantly increased in MS patients compared to healthy individuals.

Characteristics of myelin epitopes eliciting $CD8^+$ T cell responses

Given that the binding level could be a false criterion of autoimmunity, we opted to test all 188 myelin peptides selected for their capacity to induce a cellular response in the IFN-γ ELISPOT assay, regardless of the binding test results. Among the 188 myelin-derived peptides, 71 peptides elicited a response by the PBMC from either MS patients or HC, indicating that the human T cell repertoire displays TCR reacting against myelin autoantigen and that human $CD8^+$ T cells interact with a high

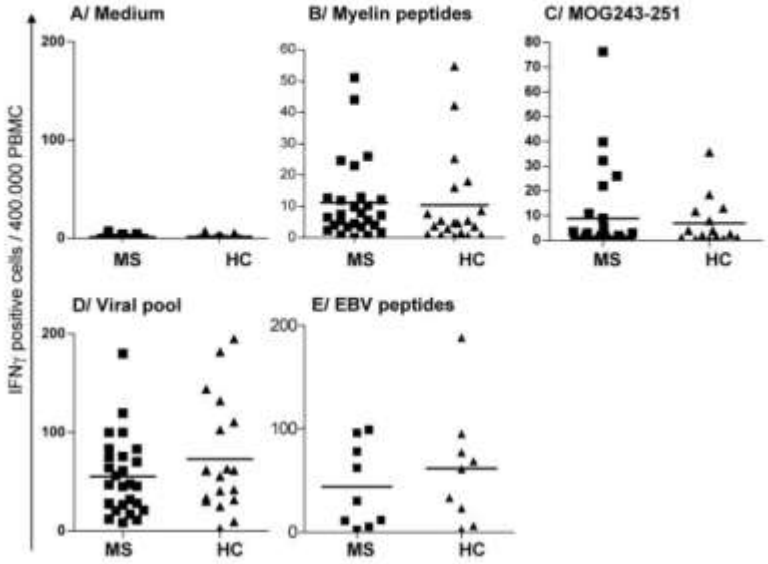

Figure 2. IFN-γ ELISPOT assays in 27 MS patients and 19 HC. Fresh PBMC (4×10^5/well) in duplicate were incubated for 15 h, in the presence of (A) medium or (B) myelin peptides or (C) in a representative experiment using a single peptide $MOG_{243-251}$, (D) with the pool of viral peptides (EBV, CMV and influenza), (E) with EBV peptides, at 10 μg/mL. For (E), MS patients (n=10) and HC (n=10). For (B), the graph represents the mean of IFN-γ-positive cells obtained with the myelin-derived epitopes.

diversity to these epitopes. Given that an enhanced $CD4^+$ T cell response has been described in MS [28], it has been suggested that citrullinated MBP could be preferentially recognized by autoreactive lymphocytes. However, stimulation with citrullinated peptides did not induce $CD8^+$ T cell responses in MS patients or HC (data not shown). Figures 3 shows the overall distribution of the epitopes and the HLA class I restriction. The MOG protein appeared to be the most immunogenic with 37 epitopes focused predominantly in the extracellular part (1–125) as well as a part that may be an intracellular domain (218–260) (Fig. 3). Among the PLP sequence, ten epitopes were spread out, being located in the intracellular, extracellular and membrane domains of the protein. For MBP, 24 epitopes were detected and the 151–192 region seemed to be highly recognized with eight epitopes present. Concerning the HLA restriction of the epitopes, most of them were HLA-A3 binders (nine from MBP, six from MOG, one from PLP). Eleven epitopes bound to HLA-A2 (two MBP, seven MOG, two PLP), nine to HLA-B27 (five MBP, four MOG), six to HLA-B7 (two MBP, three MOG, one PLP) and two to HLA-B44 (one MBP, one PLP).

Recognition of myelin peptides processed by human cells

In addition, although only $CD45RO^+$ (memory) T cells were responsive to peptides (see above, Fig. 1A), we wanted to ensure that the peptides triggering IFN-γ production were physiologically generated by processing. We thus used dendritic cells (DC) derived from monocytes as antigen-presenting cells and incubated them with MBP protein. $CD8^+$ T cells stimulated with $MBP_{200-208}$ peptide for 7 days produced more IFN-γ in the presence of MBP-pulsed DC than of non-pulsed DC (Fig. 1C). Production of IFN-γ by $CD8^+$ T cells reached the same level as when the cells were alone or incubated with non-pulsed DC (Fig. 1C). These $CD8^+$ T cells stimulated with $MBP_{200-208}$ peptide were highly responsive (Fig. 1C). Thus, $MBP_{200-208}$-specific $CD8^+$ T cells can recognize endogenously processed MBP, indicating that this peptide is naturally processed by human cells.

Individual-specific response patterns and fluctuations over time

Having characterized the myelin epitopes, we next studied the distribution of the responses to these epitopes. Each individual (either MS patients or HC) presented a specific response pattern inasmuch as they responded to a given combination of epitopes (Fig. 4A). Some epitopes appeared more immunogenic with a relatively high frequency of responding cells and a high number of MS patient and HC responders (such as MOG_{97-105} and $PLP_{202-230}$ in Fig. 4A). To determine time-dependent fluctuations of the $CD8^+$ T cell responses to myelin peptides during the disease evolution, we tested the reactivity of the MS patients (n=17) and HC (n=10) every 3 months over a period of 1 year and at each clinical relapse, using the IFN-γ ELISPOT assay. $CD8^+$ T cell production of IFN-γ upon viral stimulation was stable over time, confirming the experimental stability of the IFN-γ ELISPOT assay (Fig. 4B). Most

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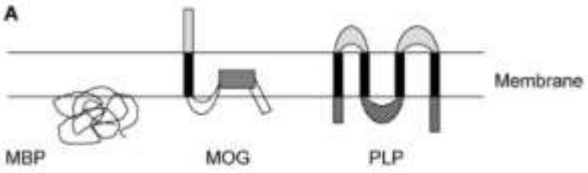

B

MBP	MOG		PLP	
SLDMASQK ~130-138 (A3)	Extracellular domain (1-149)	VIGPRDPIR ~34-42 (A3)	Intracellular domain (1-11)	LLECCARCL ~3-11 (A2)
MASQKRPSQR ~134-143		GPRHPURAL~36-44 (B7,B35,B51)		
KRPSQRHGSK~38-147 (B27)		ALVGDEVEL ~43-51(A2)	Extracellular domain (35-65)	EALTGTEK~38-45 (A3)
SQRHGSKYL~141-149 (B27)		ATGMEVGWYR~61-69		ALTGTEKLI~39-47
TASTMDHAR~151-159 (A3)		GMEVGWYR~65-70		YEYLDNVI~58-65 (B44)
MDHARHGFL ~155-163		SRVVHLYR~74-81	Transmembrane domain (65-90)	NVIHAFQYVI~63-72 (A2)
HARHGFLPR~157-165		RVVHLYRNGK~75-84 (A3)		
ARHGFLPRHR~158-167(B27)		YRGTELLK~94-102 (B27)	Transmembrane domain (150-176)	GITYALTVV~154-162
LPRHRDTGI~163-171(B7, B35, B51)		RTELLKDAI~97-105 (A2)		
DTGILDSIGR~168-177 (A3)		AIGEGKVTL~104-112 (A2)	Extracellular domain (176-238)	ADARMYGV~202-210
SIGRFFGGDR~174-183 (A3)		VTLRIRNVR~110-118 (A3)		NLLSICKTA~223-231(A2)
GAPKRGSGK~184-192 (A3)		LRIRNVRF~112-119 (B27)		LSICKTAEF~225-233(B35,B51)
TAHYGSLPQK~200-209 (A3)		VRFNDEGGF~117-125 (B27)	Intracellular domain (262-276)	FAVLKLMGR ~265-273
SLPQKSHGR~205-214 (A3)		KVEDPFYWV~143-151 (A2)		
QDENPVVHF~215-223 (B44)	Transmembrane domain(150-169)	DPFYWVSPGV~146-155(B35,B51)		
NPVVHFFKNI~218-227 (B35)		LLAVLPVLLL~157-166 (A2)		
VVHFFKNIV~220-228 (A2)		VLLLQITV~164-171 (A2)		
PRTPPPSQGK~230-239 (B27)		FLCLQYRLR ~175-183 (A3)		
GRGLSLSRF ~240-248 (B27)		LQYRLRGKL ~178-186 (B27)		
SLSRFSWGA~244-252 (A2)		KLRAEIENI~185-193 (A2)		
RASDYKSAHK~262-272 (A3)		LRAEIENLHR ~186-195		
GVDAQGTLSK~274-283 (A3)	Membrane associated domain (205-226)	FLRVPCWK~201-208		
GTLSKIFK ~282-289 (A3)		LRVPCWKI~202-209 (B27)		
SRSGSPMARR~295-304 (B27)		RVPCWKITL~203-211 (A2)		
		GPLVALICY~218-228		
		ALICYNWL~222-230		
		IICYNWLHR ~225-232 (A3)		
		HRRLAQQFL ~232-240		
		GQFLEELRKF~237-246		
		LRKFSSLCY~243-251 (B27)		
		SLCYKQRI~248-255 (A2)		
		KQRIKSQER~252-260		
		EATRGRGGL ~263-271 (A2)		
		TRGRGGLLR~265-273 (B27)		
		GRGGLLRDHI ~267-276		
		LLRDHPRGK~271-281 (A3)		
		IPRGKEEL~276-283(B7, B35, B51)		

Figure 3. (A) Schematics of MBP, MOG and PLP proteins and their arrangement in the cell membrane. For MBP, no precise 3D structure is known; this protein may be an associated membrane protein and the epitopes are positioned according to the sequence mapping. The different protein domains (intracellular ▬, extracellular ▭, membrane-associated domain ▬, and membrane ▬) for MOG and for PLP are described in [46] and [47], respectively, are indicated. Blank domains (▭) correspond to unknown conformations. (B) List of epitopes. Each epitope is represented by the amino-acid position in the corresponding protein sequence, the restricting HLA class-I molecule as defined in the binding test is indicated in brackets.

of the CD8$^+$ T cell responses to myelin peptides were stable during the 1-year follow-up (Fig. 4B). Some peptides never elicited a response in a given patient (as $MBP_{168-177}$ and PLP_{38-45} for patient MS-14; Fig. 4B) whereas others elicited stable positive responses (such as $MBP_{138-147}$, MOG_{97-105} and $PLP_{202-210}$ for patient MS-14). Some individuals presented new reactivity against some peptides during the follow-up ($MOG_{225-232}$ and $MOG_{252-260}$) whereas reactivity disappeared in others (such as $MOG_{178-186}$). In addition, no increased reactivity against myelin autoantigens was observed in the blood of MS patients during the exacerbating phase of the disease in the seven MS patients who presented a relapse during the study period (Table 1).

The immunodominant peptides were weak HLA binders

To determine whether the binding of myelin epitopes to the presenting HLA class I molecules could be linked to the magnitude of the response of the CD8$^+$ T cells, we analyzed the relationship between the number of IFN-γ-positive spots in the ELISPOT assay and the binding index. Figure 5A shows that the epitopes eliciting the strongest IFN-γ response were not the more efficient HLA binders. Although the most immunogenic HLA class I-restricted peptides were effective HLA class I binders, their binding index was low. This is illustrated in Fig. 5A for the HLA-A3-restricted peptides and was also observed for the other restricting alleles (HLA-A2, -B27, -B7 and -B44). Moreover, the myelin-derived peptides globally exhibited a tenfold higher efficient dose to reach 50% of the maximal response (ED_{50} = 5 μg/mL) than viral peptides (ED_{50} = 0.5 μg/mL) in both MS patients and HC (Fig. 5B).

A panel of cytokines involved in the CD8$^+$ T cell response to myelin peptides

IFN-γ is the most commonly produced cytokine following stimulation of CD8$^+$ T cells. However, measurement of only IFN-γ in ELISPOT assays may underestimate the CD8$^+$ T cell response. In addition, healthy individuals could be more prone to producing anti-inflammatory cytokines instead of the Th1 cytokines involved in autoreactive responses. We thus measured the production of IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17 and TNF-α in the undiluted supernatants 15 h after stimulation with the peptides. With myelin epitope stimulation, the levels of IL-4, IL-5 and IL-17 remained low

Table 1. Clinical characteristics of the patients and HLA typing

Code	Age (years)	Gender	HLA class I[a)]	Disease duration (years)	Relapse during study	EDSS score	Treatment[b)]
MS1	47	F	**-A2-A3-B7-B27**	6	2	4	IFN-β
MS2	33	M	-A1-**A31-B44**-B40	6	1	0	GA
MS3	41	F	-A1-**A3**-B8-**B14**	5	2	2	GA
MS4	42	M	-A24-**A32-B35-B51**	4	0	2	IFN-β
MS5	39	F	**-A2**-A23-B13-B15	14	2	3	IFN-β
MS6	27	F	**-A2-B51-B14**	2	0	0	GA
MS7	39	F	**-A2-A3-B7**-B57	9	2	2	IFN-β
MS8	21	F	**-A2-A3**-B18-**B51**	2	0	0	None
MS9	25	M	**-A3**-A74-**B7**-B58	1	0	0	IFN-β
MS10	37	F	**-A2-A3-B7-B27**	4	0	1.5	IFN-β
MS11	31	M	**-A2**-A24-**B51**-B45	1	1	1	IFN-β
MS12	33	M	**-A2-A11-B7-B27**	10	0	1.5	None
MS13	36	F	**-A3-A11-B35-B44**	6	0	2.5	IFN-β
MS14	24	F	**-A3-A32-B7**	3	1	1	IFN-β
MS15	42	M	**-A3-B7**	13	1	3.5	IFN-β
MS16	38	M	-A24-**A29-B7-B44**	1	0	0	IFN-β
MS17	31	F	**-A2**-A26-**B7-B44**	7	0	1.5	IFN-β
MS18	28	F	-A1-**A3**-B8-**B14**	6	1	1.5	GA
MS19	41	M	**-A2**-A23-**B44**-B45	10	0	1	GA
MS20	37	F	**-A2**-A68-**B51**-B57	2	1	2	None
MS21	35	F	**-A3-B7-B35**	3	0	1	None
MS22	44	F	**-A3-A32-B7**-B15	2	0	0	None
MS23	21	F	-A24-A80-**B7**	1	0	2.5	None
MS24	56	F	-A24-**A29**-B62-**B49**	2	1	1.5	None
MS25	55	F	-A1-**A2**-B8-B62	17	1	3.5	None
MS26	26	F	**-A2-A11-B35**-B57	5	0	2.5	None
MS27	40	M	**-A2-B7-B44**	1	0	1	None
HC1	28	F	**-A2-B7-B27**				
HC2	35	F	**-A2**-A24-B57-**B44**				
HC3	33	F	-A1-**A3-B35**				
HC4	28	F	-A1-**A2-B35**-B58				
HC5	25	F	**-A2-A3-B35-B44**				
HC6	32	F	-A1-**B7-B37**				
HC7	32	M	**-A11**-A24-B62-B57				
HC8	36	F	-A24-**A26-B7-B39**				
HC9	44	M	**-A3-A30**-B13-**B35**				
HC10	40	F	**-A2**-A24-**B7-B35**				
HC11	31	M	**-A2-A11-B49-B51**				
HC12	46	F	**-A2**-A29-**B7-B44**				
HC13	35	M	**-A11**-A24-B15-**B27**				
HC14	32	F	**-A3**-A24-**B35-B49**				
HC15	40	M	**-A2**-B15-B57				
HC16	26	M	-A1-**A2**-B63-B38				
HC17	26	F	**-A2**-A29-**B7-B44**				
HC18	29	M	**-A2-A11-B35-B44**				
HC19	50	F	-A1-B8-**B44**				

a) Bold HLA types belong to the HLA supertypes: HLA-A3 (-A3, -A11, -A31), HLA-A2 (-A2, -A28, -B70), HLA-B7 (-B7, -B35, -B51), HLA-B27 (-B27, -A32, -B14, -B39), and HLA-B44 (-B44, -A29, -B37, -B45, -B50).

b) GA, Glatiramer acetate (Copaxone®). IFN-β treatments consisted of Rebif® or Avonex®.

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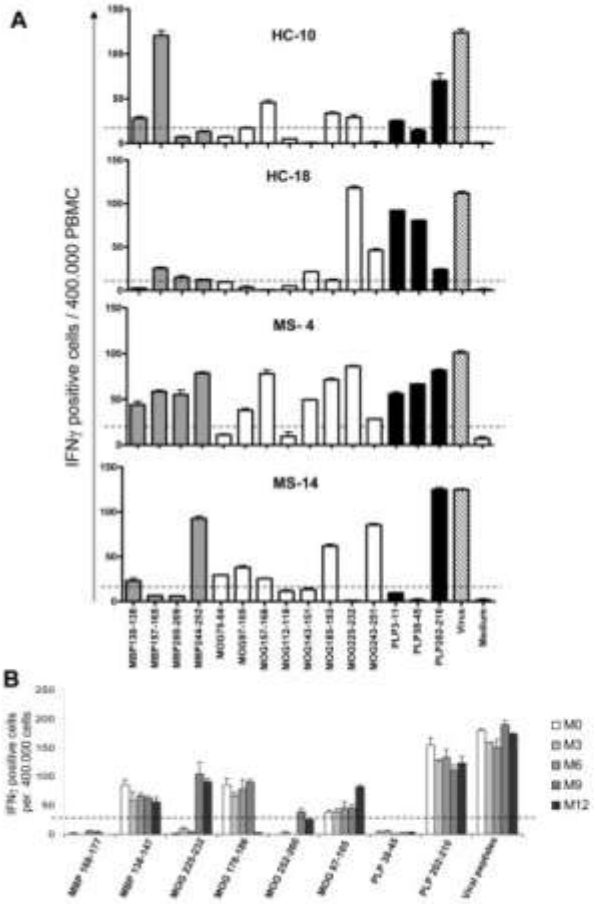

Figure 4. Patterns of T cell reactivity to myelin-derived epitopes. (A) Pattern of T cell reactivity to 18 myelin-derived epitopes and a pool of viral peptides. Fresh PBMC (4×10^5/well) were incubated in duplicate for 15 h, in the presence of a pool of viral peptides or each myelin epitope at 10 μg/mL. The results from four representative individuals (two MS patients and two HC) tested in an ELISPOT assay against myelin epitopes and the positive viral control, are given. The dotted line indicates the threshold of the positive response. (B) Time dependence of ELISPOT responses. Every 3 months for 1 year (M0, M3, M6, M9 and M12), fresh PBMC (4×10^5/well) were stimulated in duplicate for 15 h in the ELISPOT assay with myelin peptides at 10 μg/mL. The dotted line indicates the threshold of positive responses. In (B), the kinetics of reactivity for the patient MS-4 is shown.

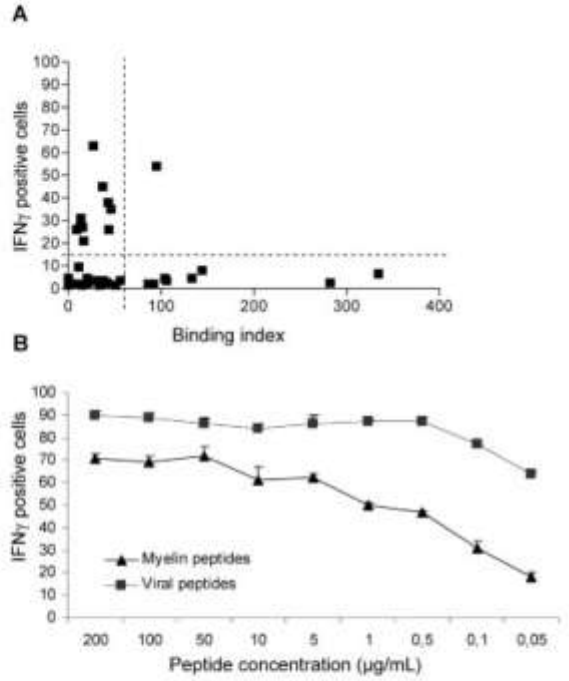

Figure 5. HLA binding efficiency of peptides and T cell responses. (A) HLA binding and magnitude of ELISPOT responses for HLA-A3. Peptides which can potentially bind HLA-A3 were tested in a binding assay and in an ELISPOT assay. The binding index, on the x axis, is the percentage of fluorescence obtained with the tested peptide, normalized with the fluorescence obtained with the positive control peptide in the binding test. The mean number of spots obtained for the (HLA-A3-restricted) individuals stimulated with peptides in the ELISPOT assay is indicated on the y axis. Points on the left of the vertical dotted line correspond to myelin peptides with low binding index for HLA-A3 and a strong IFN-γ ELISPOT response. Points under the horizontal dotted line correspond to myelin peptides with a high binding index for HLA-A3 but unable to induce an IFN-γ response. (B) T cell responses in ELISPOT assay and different peptide concentrations. Fresh PBMC (4×10^5/well) were incubated in duplicate for 15 h in complete RPMI medium (with 10% AB serum), in the presence of a pool of viral peptides and a pool of myelin peptides. The dilution ranged from 200 to 0.05 μg/mL. The graph shows a representative example (patient MS-4) of peptide dilution.

whereas an increase in IL-1β, IL-6, IL-8, IL-10 and TNF-α levels was associated with IFN-γ production in MS patients and HC (Fig. 6). HC did not produce more IL-4, IL-5 or IL-10, or less pro-inflammatory cytokines (IL-1β, TNF-α and IFN-γ), in response to autoreactive epitopes than MS patients.

Discussion

There is growing evidence suggesting a role for $CD8^+$ T cells in MS pathology, with a potential involvement of $CD8^+$ effectors in brain lesions [6, 12, 15]. Nevertheless, little attention has been paid to HLA class I-restricted epitopes derived from myelin proteins. Having compared the peripheral blood $CD8^+$ T cell reactivity against putative myelin autoantigens in 27 MS patients and 19 HC, we report here 69 new myelin-derived peptides able to stimulate blood $CD8^+$ T cells from MS patients and HC. We also confirmed $CD8^+$ T cell reactivity towards the $MBP_{220-228}$ peptide (referred to as MBP_{87-95} by Zang *et al.* [21]) and $MBP_{244-252}$ peptide (known as $MBP_{110-118}$ or $MBP_{111-119}$, depending on database sequences) [17, 21]. Moreover, using one of our synthetic peptides ($MBP_{200-208}$), we showed that $MBP_{200-208}$ is generated by human antigen-presenting cell processing and can be presented. The $MBP_{244-252}$ peptide has also been shown to be generated by endogenous processing [17]. We cannot, however, demonstrate that all of the peptides described are processed naturally.

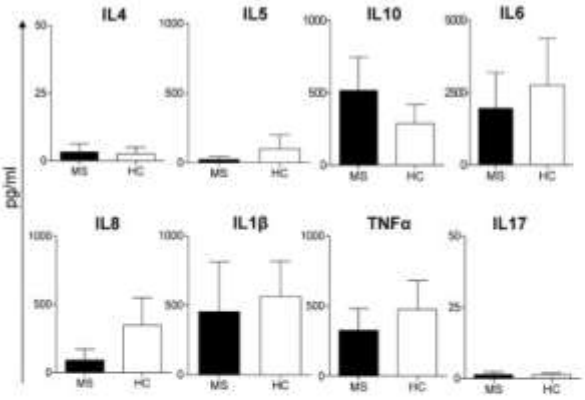

Figure 6. Production of cytokines following myelin epitope stimulation. Supernatants of IFN-γ ELISPOT assays were analyzed for IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17 and TNF-α production using a Lincoplex kit. Six MS patients versus six HC were studied. The variation in cytokine production after myelin peptide stimulation was obtained after subtracting the values obtained with no stimulation.

Nevertheless, the fact that they stimulate blood CD8 memory cells strongly suggests that this is the case.

The highest $CD8^+$ T cell reactivity for both MS patients and HC was observed using MOG-derived peptides. When testing reactivity to MBP, PLP and MOG proteins, Diaz-Villoslada *et al.* also showed the same trend for a prevalent response to MOG [29]. Interestingly, Mars *et al.*, using a HLA-A2 humanized mouse, detected an *in vivo* response to $MOG_{143-151}$ (referred to in their article as $MOG_{114-122}$) [30]. It is also important to notice that the MBP, PLP and MOG proteins could theoretically induce different levels of self tolerance [31]. Indeed, the MOG protein is only expressed in the central nervous system whereas MBP and PLP are also expressed in the thymus [32].

Comparing the binding index of peptides and the magnitude of $CD8^+$ T cell responses in the ELISPOT assay, we found that $CD8^+$ T cells do not preferentially react with high-binding epitopes. These results suggest that epitopes with a high-binding index might have induced apoptosis of autoreactive cells.

The HLA-A2 allele is commonly found in Caucasians, and most epitopes described previously in MS were, for the majority, restricted to this HLA allele. However, the importance of HLA-A3, which is associated with MS [18, 19], might have been underestimated. In this study, we focused on the representative HLA molecules present in our cohort of MS patients. Interestingly, a majority of epitopes (36%) were HLA-A3 restricted, suggesting that HLA-A3 restriction may be involved in MS autoimmunity.

Tranquill *et al.* suggested that MS patients could present enhanced T cell responsiveness to citrullinated peptides from MBP [28]. Nevertheless, the latter results were obtained in a small cohort (n=5), using a protocol including an *in vitro* stimulation step and with 17- to 20-mer peptides. Here, we tested reactivity against nine citrunillated peptides derived from MBP, but were unable to detect *ex vivo* responses from $CD8^+$ T cells in both MS patients and HC.

Analysis of the 188 myelin-derived peptides in an IFN-γ ELISPOT assay detecting only $CD45RO^+CD8^+$ T cell responses did not reveal differences in the frequency of IFN-γ-producing $CD8^+$ T cells reactive against myelin-derived peptides between MS patients and HC. Moreover, autoreactive IFN-γ-producing $CD8^+$ T cells from MS patients and HC produced similar amounts of IL-1β, IL-6, IL-8, IL-10 and TNF-α. Tsuchida *et al.* also found that both MS patients and normal individuals present autoreactive $CD8^+$ T cell precursors [17]. However, Zang *et al.* reported an increased frequency of $CD8^+$ T cell precursors reacting to MBP_{87-95} and $MBP_{111-119}$ in MS patients compared to HC, but this difference was observed after a 7-day stimulation *in vitro* [21]. Using the ELISPOT assay, Moldovan *et al.* tested MBP- and PLP-derived overlapping 9-mer peptide reactivity and found that MS patients presented higher cumulative responses, but after a 48-h incubation [33]. Our results based on *ex vivo* responses with a 15-h stimulation indicate that MS patients and HC have the same frequency of circulating autoreactive $CD8^+$ T cells. These data mimic results that have been reported for $CD4^+$ T cell reactivity to myelin autoantigens in ELISPOT assays [34]. Therefore, the presence of autoreactive T cells in the blood may not be specific to MS. Moreover, it is possible that these cells may simply not be involved in MS pathology.

However, results of *in vitro* assays generating autoreactive T cell lines against myelin-derived autoantigens support the concept that autoreactive T cells from the blood of MS patients may present a different degree of activation as compared to those derived from HC [34, 35]. T cells from MS patients seem to produce more pro-inflammatory cytokines as TNF-α, IFN-γ and IL-1β during the relapse phase [36]. Autoreactive T cell lines also appear less sensitive to apoptosis [37, 38] and have increased $CD8^+$ T cell adhesive capacities to endothelium [39]. Therefore, if autoreactive T cells are found at similar frequencies in MS patients and HC, cells from MS patients, unlike those from healthy individuals, may be involved in an active immune process. In addition, the truly active $CD8^+$ T cells might have already migrated to the central nervous system lesion site, and their frequency may be too low in the periphery for a significant detection. Another question concerning the "paradoxical" absence of a difference in frequencies of autoreactive $CD8^+$ T cells between MS patients and HC is that, although MBP, MOG and PLP are the major components of myelin, they might not be the recognized autoantigens in the human pathological process. In fact, several other antigens compose the myelin sheath and are also specific to the central nervous system, such as the oligodendrocyte-specific protein (OSP) or the myelin-associated oligodendrocyte basic protein (MOBP). For instance, Niland *et al.* found an increased response in MS patients compared to normal controls for an HLA-A2-restricted peptide derived from the transaldolase protein [40].

Taken together, our data show that autoreactive $CD8^+$ T cells are present in the blood of MS patients and HC. However, $CD8^+$ T cells from MS patients could be in an active state that induces pathological processes. By providing a better knowledge of the $CD8^+$ T cell repertoire of MS peptides, our data may offer the opportunity to better explore the role of these cells in MS,

particularly by comparing the blood pattern with that found within the cerebrospinal fluid.

Materials and methods

MS patients and HC

Twenty-seven patients with RR-MS and nineteen HC were included and monitored every 3 months for a period of 1 year. RR-MS was clinically defined using the McDonald criteria [41]. All individuals gave their informed consent before participating in this study, which was approved by the Ethical Committee. No patient was under immunosuppressive therapy for at least 6 months before analysis; twelve patients were treated with IFN-β and five with glatiramer acetate (Table 1). Typing of HLA class I molecules was performed by PCR (Olerup SSP™ HLA-A, -B low resolution; Saltsjöbaden, Sweden). MS patients and HC were sex matched and age matched and displayed a roughly similar frequency of the supertypes HLA-A2, -A3, -B44 and -B7.

Sequence analysis of myelin antigens and peptide selection

To select peptides able to bind to HLA molecules, a search for HLA binding motifs, as defined by Sette *et al.* [23], was performed by the sequence analysis of human MBP [42], MOG [43] and PLP [44]. A total of 188 peptides (8- to 11-mers) containing anchor residues required for binding to five HLA class I supertypes were selected. All peptides were synthesized (Mimotopes, Clayton Australia) and dissolved in 4% DMSO. Nine peptides from MBP containing the arginyl residue, a site of potential citrullination, were selected (citrullinated sequences described in [28]). These peptides were produced with the arginine residues replaced by citrullinyl residues (Cambridge Research Biochemicals, UK). When indicated, peptides from EBV, CMV and influenza virus were used as positive controls in the ELISPOT assay.

Peptide/HLA class I binding assay

Wells of plates (Nunc Maxisorp, Taastrop, Denmark) were coated with monoclonal anti-HLA antibodies at a concentration of 10 μg/mL in PBS for 2 h at 37°C. The following antibodies were used: BB7.2 to detect the HLA-A2 molecule, A11.1M and GAP-A3 for HLA-A3, B1.23.2 or PA2.6 for HLA-B7/35/51, HLA-B27 and HLA-B44. All antibodies were obtained from ATCC, except for B1.23.2 [45]. The plates were washed three times with PBS-Tween and saturated overnight with PBS-Tween/1% BSA at 4°C. HLA class I molecules were purified from human lymphoblastoid cell lines transformed by EBV and denatured. HLA molecules (2 μg/100 μL) were incubated with each peptide (10^{-4}, 10^{-6} and 10^{-8} M) and 2 μg/mL β2-microglobulin in PBS-Tween/1% BSA, 1 mM *p*-amidino-phenyl-methyl-sulfonyl-fluoride, 10 μg/mL trypsin inhibitor for 2 h at room temperature. The HLA molecules pre-incubated with peptides were then added in duplicate to antibody-coated wells for 90 min at 37°C and the plates were washed three times with PBS-Tween. To detect the formation of correctly folded HLA/peptide complexes, the anti-β2-microglobulin antibody M28, coupled to alkaline phosphatase, was added for 1 h at 37°C. The enzymatic activity was then revealed by adding the MUP substrate. The fluorescence was measured at 355/460 nm with a Fluoscan reader (VICTOR; Wallac, Evry, France). The positive control was a viral epitope: M 58–66 from influenza virus for HLA-A2; Nef 73–82 from HIV-1 for HLA-A3/11; Nef 68–76 and 71–79 for HLA-B7/35; NP 383–391 from influenza virus for HLA-B27; and Vpr 47–54 from HIV-1 for HLA-B44. Reactions were considered positive for a given peptide if fluorescence exceeded at least 30% of those obtained with viral control peptides. The percentage of fluorescence obtained with a myelin peptide in comparison with the fluorescence of a positive control is described as the binding index.

IFN-γ ELISPOT assay and cytokine detection in culture supernatants

Fresh PBMC from MS patients and HC were tested using IFN-γ ELISPOT. PBMC were isolated from 100 mL of blood, then purified on a gradient Ficoll layer (Eurobio, Les Ulis, France) and directly assayed with a human IFN-γ ELISPOT kit according to the manufacturer's recommendations (Endogen/Pierce, Rockford, IL). PBMC (4×10^5/well) or purified cells (4×10^4/well) were incubated with 10 μg/mL peptide for 15 h at 37°C, in complete RPMI medium supplemented with 10% AB serum in antibody-coated plates. The supernatants were then collected and frozen for cytokine quantification. The spots were counted by the ELISPOT reader system (AID, Strassberg, Germany). The criteria in the ELISPOT assay for epitope selection were: more than 20 spots per 4×10^5 PBMC and a number of spots at least three times the background.

Cytokines (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17 and TNF-α) contained in the ELISPOT supernatants (see above) were quantified using a multiplex fluorescent bead immunoassay (Lincoplex, Linco, MO) and measured with a Luminex® device.

Cell purification and culture

B cells, monocytes, $CD4^+$ and $CD8^+$ $CD45RO^+$ or $CD45RO^-$ cells as well as $CD8^+$-depleted PBMC were separated from PBMC using a high-speed cell sorter (ARIA; BD Biosciences, San Jose, CA). Cell samples (approximately 1×10^8) were incubated for 15 min with monoclonal antibodies: 20 μL of anti-CD8-PerCP-Cy5.5, 20 μL of anti-CD45RO-ECD, 20 μL of anti-CD14-FITC and 20 μL of anti-CD19-PE-Cy7 (purchased from BD Biosciences). Purity was >95% for all sorted subpopulations. For the generation of DC, $CD14^+$ cells were cultivated in complete RPMI medium supplemented

with 10% AB serum, GM-CSF (500 IU/mL; AbCys, Paris, France) and IL-4 (100 U/mL; AbCys) for 5 days. DC maturation was induced by adding LPS (1 μg/mL) with or without MBP protein (100 μg/mL) to culture medium and culturing cells for a further 2 days (days 5–7). $CD8^+$ T cells were stimulated with the peptide $MBP_{200–208}$ (10 μg/mL) at days 0 and 5 and received IL-2 stimulation at days 3 and 5. At day 7, DC and $CD8^+$ T cells were washed and incubated in ELISPOT plates.

Statistical analyses

Non-parametric Mann-Whitney tests were performed for the comparison of ELISPOT assay responses and phenotypic analyses between the MS patient and HC groups and a p value of <0.05 was considered significant.

Acknowledgements: This investigation was supported by ARSEP (French association for research on multiple sclerosis) and Progreffe (French foundation for immunointervention research in transplantation). The authors acknowledge Joanna Ashton-Chess for manuscript editing.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **HC:** healthy control · **MBP:** myelin basic protein · **MOG:** myelin oligodendrocyte glycoprotein · **MRI:** magnetic resonance imaging · **PLP:** proteolipid protein · **RR-MS:** relapsing-remitting multiple sclerosis

Full correspondence: Prof. Jean-Paul Soulillou, INSERM U643,
30 Bd Jean Monnet, CHU Hotel-Dieu, 44093 Nantes Cedex 1, France
Fax: +33-2-40087411
e-mail: Jean-Paul.Soulillou@univ-nantes.fr

Received: 21/11/07
Revised: 13/3/08
Accepted: 17/4/08

REVUE I : Y-a-t-il une augmentation de la fréquence des cellules T auto-réactives circulantes dans le sang des patients atteints de Sclérose en Plaques de forme récurrente-rémittente ?

Par : Pettré S; Bahbouhi B, Brouard S*, Laplaud DA*, Soulillou JP*

(*les auteurs ont contribués de manière égale à ce travail)

Revue en préparation

Frequency of circulating auto-reactive T cells in the blood of relapsing-remitting Multiple Sclerosis patients

Ségolène Pettré[1]; Bouchaib Bahbouhi[1], Sophie Brouard[1 *], David-Axel Laplaud[1, 2, 3 *] and Jean-Paul Soulillou[1 *]

[1] INSERM, UMR 643, Nantes, F44093 France; CHU Nantes, Institut de Transplantation et de Recherche en Transplantation (ITERT) Nantes, F44000 France; Université de Nantes, Faculté de Médecine, Nantes, F44000 France

[2] INSERM CIC004, University Hospital, Nantes, 44093

[3]Department of Neurology, CHU Nantes, F-44093 Nantes France

[*] Both authors have similarly contributed and are listed by alphabetic order.

Running Title: Frequencies of auto-reactive T cells in multiple sclerosis

Corresponding author: Jean-Paul Soulillou, Inserm UMR 643, 30 Boulevard J Monnet, 44093 Nantes Cedex 1.

E-mail: jean-paul.soulillou@univ-nantes.fr

Abstract

Multiple Sclerosis (MS) is considered as an autoimmune disease in which T cell reactivity to self-antigens expressed in the brain, particularly myelin antigens, play a pivotal role. Various myelin derived peptides, including peptides of myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) have been studied as putative target in MS. However, CD4+ and CD8+ T cells recognizing auto-antigens such as MBP and PLP have been demonstrated in the blood of MS patients and also found in the blood of normal individuals. Here we review and discuss the studies focused on the assessment of auto-reactive T cells frequency using different assays such as LDA, IFNγ-ELISPOT and TRAP (T cell Recognition of Antigen by Protein transfer) allowing to assess the frequency of cells responding to a given antigen.

Introduction

Despite strong evidences of the inflammatory nature of Multiple Sclerosis (MS) such as the presence of T cell and monocyte/macrophage infiltrates at the border or within the active lesions, the autoimmune etiology of the disease is still debated (Lucchinetti et al. Brain Pathol 1996, Fabrieck et al. J Neuroimmunol 2005). According to current hypotheses, mainly based on the animal model which are different from the human disease (Experimental Autoimmune Encephalitis, EAE), MS is considered in many aspect as an autoimmune disorder in which T cell reactivity to self-antigens expressed in the brain, particularly myelin derived peptides, plays a pivotal role in disease progression and possibly as an exclusive etiology. The current physiopathological concept sustaining this hypothesis in MS lies on the presence within the peripheral blood of activated and memory auto-reactive T cells directed against myelin components. Auto-reactive T cells from the blood of MS patients are able to transmigrate the Blood Brain Barrier (BBB) endothelium and may gain access to the Central Nervous System (CNS) and trigger, or contribute to, demyelinating lesions and axonal insuet (Prat et al. Ann Neurol 1999, Battistini et al. Blood 2003). Various myelin antigens, including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) have been studied as putative target auto-antigen in MS.

However, both CD4+ (Laplaud et al. Brain 2004) and CD8+ (Laplaud et al. Brain 2004, Skulina et al. PNAS 2004, Moldovan et al. J Neuroimmunol 2003) circulating T cells recognizing sequestered auto-antigens such as MBP and PLP have been demonstrated in normal individuals and in MS patients. Using different approaches such as tissue culture based techniques (Ota et al. Nature 1990; Martin et al. Ann Neurol 1993), quantitative polymerase chain reaction (Muraro et al. Brain 2003, Hong et al. Eur J Immunol 2004), flow cytometry (Crawford et al. Blood 2004) or tetramer (Olsson et al. Eur J Immunol 1992), many teams have tried to demonstrate a higher frequency of MBP, MOG and PLP auto-reactive CD4+ and/or CD8+ T cells in the blood of MS patients. Several groups, including our own (Berthelot et al. Eur J Immunol 2008), have shown no difference in terms of number of circulating auto-reactive T cells in MS as compared to aged and gender healthy controls (Jingwu et al. Ann Neurol 1992, Hellings et al. J Neurosci Res 2001). Whereas, qualitative differences have been observed leading to the concept that the circulating auto-reactive T cells from MS patients may have a different state of activation than the one observed in healthy individuals (Chou et al. J Neuroimmunol 1992; Zhang et al. J Exp Med 1994) allowing a better BBB crossing (Dressel et al. Acta Neurol Scand 2007). Nevertheless, other groups have

also suggested an increased frequency of myelin T cells in the blood of MS patients (Pender et al. J Immunol 2000, Olsson et al. J Clin Invest 1990, Wallström et al. Eur J Immunol 1998). Thus, whether higher numbers of myelin-specific T lymphocytes prevail in the peripheral blood mononuclear cells (PBMC) of MS patients in comparison to healthy volunteers (HV) is still a debated question. Here, we will revue the reports on the frequencies of auto-reactive T cells in MS and particularly, we will discuss the results in the context of the classical methods such as Limiting Dilution Assay (LDA) or Enzyme Linked ImmunoSPOT (ELISPOT) assay and of a new method allowing to reassess the frequency of cells responding to a given antigen without depending on the peptide, the restricting MHC molecule or the type of cytokine produced (Beadling et al. Nat Med 2006).

Assessment of auto-reactive T cells frequency using Limiting dilution assay (LDA)

T cells with high affinity receptors recognizing MBP (or MBP derived synthetic peptides) and PLP are part of the normal T cell repertoire and are present in the blood of MS patients as well as in healthy individuals, most of the studies based on the establishment of MBP specific T cell lines have shown that there are similar frequencies of 1 in 10^5-10^6 T cells in both groups (Martin et al. J Immunol 1990, Ota et al. Nature 1990, Pette et al. PNAS 1990). Moreover, Bieganowska et al. tested two methods in order to evaluate MBPp85-99 specific T cells frequency. In contrast to frequencies of 1 in 10^5-10^6 as measured by LDA, they observed an anti-MBP T cell frequency of 1 in 300 PBMC using a T cell receptor sequence-based approach (Bieganowska et al. J Exp Med 1997).

A. Auto-reactive precursor frequency in unmanipulated fresh T cells in MS

LDA was the first method to calculate the precursor frequency of antigen responsive T cells. LDA allows to detect an all-or-none (positive or negative) immunoresponse in a dose-dependant manner in each individual culture within replicates that vary in the number of responder cells tested. This procedure requires *in vitro* culture and according to the read-out implies a selection of cells with preferential properties (for instance: proliferation rather than cells that respond by producing effectors molecules). Therefore, the frequency of anti-myelin reactive T cells *in vivo* is potentially much higher than the one appreciated from the proliferation based techniques. The frequency of positive cultures is depending on the number of precursors in the culture well that are given the positive response. The negative response demonstrates that there are no precursors of a given specificity. The evaluation of the cell

frequency in the original population is possible by determining the number of cultures that are negative in the experiment. The percentage of negative cultures is then converted to its negative logarithm plotted graphically. In addition, the LDA technique, which requires sophisticated experimentation and a relatively large amount of blood lymphocytes and makes it difficult to study T cell frequencies of various myelin reactive T cell populations in individual patients.

Chou and colleagues have used LDA to determine the frequency of MBP and PLP peptide specific T cells in blood from eight MS patients (stable relapsing-remitting MS patients - RRMS- and MS patients with chronic progressive disease) and six HV (Chou et al. J Neuroimmunol 1992). PBMC were cultured with whole MBP (50µg/ml) or $PLP_{139-151}$ (50µg/ml). Circulating MBP reactive T cells from MS patients had a mean frequency of 0.61 cells per 10^5 PBMC that was significantly higher than that of normal individuals (0.10 cells per 10^5 PBMC). In contrast, no difference was found in circulating T cell frequencies to $PLP_{139-151}$ (Chou et al. J Neuroimmunol 1992).

Using the same method, Jingwu and colleagues analyzed the frequency of blood CD4+ T cells reactive to whole MBP (40µg/ml) in eight MS patients and four normal donors. However, no information was available on the treatment and disease state at the time of sampling. MBP specific T cells occurred with mean of frequencies 6.7 cells per 10^7 PBMC in MS patients and 5.6 cells per 10^7 PBMC in HV with no significant difference between the two groups (Jingwu et al. Ann Neurol 1992).

Still using LDA, Zhang and colleagues reported the frequencies of MBP and PLP reactive T cells using MBP (40µg/ml) or PLP (50µg/ml). No information was given concerning disease form, phase or treatment. Frequencies of MBP reactive T cells were similar in 19 MS patients (1.1 per 10^6 PBMC) and 17 HV (0.9 per 10^6 PBMC) (Zhang et al. J Exp Med 1994). In the same study, 16 different MS patients and 10 HV were examined for PLP reactivity. No statistical difference was reported for PLP auto-reactivity (Zhang et al. J Exp Med 1994).

Pender et al. have tested the peripheral blood T cell response to PLP_{41-58}, $PLP_{184-199}$, $PLP_{190-209}$, MBP_{82-100} (20µg/ml) and MBP (30µg/ml) using LDA, in five patients with RRMS and four HV. These patients received no treatment at the beginning of the study but three of them started IFNβ during the study and two received treatments for a relapse. For instance, T cell frequencies for PLP_{41-58} were significantly higher in MS compared to HV (1.34 in MS *vs* 0.7 in HV per 10^6 PBMC, p =0.019) and similar T cell frequencies for $PLP_{184-199}$ were observed between MS patients and HV (2.0 MS *vs* 0.93 HV per 10^6 PBMC, p =0.084). The mean frequencies of T cells reacting to the 41-58 and 190-209 of PLP residues were also

significantly higher in the MS patients than in the normal controls. However; the mean frequencies of T cells reactive to $PLP_{184-199}$, MBP_{82-100} or whole MBP in MS patients were not significantly different from those of HV (Pender et al. J Immunol 2000). Results obtained for $PLP_{190-209}$, MBP_{82-100} and MBP were summed up in Table 1.

Tejada-Simon et al. also compared MBP and PLP reactivity in RRMS patients (n=11), chronic progressive MS (n=5) and HV (n=9) (Tejada-Simon et al. Eur J Immunol 2001). The patients did not receive immunosuppressive or immunomodulating agents before the study. T cells recognizing whole human MBP (40µg/ml in the test) was not statistically significant. T cell response to a complete panel of overlapping peptides of PLP of MS patients (n=15) and HV (n=9) were tested. Synthetic peptides of MBP and PLP were used at a concentration of 12µg/ml. Again, no statistical difference was reported between MS patients and normal controls (Tejada-Simon et al. Eur J Immunol 2001).

In another study by Van der Aa et al., blood samples from nine MS patients (RRMS and with a progressive disease course) and from five HV were tested with a mixture of four synthetic MOG peptides (MOG_{1-22}, MOG_{34-56}, MOG_{64-86} and MOG_{74-96}; 10µg/ml each) (Van der Aa et al. J Neuroimmunol 2003) Although the LDA estimated frequency of MOG reactive T cell was much lower than in ELISPOT analysis (ELISPOT paragraph, see below), the frequency of MOG-reactive T cells was similar in both study groups (13.3 cells per 10^7 PBMC from MS patients and 14.2 cells per 10^7 PBMC from normal controls) (Van der Aa et al. J Neuroimmunol 2003).

B. Estimation of precursor frequency after in vitro culture step and stimulation

Because of low cells not obtained from Cerebrospinal Fluid (CSF), Chou and colleagues have estimated T cells frequencies based on the number of antigen specific IL2/IL4 reactive isolates recovered from the CSF (Chou et al. J Neuroimmunol 1992). Nine MS patients (three RRMS and six with chronic progressive disease) and six other with other neurological diseases (OND) were tested. First, cells were expanded with a medium containing IL2 and IL4 (50IU/ml for each). Then, the myelin specificity was tested. The mean estimated relative frequency of CSF T cells specific for MBP among MS patients was 22.1 cells per 10^5 CSF cells and the relative frequency of T cells specific for $PLP_{139-151}$ was 9.5 cells per 10^5 CSF cells. These frequencies were significantly higher than those observed in the CSF of OND subjects (Chou et al. J Neuroimmunol 1992).

Zhang and colleagues also used IL2 pre-stimulation PBMC or mononuclear cells from CSF before antigen incubation. LDA of CSF T cells exhibited a higher frequency of MBP reactive

T cells after a culture step of two rounds of IL2 stimulation (40U/ml) in MS patients compared with T cells from paired PBMC (5.4 cells per 10^5 CSF cells *vs* 0.32 cells per 10^5 PBMC; p <0.001). The frequency of MBP specific T cells after IL2 stimulation from CSF was also significantly higher in MS patients (5.4 cells per 10^5 CSF cells) compared with OND patients (0 cells per 10^5 CSF cells) (Zhang et al. J Exp Med 1994).

In conclusion, as shown in Table 1, only one study has shown a significantly higher whole MBP-specific T cell frequency in MS using LDA (Chou et al. J Neuroimmunol 1992). Whereas, only Pender et al. have obtained significantly higher PLP-specific T cell frequency in MS using two synthetic peptides: PLP_{41-58} and $PLP_{190-209}$ (Pender et al. J Immunol 2000). Thus, most of LDA studies (Jingwu et al. Ann Neurol 1992, Zhang et al. J Exp Med 1994, Tejada-Simon et al. Eur J Immunol 2001, Van der Aa et al. J Neurimmunol 2003) did not show differences in term of myelin-specific auto-reactive T cell frequency between MS patients and controls (Table 1). However, myelin auto-reactive T cells from MS patients seem to be more sensitive to IL2 stimulation, suggesting an activation step with higher expression of high affinity IL2 receptor. Thus, differences in activation threshold and may be function, instead of differences in frequency, might be involved in MS pathology.

Analysis of auto-reactive T cells frequency using ELISPOT assay

A. Analysis of precursor frequency without prestimulation

ELISPOT assay has been more extensively used to quantify T cells in fresh cells that secrete IFNγ pulsed with myelin derived antigen. A major advantage of ELISPOT is an easier procedure, which does not require *in vitro* proliferation. In addition, it requires limited amounts of blood, making it possible to analyze simultaneously T cell reactivity to a whole range of myelin antigens. Measurement of the IFNγ secretion as a read out for T cell reactivity may especially be relevant for MS studies as this cytokine is thought to play an essential role in the disease (Hermans et al. Ann Neurol 1997). However, ELISPOT is based on the secretion of only one cytokine excludes cells that may secrete other types of cytokines.
Olsson and colleagues have collected blood and CSF samples from 39 untreated MS patients and controls with or without neuroinflammatory diseases: 16 patients with acute meningitis and 25 patients with tension headache. PBMC at 2.10^5 cells per wells or CSF cells at $5\text{-}20.10^3$ cells per wells were pulsed with whole MBP, whole PLP or MBP peptides ($MBP_{132-150}$ or

$MBP_{174-191}$ at a final concentration of 10µg/ml). After 48 hours of culture, 2.7 out of 10^5 PBMC of MS patients responded to whole MBP, 5.2 out of 10^5 PBMC responded to $MBP_{132-150}$, 4.9 out of 10^5 PBMC responded to $MBP_{174-191}$ and 4.7 out of 10^5 PBMC responded to PLP. Frequencies of these auto-reactive T cells were significantly increased compared to controls (p <0.001 for each tested antigen) (Olsson et al. J Clin Invest 1990) (Table 1). In a following step of this study, Olsson et al. have estimated auto-reactive T cells frequency in CSF cells. Due to the limited numbers of CSF cells available for analysis, only MBP reactive specific T cells were examined. The authors had observed 185 out of 10^5 CSF cells responded to MBP in MS patients. T cells auto-reactive to different myelin components and significantly increased in peripheral blood were also strongly enriched in CSF of MS patients compared with the two control groups (Olsson et al. J Clin Invest 1990).

Sun et al. have studied MOG reactive T cells in CSF and blood from 16 untreated MS patients and 18 controls (Sun et al. J Immunol 1991). The control group was constituted with nine aseptic meningitis patients, seven patients with muscular tension headache and 2 other patients suffering from other neurologic diseases. CSF cells at 4 to 10.10^3 cells per wells or PBMC at 2.10^5 cells per wells were stimulated with MOG (10µg/ml). After a 48 hours ELIPSOT, the mean value of MOG reactive T cells in MS patients was significantly increased compared to controls (p <0.05) (22.1 per 10^5 PBMC in MS patients *vs* 7.6 per 10^5 PBMC in controls). Mean values of auto-reactive T cells in CSF were 804 per 10^5 cells in MS patients and 346 per 10^5 cells in controls. This suggests that MS patients have higher numbers of MOG reactive T cells in blood and in CSF compared to controls (Sun et al. J Immunol 1991).

Wallström and colleagues have investigated blood T cell responses to synthetic overlapping MOG class II peptides in 20 untreated MS patients and 14 HV with the MS-associated HLA haplotype DR2 (Wallström et al. Eur J Immunol 1998). After a 40 hours ELISPOT, there were a significant increased responses in MS patients compared to normal individuals. MOG_{63-87} evoked the strongest response: 3.5 specific IFNγ secreting cells per 10^5 PBMC in MS patients *vs* 0.3 in normal individuals (Wallström et al. Eur J Immunol 1998).

Pelfrey et al. examined reactivity using peptides spanning the entire PLP molecule (Pelfrey et al. J Immunol 2000). MS patients (n=22) with RRMS (n=9), secondary progressive (SPMS, n=8) or primary progressive (PPMS, n=5) form of disease were compared with 22 HV. Some patients were under IFNβ or glatiramer acetate therapy. PBMC at $1.5.10^5$ cells per well were incubated for 24 hours with 7 µM PLP peptides. These peptides were nonamer peptides MHC class I molecules restricted, in addition to class II molecules, bypassing intracellular processing. Total number of IFNγ positive PLP peptides was 4 times higher in MS patients

than in HV (6.5 *vs* 1.5 respectively; p =0.022). Whereas no difference was observed between MS and HV in IL5-ELISPOT assay (1.5 *vs* 0.6 respectively) (Pelfrey et al. J Immunol 2000).

Hellings et al. have analysed the T cell reactivity to MBP, MOG and to a panel of synthetic MBP, MOG and PLP peptides in 36 MS patients and 31 HV using IFNγ-ELISPOT. MS patients comprised patients with RRMS (n=24) or progressive disease course (n=12). PBMC at 2.10^5 cells per wells were incubated in a 20 hours ELISPOT in the presence of MBP (40µg/ml), MOG (10µg/ml), synthetic myelin peptides (MBP_{84-102}, $MBP_{143-168}$, MOG_{1-22}, MOG_{34-56}, MOG_{64-86}, MOG_{74-96}, PLP_{41-58}, $PLP_{184-199}$, and $PLP_{190-209}$; 10µg/ml). The mean frequency of MBP reactive T cells was comparable for MS patients ($4.1.10^{-5}$) and HV ($4.4.10^{-5}$). Similar mean frequencies for anti-MOG T cells were also found for both groups (4.0 per 10^5 PBMC in MS patients *vs* $4.2.10^{-5}$ per 10^5 PBMC in HV). In contrast, no significant differences in reactivity were observed between MS patients and control subjects to any of these myelin antigens and peptides (Hellings et al. J Neurosc Res 2001).

Myelin associated glycoprotein (MAG) is a minor component of myelin which counts for less than 1% of the total myelin protein in the CNS. It is also present, but in lower proportion, in the peripheral nervous system (Sospedra et al. Annu Rev Immunol 2005). As shown in Table 1, Andersson and colleagues examined the T cell response to MAG or five selected MAG peptides of 16 and 20 amino acids to elicit CD4+ response. Eleven of the MS patients were tested during disease exacerbation and fifteen patients were tested during remission. Two control groups consisted in 23 patients with OND and 17 HV. PBMC (2.10^5 per wells) were added with either whole MAG or MBP, or MAG peptides (MAG_{20-40}, MAG_{41-63}, $MAG_{241-260}$, $MAG_{596-612}$ and $MAG_{609-626}$) in a 48 hours ELISPOT test. MS patients had significantly higher numbers of MAG reactive T cells in peripheral blood than HV (2.5 per 10^5 PBMC in MS patients *vs* 0.6 per 10^5 PBMC in HV). Similar results were obtained for MBP (2.2 per 10^5 PBMC in MS patients *vs* 0.6 per 10^5 PBMC in HV). MS patients had higher numbers of T cells recognizing three of MAG peptides tested (MAG_{41-63}, $MAG_{596-612}$ and $MAG_{609-626}$), with frequencies varying from 1 to 2 cells for 10^5 PBMC in MS patients compared to 0.2 to 0.5 cells for HV. No differences were observed for MAG_{20-40} (0.5 per 10^5 PBMC in MS patients *vs* 0.4 per 10^5 PBMC in HV) and $MAG_{241-260}$ (0.2 per 10^5 PBMC in MS patients *vs* 0.2 per 10^5 PBMC in HV) (Andersson et al. Eur J Neurol 2002).

MOG specific T cell frequency was tested in 15 MS patients (including RRMS and patients with a progressive disease course) and eight HV. PBMC (2.10^5 per wells) were incubated for in a 20 hours ELISPOT with MOG (10µg/ml) (Van der Aa et al. J Neuroimmunol 2003).

Frequency of MOG reactive cells was not statistically significant (14.7 cells per 10^5 PBMC in MS patients *vs* 12 cells per 10^5 PBMC in HV) (Van der Aa et al. J Neuroimmunol 2003).

Ratts and colleagues examined on frequency of blood T cell against synthetic MBP peptides in twelve MS patients (six RRMS and six PPMS) and nine HV. PBMC at 5.10^5 cells per wells were incubated for 36 hours with 50µg/ml MBP peptides (MBP_{83-106}, $MBP_{111-129}$ and $MBP_{143-171}$). The highest frequency of myelin reactive T cells was observed in an MS patient (Ratts et al. J Neuroimmunol 2006).

Recently, we have analysed fresh blood CD8+ frequency against short myelin derived peptides of 27 RRMS patients treated or not glatiramer acetate or IFNβ and 19 HV (Berthelot et al. Eur J Immunol 2008). PBMC (4.10^5 cells per wells) or purified CD8+ cells (4.10^4 cells per wells) were incubated with 10µg/ml peptide in a 15 hours ELISPOT assay. PBMC from the two groups showed roughly similar auto-reactive responses against the myelin peptide pool (MOG_{74-81}, MOG_{97-105}, $MOG_{225-232}$, $PLP_{202-210}$, $MBP_{244-252}$ and $MBP_{138-147}$) as well as to each tested peptides. The mean value of reactive T cell was 11 per 4.10^5 cells without difference between untreated or treated MS patients. Using purified CD8+ from five MS patients and two HV, we showed that only memory CD8+ T cells produced IFNγ in response to class I restricted peptides derived from myelin. Similar response was observed between MS patients and HV (Berthelot et al. Eur J Immunol 2008). Finally, as Tranquill et al. suggested that MS patients could present enhanced T cell responsiveness to citrunillated peptides from MBP (Tranquill et al. Mult Scler 2000) citrullinated peptides derived from MBP were tested but did induced CD8+ T cell responses neither in MS patients nor HV (Berthelot et al. Eur J Immunol 2008).

B. ELISPOT estimated of precursor frequency following ex vivo culture step

Transaldolase is expressed at selectively high levels in oligodendrocytes and targeted by autoreactive T cells of MS patients (Banki et al. J Exp Med 1994). These patients have transaldolase antibodies in their blood and CSF (Colombo et al. J Clin Invest 1997). Niland et al. analysed 14 patients with MS (RRMS or PPMS or SPMS) and eight patients with OND for transaldolase committed blood T cells. PBMC at 2.10^5 cells per wells were prestimulated for 1 week with 5µg/ml of whole transaldolase or transaldolase peptide. Then, PBMC were tested for a 20 hours ELISPOT either with the protein or peptide. Frequency of IFNγ producing T cells was significantly increased in HLA-$A2^+$ MS patients (whole: $40.4.10^{-5}$ or peptide: $48.5.10^{-5}$) compared to HLA-$A2^+$ OND patients in response to stimulation by transaldolase (Niland et al. J Immunol 2005).

LDA and ELISPOT assays are the two most common techniques that have been used to analyse the frequency of myelin reactive cells in MS. LDA is usually based on the proliferative response to myelin of auto-reactive T cells whereas ELISPOT is based on the detection of cytokine secreting cells, usually memory cells according to test parameter or purified memory cells, upon antigen stimulation. Few report compared the two techniques. Although both techniques did not reveal frequency differences between MS and controls numbers of cells responsive to myelin were underestimated by LDA (Van der Aa et al. J Neuroimmunol 2003). Time of incubation, final concentrations of antigen differ between studies.

Another difficulty in interpreting differences of frequency is related to the different clinical status of the patient between MS and control cohorts. Indeed, MS cohorts of the different studies reported in this review are heterogeneous in term of disease forms, treatment and the size often small of cohorts particularly for comparison. Moreover, the control groups are not homogeneous, some authors using healthy volunteers, others OND patients.

Measurement of auto-reactive T cells frequency using protein transfer

Trogocytosis is an active membrane transfer of lymphocytes having engaged an immune synapse with an antigen presenting cells (APC) (Hudrisier et al. Elso Gaz 2002). Trogocytosis, which involves the transfer of plasma membrane fragments from the presenting cell to the lymphocyte, has been documented in T, B and natural killer cells, *in vivo* and *in vitro* (Huang et al. Science 1999;,Hudrisier et al. J Immunol 2001, Stinchcombe et al. Immunity 2001). Protein transfer is specifically dependant on antigen receptor signalling and might be involved in the immune response and possibly for the control of other cellular systems (Joly et al. Nat Immunol 2003).

Recently, transfer protein has been used as a global method to quantify antigen reactive T cells. This method, so-called TRAP (T cell Recognition of Antigen by Protein transfer) based on trogocytosis, has been set up in a model of induced response against CMV and proposed as a sensitive method to count antigen-reactive lymphocytes. When used with autologous APC, TRAP allow to be free from MHC restriction or of the peptide presented, whatever the proteins tested (Beadling et al. Nat Med 2006). By labelling the plasma membrane of APC with fluorescent dyes detectable by flow cytometry, it is possible to measure the number of T lymphocytes that specifically interacted with APC by quantifying T cells which acquire APC labelled membrane material. The read out is thus not restricted to one of cytokines as in

ELISPOT assay. An advantage of TRAP is to provide a "global" read out which san obligatory step of T cell response. The TRAP assay provides the opportunity to test the response of T cells to all epitopes present in a whole protein processed by APC *in vitro*.

Using this novel technique, we have recently reassessed the frequency of both $CD4^+$ and $CD8^+$ T cells engaging immune synapses with autologous APC, in the presence of myelin autoantigens. Preincubated APC with whole native human myelin was used to stimulate syngenic memory $CD45RO^+$ lymphocytes overnight, and TRAP was measured in the $CD3^+$ T lymphocytes. At this stage of the study, in the presence of whole native human myelin, the frequency of $TRAP^+$ cells was 3,6±2,7% in the 13 untreated relapsing-remitting MS patients and 1,7±2,06% in the 5 HV (p <0,03). The background signal, without the autoantigen, was comparable between the two groups (1,2±.1% and 1,5±1,9% in the MS and HV; respectively). All the subjects positive for myelin by the INFγ-ELISPOT assay were also positive by the TRAP assay, but TRAP detected significantly more reactive cells than the ELISPOT assay (3,6 ± 2,7% vs 0,06 ± 0,03%, respectively, p <0,0001). Furthermore, in the presence of the human myelin, TRAP signal was positive in two MS patients and two HV who tested positive previously for myelin-derived HLA-I restricted peptides in the INFγ-ELISPOT assay. Finally, 70-80% of the $TRAP^+$ cells were also positive for CD154 (CD40L), confirming their status of activated cells susceptible to interact with APC *in vitro*.

By using two different methodological approaches: whole native human myelin as an auto-antigen and the TRAP assay to recount T cells able to engage immune synapses with APC, they have found a higher frequency of autoreactive T cells in MS patients as compared to HV. Further, these autoreactive cells were both $CD4^+$ and $CD8^+$ lymphocytes with a memory phenotype and activation markers as CD40L. These two new approaches may yield a new assessment of the actual level of autoimmunity in MS (Bahbouhi et al. 2009).

Conclusion

Frequencies of auto-reactive T cells in MS patients and HV appear to vary greatly depending on the methodology. These different studies may suggest that the peripheral autoreactive T cells in MS patients have qualitative differences rather than being more numerous in comparison to HV. However, new methods that take into account a more global read out of immune activation than the ELISPOT (based on a few cytokine releases) or the proliferation assays are needed.

Legends

Table 1: Auto-reactive T cell frequencies in peripheral blood from MS patients (NS: No significant difference; ND: No determinated)

Figure 1: Trogocytosis between myelin loading monocytes and autologous lymphocytes T.

1. Purified monocytes were incubated with myelin antigens. Then, they were labelled with antibodies conjugated with fluorochrome. **2.** Memory T lymphocytes were mixed with labelled monocytes. **3.** At the end of the incubation, myelin loading monocytes had exchange labelled plasma membrane proteins with auto-reactive T lymphocytes. These lymphocytes were analysed for the acquisition of monocyte-fluorescence.

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Table 1

	References	Similar auto-reactive T cell frequency	Higher auto-reactive T cell frequency in MS	*p*
LDA	Chou et al. 1992		whole MBP	< 0.01
		$PLP_{139-151}$		NS
	Jingwu et al. 1992	whole MBP		NS
	Zhang et al. 1994	whole MBP		NS
		whole PLP		NS
	Pender et al. 2000	whole MBP		NS
		MBP_{82-100}		0.39
		$PLP_{184-199}$		0.084
			PLP_{41-58}	0.019
			$PLP_{190-209}$	0.003
	Tejada-Simon et al. 2001	whole MBP		NS
		synthetic MBP peptides		ND
		synthetic PLP peptides		NS
	Van der Aa et al. 2003	MOG_{1-22} + MOG_{34-56} + MOG_{64-86} + MOG_{74-96}		NS
IFNγ ELISPOT	Olsson et al. 1990		whole MBP	< 0.001
			$MPB_{132-150}$	< 0.001
			$MBP_{174-191}$	< 0.001
			whole PLP	< 0.001
	Sun et al. 1991		whole MOG	< 0.05
	Wallström et al. 1998		MBP_{80-102}	< 0.01
			synthetic MOG peptides	< 0.05
	Pelfrey et al. 2000		synthetic PLP peptides	0.022
	Hellings et al. 2001	whole MBP		NS
		whole MOG		NS
		synthetic myelin peptides		NS
	Andersson et al. 2002		whole MAG	< 0.01
			whole MBP	< 0.05
		MAG_{20-40}		NS
			MAG_{41-63}	< 0.05
		$MAG_{241-260}$		NS
			$MAG_{596-612}$	< 0.05
		$MAG_{609-626}$		NS
	Van der Aa et al. 2003	whole MOG		NS
	Niland et al. 2005		whole transaldolase	$2.3.10^{-8}$
			transaldolase peptide	$2.4.10^{-6}$
	Ratts et al. 2006		MBP_{83-106} + $MBP_{111-129}$ + $MBP_{143-171}$	ND
	Berthelot et al. 2008	synthetic myelin peptides		NS

Figure 1

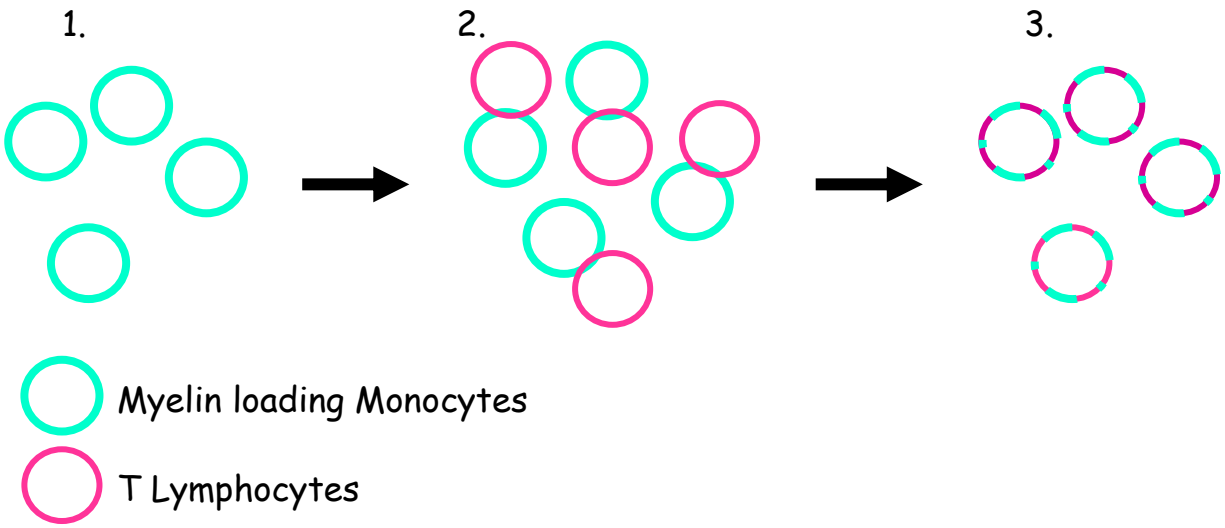

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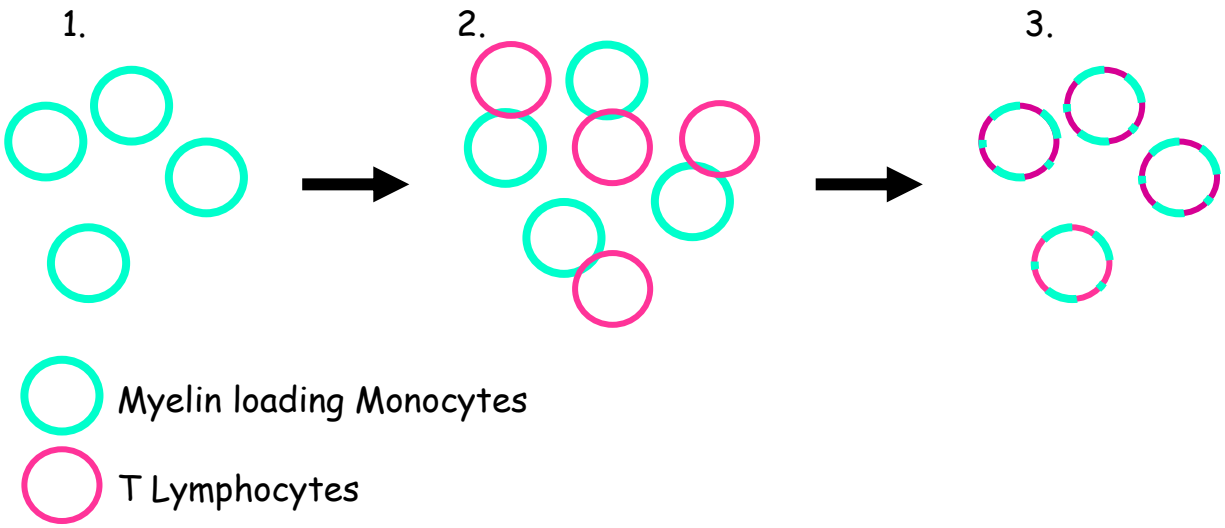
Figure 1



ARTICLE II : Les patients atteints de Sclérose en Plaques de forme rémittente ont une fonction régulatrice T normale quand les cellules exprimant la chaine α du récepteur à l'IL7 sont exclues de l'analyse.

Par : Pettré S*, Michel L*, Berthelot L*, Wiertlewski S, Lefrère F, Braudeau C, Brouard S*, Soulillou JP*, Laplaud DA*

(*les auteurs ont contribués de manière égale à ce travail)

Publié dans : Journal of Clinical Investigation. 2008.118(10) : 3411-9

Research article

Patients with relapsing-remitting multiple sclerosis have normal Treg function when cells expressing IL-7 receptor α-chain are excluded from the analysis

Laure Michel,[1,2] Laureline Berthelot,[1] Ségolène Pettré,[1] Sandrine Wiertlewski,[2,3] Fabienne Lefrère,[3] Cécile Braudeau,[1] Sophie Brouard,[1] Jean-Paul Soulillou,[1] and David-Axel Laplaud[1,2,3]

[1]INSERM U643, CHU Nantes, Institut de Transplantation et de Recherche en Transplantation (ITERT), and Faculté de Médecine, Université de Nantes, Nantes, France. [2]Service de Neurologie and [3]INSERM CIC004, CHU Nantes, Nantes, France.

Multiple sclerosis (MS) is a chronic inflammatory disease that results in demyelination in the central nervous system, and a defect in the regulatory function of CD4$^+$CD25^{high} T cells has been implicated in the pathogenesis of the disease. Here, we reanalyzed the function of this T cell subset in patients with MS, but we depleted cells expressing IL-7 receptor α-chain (CD127), a marker recently described as present on activated T cells but not Tregs. Similar to other studies, we observed a marked defect in the suppressive function of unseparated CD4$^+$CD25^{high} T cells isolated from MS patients. However, when CD127^{high} cells were removed from the CD4$^+$CD25^{high} population, patient and control cells inhibited T cell proliferation and cytokine production equally. Likewise, when the CD25 gate used to sort the cells was stringent enough to eliminate CD127^{high} cells, CD4$^+$CD25^{high} T cells from patients with MS and healthy individuals had similar regulatory function. Additional analysis indicated that the CD127^{high} cells within the CD4$^+$CD25^{high} T cell population from patients with MS appeared more proliferative and secreted more IFN-γ and IL-2 than the same cells from healthy individuals. Taken together, we conclude that CD4$^+$CD25^{high}CD127^{low} Tregs from MS patients and healthy individuals exhibit similar suppressive functions. The decreased inhibitory function of unfractioned CD4$^+$CD25^{high} cells previously observed might be due to abnormal activation of CD127^{high} T cells in patients with MS.

Introduction

In the adaptive immune system, the balance between the efficient recognition of pathogens and the control of autoimmune diseases is assumed by deletion of autoreactive clones and mechanisms of peripheral tolerance in which Tregs have a key role (1, 2). Such a role for Tregs was first described by Sakaguchi and colleagues, opening the way for the description of different types of Tregs (3, 4). The same group identified the transcription factor FOXP3 as the hallmark of regulatory function (5–7). However, FOXP3 cannot be used to isolate living Tregs because of its intracellular expression. In addition, FOXP3 can also be expressed by activated cells (8–10). Natural Tregs also express IL-2 receptor α-chain (IL-2Rα chain, also known as CD25), a cell surface marker commonly used to distinguish among regulatory (CD25^{high}), activated (CD25^{int}), and naive (CD25^{low}) T cells (11, 12) in humans. However, despite CD25 being a useful marker, the level of CD25 expression alone does not enable a precise estimation of the content of Tregs within a biological sample. Recently, Seddiki et al. (13) and Liu et al. (14) showed that, in humans, low expression of IL-7Rα chain (CD127) combined with high expression of CD25 enables better isolation and purification of Treg populations among CD4$^+$CD25$^+$ T cells. In functional assays, CD4$^+$CD25^{high}CD127^{low} T cells are highly suppressive. Furthermore, expression of CD127 negatively correlates with FOXP3 content, since FOXP3 interacts with the CD127 promoter, contributing to the low expression of CD127 in CD4$^+$CD25^{high} Tregs (14).

MS is a chronic inflammatory and demyelinating disease of the central nervous system. This disorder is thought to be initiated by autoreactive T cells recognizing peptides from myelin sheath proteins (15, 16). However, there is no compelling evidence that the frequency of autoreactive cells is increased in MS versus age-matched controls (17). In an initial study, the frequency of CD4$^+$CD25^{high} T cells was found to be normal, but the authors did not assess functional suppression (18). Several studies have sought to prove the hypothesis of a reduced suppressive function of this T cell subset in MS (19–21). Viglietta et al. (21) reported a decrease in the regulatory function of CD4$^+$CD25^{high} T cells from the peripheral blood of patients with relapsing-remitting MS (RR-MS) compared with healthy individuals (HIs). In addition, the levels of FOXP3 have also been reported as decreased, both at the single cell level and in the CD4$^+$CD25$^+$ population (22, 23). Hence, a defect in the control of the in vitro proliferative response of MS patient CD4$^+$ T cells to myelin proteins has also been reported (19, 20). However, in all of these studies, a single-step CD25 enrichment protocol was used to isolate the T cell populations tested in a coculture system in which the regulatory potency assessment was based on the inhibition of CD4$^+$CD25$^-$ cell growth following polyclonal stimulation.

In this study, we took advantage of new CD4$^+$CD25^{high} markers to revisit CD4 T cell regulatory function in MS patients. For this purpose, we used CD127-depleted cells to more precisely characterize the regulatory properties of CD4$^+$CD25^{high} T cells from MS

Nonstandard abbreviations used: HI, healthy individual; RR-MS, relapsing-remitting MS.

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

Citation for this article: *J. Clin. Invest.* **118**:3411–3419 (2008). doi:10.1172/JCI35365.

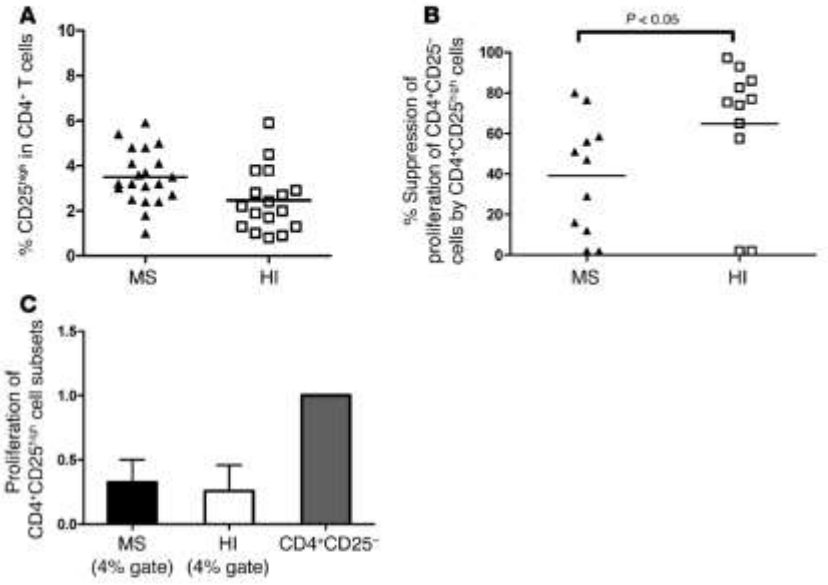


Figure 1

Frequency, proliferation, and suppressive activity of the top 4% of sorted CD4+CD25^{high} T cells from MS patients and HIs. (**A**) Comparison of the percentage of CD4+CD25^{high} T cells from MS patients and controls. The cut-off for high-staining CD25 was placed at 6 × 10^2 of mean fluorescence intensity. No statistical difference can be shown between the groups (patients, n = 21; HI, n = 17). (**B**) Regulatory properties of CD4+CD25^{high} cells were examined in 11 untreated patients with RR-MS and 11 healthy controls. Cocultures of CD4+CD25$^-$ and CD4+CD25^{high} cells were performed at a 1:1 ratio and under anti-CD3 stimulation. Proliferation was measured by incorporation of ^{3}H-thymidine after 5 days of incubation. The percentage of suppression of responding cell proliferation (CD4+CD25$^-$) by CD4+CD25^{high} cells was determined as 1 – (proliferation of coculture / proliferation of responder population alone) × 100, where proliferation is expressed as cpm. CD4+CD25^{high} T cells from MS patients exhibited less suppressive activity when the gate for sorting was positioned as shown in Supplemental Figure 1 ($P < 0.05$, Mann-Whitney U test). Mean values in **A** and **B** are indicated by horizontal lines. (**C**) Comparison of the proliferation of CD4+CD25^{high} T cells in MS patients and HIs relative to the proliferation of the CD4+CD25$^-$ T cell subset. Proliferation of CD4+CD25^{high} cells was not significantly different between patients (n = 11) and HIs (n = 11). When the top 4% of CD4+CD25^{high} T cells were sorted, the cells were not fully anergic, suggesting the presence of activated T cells. Data represent mean ± SD.

patients compared with HIs. Based on a study of 34 patients and 25 healthy volunteers, we now report that the regulatory function of the CD4+CD25^{high}CD127^{low} T cells is similar in MS patients and HIs. We also show that the isolated CD4+CD25^{high}CD127^{high} T cell subset of MS patients may proliferate more and produce more mitogenic lymphokines in coculture assays, resulting in an apparent peripheral defect of CD4+CD25^{high} regulation in MS patients.

Results

Suppressive function of the top 4% of sorted CD4+CD25^{high} T cells from MS patients. We first studied the frequency of CD4+CD25^{high} T cells in MS patients and HIs using flow cytometry. Figure 1A shows that the mean frequency of CD4+CD25^{high} cells within the CD4+ T cell population was 2.5% ± 1.4% for HIs and 3.5% ± 1.2% for MS patients (P = NS), confirming that there is no difference in the frequency of CD4+CD25^{high} T cells between MS patients and HIs.

Next, in order to confirm the reported suppressive defect of the CD4+CD25^{high} T cell population, we sorted these cells from the peripheral blood of patients (n = 11) and HIs (n = 11) using a high-speed FACS sorter and compared their inhibitory properties. The gate was set up to include 4% of the CD4+ T cells (based on a reference umbilical cord population) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI35365DS1). A regulatory function assay was then performed based on the capacity of the cells to inhibit polyclonal proliferation of autologous CD4+CD25$^-$ cells. Figure 1B shows the results obtained under these conditions, when CD4+CD25^{high} T cells sorted from MS patients were added to the coculture system, indicating an apparent defective regulatory function as compared with HIs (39.0% ± 28.4% suppression in MS patients versus 64.7% ± 33% in HIs; P = 0.048, Mann-Whitney U test). However, Figure 1C shows that under these sorting conditions, the isolated CD4+CD25^{high} cells of both MS patients and normal individuals were not fully anergic, with a proliferation of 32.7% in MS patients and 26.3% in HIs (the proliferation of CD4+CD25$^-$ T cells from each group was used as the reference). The fact that the cells were not fully anergic under this gating condition led us to explore the possibility that contaminating cells were interfering with the proliferation assay. To do this, Tregs were sorted either by additionally taking into account their expression of CD127 or by using more stringent gating to select cells at the extreme end of CD25 positivity.

Similar suppressive activity of the top 2% of sorted CD4+CD25^{high} T cells in MS patients and HIs. Because contrasting expression of CD25 and CD127 markers has been reported (13, 14), we sorted the CD4+CD25^{high} T cells using a more stringent threshold for CD25 expression (CD25^{high}; less than 2% of the CD4+ T cells) to compare their inhibitory properties in patients and controls. Figure 2A shows that the gating stringency was indeed associated with a disappearance of CD127^{high} T cells among the purified CD4+CD25^{high} T cells. Figure 2B shows that when sorting the top 2%, the suppression of CD4+CD25$^-$ T cell proliferation was roughly similar between the 2 groups (73.3% ± 17.8% in MS patients, n = 12, and 76.5% ± 20.5% in healthy controls, n = 10). Furthermore, in this sorting condition, the purified CD4+CD25^{high} T cells were fully anergic (Figure 2C). Finally, we analyzed the intracellular FOXP3 expression in this CD4+CD25^{high} subset. No significant difference was noted between MS patients (n = 10) and HIs (n = 9), with a mean expression of 83.2% ± 9.5% in patients and 78.5% ± 6% in HIs when gating only on the top 2% of CD4+CD25^{high} cells (Figure 2D).

Thus, these experiments further support the idea that in MS patients, the presence of contaminating CD127^{high} cells within the CD4+CD25^{high} T cell subset may explain an apparent alteration in their regulatory function.

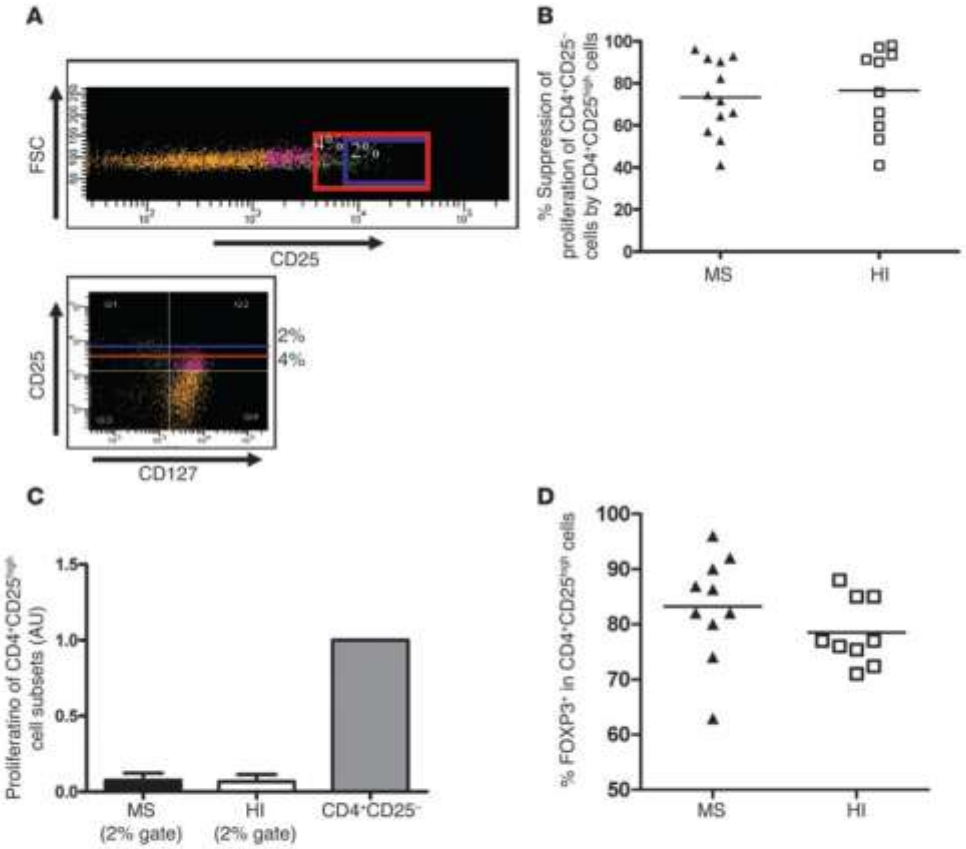


Figure 2

Suppressive activity and proliferation of the top 2% of sorted $CD4^+CD25^{high}$ cells from MS patients and healthy controls. (**A**) $CD4^+$ lymphocytes obtained from the peripheral blood of MS patients and healthy controls were stained with Pe-Cy7–conjugated anti-CD3, FITC-conjugated anti-CD8, Alexa Fluor 647–conjugated anti-CD25, and PE-conjugated anti-CD127. Sorting was performed on the $CD4^+CD25^{high}CD127^{low}$ and $CD4^+CD25^{high}CD127^{high}$ populations. Sorting was also performed on the $CD4^+CD25^{high}$ population with 2 different gates. In the example provided for 1 MS patient, $CD4^+CD25^+$ T cells appear in orange, $CD4^+CD25^+CD127^{low}$ T cells appear in green, and $CD127^{high}$ cells appear in violet. The presence of $CD127^{high}$ activated cells can be observed in the $CD4^+CD25^{high}$ sorted T cells when the gate is not stringent enough (4% gating stringency, area above the red line), while in the case of a 2% gating stringency (area above the blue line), only a few $CD127^{high}$ cells remain within the $CD4^+CD25^{high}$ T cell subset. FSC, forward scatter. (**B**) $CD4^+CD25^-$ responder cells were stimulated with anti-CD3 antibody (0.1 μg/ml) in coculture with the top 2% of $CD4^+CD25^{high}$ sorted cells. Data are the mean of duplicate wells. Regulatory properties of $CD4^+CD25^{high}$ cells are comparable in both HIs (n = 10) and patients (n = 12). (**C**) Comparison of the proliferation of the top 2% of sorted $CD4^+CD25^{high}$ T cells in MS patients and HIs relative to the proliferation of the $CD4^+CD25^-$ T cell subset. The proliferation of $CD4^+CD25^{high}$ T cells was minimal and almost null in both patients and controls. Data are mean ± SD. (**D**) Intracellular FOXP3 staining was performed on the top 2% of sorted $CD4^+CD25^{high}$ T cells in 10 patients and 9 HIs. In **B** and **D**, the mean values for each group are indicated by horizontal lines.

Similar frequency and suppressive activity of $CD4^+CD25^{high}CD127^{low}$ cells in MS patients and HIs. Because CD25 is not a specific marker of Tregs (activated and memory T cells can also express CD25), we used expression of CD127 to discriminate activated and memory T cells ($CD4^+CD25^{high}CD127^{high}$ T cells) from regulatory cells ($CD4^+CD25^{high}CD127^{low}$ T cells) among the $CD4^+CD25^{high}$ subset (13). We compared the regulatory properties of CD127-depleted $CD25^{high}$ T cells from MS patients and age-matched individuals. T cells were thus labeled with anti-CD127, and the $CD4^+CD25^{high}CD127^{low}$ cells were sorted (Figure 2A). Figure 3A shows that there was no difference in the percentage of $CD25^{high}CD127^{low}$ cells within the $CD4^+$ T cell populations of MS patients compared with HIs, with a mean frequency of 6.8% ± 1.8% for MS patients and 6.2% ± 1.6% for HIs (P = NS, Mann-Whitney U test). The cells were then tested for their ability to suppress the proliferation of $CD4^+CD25^-$ cells in response to irradiated autologous PBMC and anti-CD3 activation over 5 days. Under these conditions, no significant difference in suppression of autologous $CD4^+CD25^-$ proliferation was observed between the $CD4^+CD25^{high}CD127^{low}$ cells of RR-MS patients (n = 25) and those of healthy controls (n = 23) (Figure 3B). Proliferation of $CD4^+CD25^-$ T cells was inhibited by a mean of 75.3% ± 20.9% in MS patients and 78.3% ± 15.0% in age-matched HIs. Thus, an improvement in the suppressive function of this cell subset was observed in both MS patients and HIs, as compared with the use of CD25 alone as a marker of Tregs. The proliferation of these cell subsets was then tested and compared with

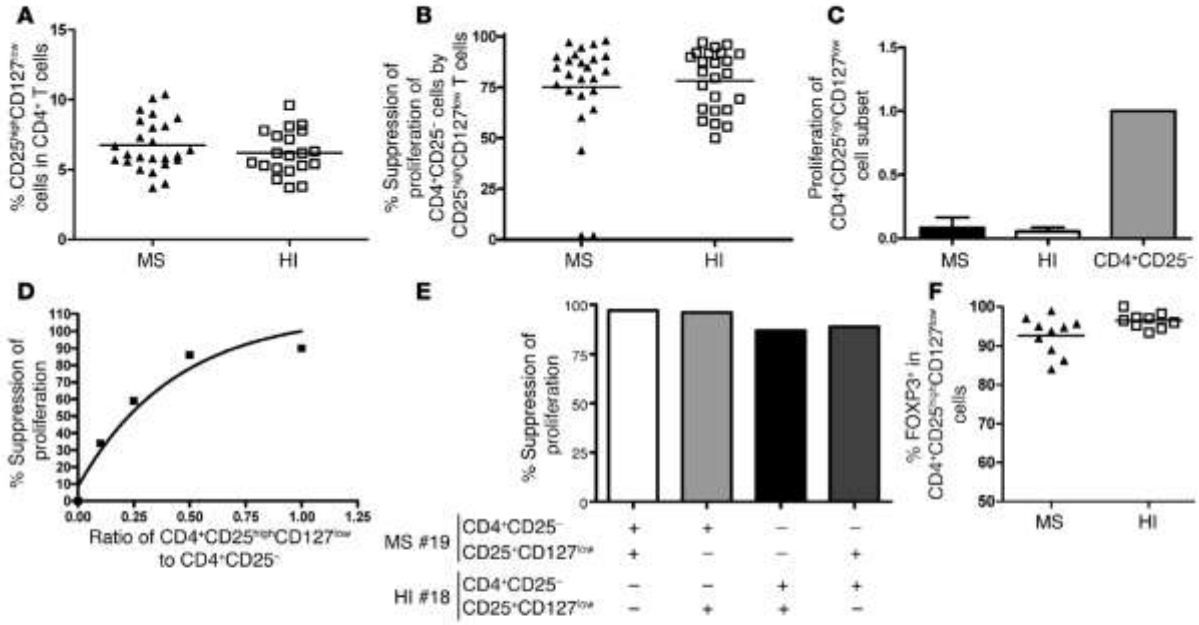


Figure 3

Frequency, proliferation, and suppressive activity of CD4+CD25^{high}CD127^{low} cells from MS patients and healthy controls. (**A**) Percentages of CD25^{high}CD127^{low} cells within the total CD4+ T cell population were determined by flow cytometry analysis of PBMCs. No statistical difference was found between the groups (25 patients and 20 HIs). (**B**) Regulatory properties of CD4+CD25^{high}CD127^{low} cells were comparable in both HIs (n = 24) and patients (n = 25). (**C**) Comparison of the proliferation of CD4+CD25^{high}CD127^{low} T cells in MS patients and HIs normalized against the proliferation of the CD4+CD25- T cell subset. The proliferation of CD4+CD25^{high}CD127^{low} T cells was minimal and approaching 0 in both patients and controls. (**D**) Example in 1 patient of variations of the suppression of proliferation by CD4+CD25^{high}CD127^{low} cells in the cocultures at varying ratios of CD4+CD25^{high}CD127^{low} to CD4+CD25-. Decreasing the number of CD4+CD25^{high}CD127^{low} T cells resulted in less suppressor activity. (**E**) CD4+CD25^{high}CD127^{low} T cells from 1 patient with MS inhibited proliferation of responder T cells isolated from either the autologous individual or the healthy control. Conversely, Tregs from 1 HI were cocultured with responder T cells from the same subject or those from the MS patient. MS#19, MS patient 19; HI#18, HI subject 18. (**F**) Intracellular FOXP3 staining was performed on CD4+CD25^{high}CD127^{low} T cells in 10 patients and 9 HIs. In **A**, **B**, and **F**, mean values are indicated by horizontal lines.

their CD4+CD25- counterparts. Figure 3C shows that in these new conditions, the basal proliferation of the CD4+CD25^{high}CD127^{low} T cells was very low or absent (a representative example is shown in Supplemental Figure 2) and clearly differed from that of the top 4% of sorted CD4+CD25^{high} cells. In addition, this lack of proliferation was totally reversed by the addition of IL-2 (data not shown), suggesting a state of anergy. The suppressive activity was dose dependent in that dilution of the CD4+CD25^{high}CD127^{low} T cells (over a range of 1:1 to 1:10) decreased their suppressive function (Figure 3D). Finally, CD4+CD25^{high}CD127^{low} Tregs from MS patients were able to suppress effector CD4+CD25- cells from HIs to the same extent as the CD4+CD25^{high}CD127^{low} cells from HIs on CD4+CD25- T cells from MS patients (Figure 3E).

Finally, we analyzed intracellular FOXP3 expression in the CD4+CD25^{high}CD127^{low} cells. No significant difference was noted between patients (n = 10) and HIs (n = 9), with a mean expression of 92.6% ± 4.8% in MS patients and 96.4% ± 2% in HIs (Figure 3F).

Further, when considering the production of IFN-γ or IL-2 by CD4+CD25- responder cells after 3 days of polyclonal stimulation in the presence of irradiated autologous PBMCs, the same suppressive capacity of CD4+CD25^{high}CD127^{low} T cells was observed in patients and controls (n = 4; Figure 4). An 84% and an 83% reduction in IFN-γ production was observed in MS patients and HIs, respectively. Similarly, the reduction in IL-2 production was 88% and 87% in MS patients and HIs, respectively.

Taken together, these data indicate that CD4+CD25^{high}CD127^{low} Tregs from MS patients do not display a defect in their suppressive properties and that activated CD127^{high} cells within the CD4+CD25^{high} T cell population interfere with the proliferation assay.

Significantly higher proliferation of activated CD4+CD25^{high}CD127^{high} T cells in MS patients versus HIs. In order to know whether the apparently impaired suppressive function of the CD4+CD25^{high} cell subset (not depleted of CD127^{high} cells) from MS patients (Figure 1B) could be due to the presence of activated CD127^{high} cells, we first compared the frequency of CD4+CD25^{high}CD127^{high} cells between MS patients and HIs. No difference was observed between the 2 groups (9.2% ± 4.8% in MS patients and 8.2% ± 4.6% in controls) (Figure 5A). Next, the proliferation of the CD4+CD25^{high}CD127^{high} T cells of both MS patients (n = 20) and HIs (n = 20) was compared with that of CD4+CD25^{high}CD127^{low} T cells. The data were normalized against the proliferation of CD4+CD25- T cells in order to compare individuals, as the absolute values of proliferation under CD3 stimulation can be variable. This type of presentation has been reported before (14). The raw data expressed in cpm are provided in Supplemental Figures 3–5. Figure 5B shows no difference in proliferation of the CD4+CD25^{high}CD127^{low} subset between MS patients and HIs, with a

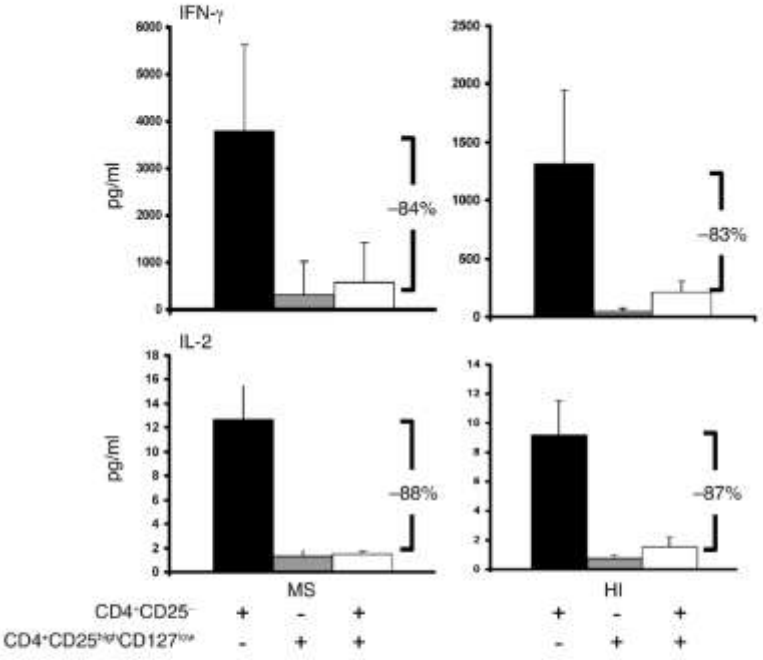


Figure 4

Suppression of cytokine production by CD4+CD25^{high}CD127^{low} T cells in patients and controls. Cytokines (IL-2 and IFN-γ) were measured in supernatants taken from each well 3 days after the initiation of coculture (CD4+CD25– T cells/CD4+CD25^{high}CD127^{low} T cells at a 1:1 ratio) using a multiplex fluorescent bead immunoassay. Three days after initiation of the coculture, the same percentage suppression of IFN-γ (top panels) and IL-2 (bottom panels) production by CD4+CD25^{high}CD127^{low} was observed in the cocultures from 4 patients and 4 HIs. Data are mean ± SD.

very low proliferation in both groups. On the contrary, the proliferation of CD4+CD25^{high}CD127^{high} T cells in MS patients was 1.9-fold higher (4.2 ± 3.9 relative to the proliferation of CD4+CD25– T cells) than in controls (2.2 ± 2.8; $P = 0.017$, Mann-Whitney U test; Figure 5B). This observation further supports the possibility that the CD127^{high} T cells within the CD4+CD25^{high} subset might interfere in the apparent defective regulation of CD4+CD25^{high} T cells in MS patients. This CD127^{high} contamination can be prevented using a selective sorting of CD127^{low} cells or more stringent gating sorting conditions of CD4+CD25^{high} cells (Figure 2A). The contribution of these activated cells to the apparent alteration in regulatory properties of the CD4+CD25^{high} cells was also demonstrated by the significant inverse correlation observed between the suppressive function of CD4+CD25^{high} cells and the presence of CD127^{high} T cells within this cell subset (Pearson correlation coefficient of $r = -0.51$; $P = 0.006$, linear regression test; Figure 5C). Finally, we analyzed intracellular FOXP3 expression in the CD4+CD25^{high}CD127^{high} cells. No significant difference was noted between MS patients ($n = 10$) and HIs ($n = 9$), with a mean expression of 17.7% ± 10.8% in patients and 16.8% ± 8.6% in controls (Figure 5D).

Cytokine secretion by the CD4+CD25^{high}CD127^{high}, CD4+CD25–, and CD4+CD25^{high}CD127^{low} populations in MS patients and HIs. The cytokines TNF-α, IFN-γ, and IL-2 were also measured in the supernatants of the proliferation assays from the CD4+CD25^{high}CD127^{high}, CD4+CD25^{high}CD127^{low}, and CD4+CD25– cell populations and their cocultures after 24 hours of proliferation. No significant difference was observed between patients ($n = 10$) and HIs ($n = 10$) for the 3 cell subsets (data not shown) after 24 hours. However, CD4+CD25^{high}CD127^{high} cells from MS patients secreted significantly higher levels of cytokines than CD4+CD25^{high}CD127^{low} and CD4+CD25– T cells (Figure 6A; $P = 0.011$, $P = 0.0001$, and $P = 0.0005$ when compared with CD4+CD25– for IFN-γ, TNF-α, and IL-2, respectively; and $P = 0.0031$, $P = 0.0039$, and $P < 0.0001$ when compared with CD4+CD25^{high}CD127^{low} for IFN-γ, TNF-α, and IL-2, respectively; Mann-Whitney U test), suggesting that these cells had a proinflammatory potential. In addition, CD4+CD25^{high}CD127^{low} T cells did not produce IL-2 or IFN-γ when stimulated by anti-CD3, as would be expected from their regulatory phenotype.

Hence, to study their production of cytokines, CD4+CD25^{high}CD127^{high} cells from MS patients ($n = 4$) and HIs ($n = 4$) were cultured under CD3 polyclonal stimulation in the presence of irradiated PBMCs for 3 days. IFN-γ and TNF-α were subsequently measured using a multiplex fluorescent bead assay. As shown in Figure 6B, 6,580 ± 2,093 pg/ml of IFN-γ was detected in the cultures of MS patient cells as compared with 2,567 ± 1,092 pg/ml for HIs. Concerning TNF-α production, the cells from MS patients produced 3,107 ± 346 pg/ml compared with 1,571 ± 629 pg/ml for HIs. IL-2 production was also increased in MS patients as compared with HIs (14 ± 3 versus 6 ± 2 pg/ml, respectively). The CD4+CD25^{high}CD127^{high} cells from MS patients produced nearly 2-fold more proinflammatory cytokines compared with the same cells from HIs. While statistical significance was not achieved, a trend toward significance was observed (IFN-γ, $P = 0.06$, Mann-Whitney U test; TNF-α, $P = 0.09$; IL-2, $P = 0.1$) despite the very low number of patients and controls studied ($n = 4$).

FOXP3 content of CD4+CD25^{high} and CD4+CD25^{high}CD127^{low} populations in MS patients and HIs. We compared intracellular FOXP3 expression between CD4+CD25^{high} and CD4+CD25^{high}CD127^{low} cell subsets in patients ($n = 10$) and controls ($n = 9$). The use of anti-CD127 antibody provided a purified CD4+CD25+CD127^{low} population expressing more FOXP3 in MS patients and HIs (92.6% ± 4.8% and 96.4% ± 2%, respectively), as compared with 83.2% ± 9.5% in patients and 78.5% ± 6% in HIs when gating only on the top 2% of the CD4+CD25^{high} population ($P < 0.0001$, Mann-Whitney U test; Figure 7).

Discussion

CD4+CD25+FOXP3+ regulatory T lymphocytes have been shown to play a key role in controlling potentially harmful responses to self-determinants in mice (24) and humans (1). Recently, a possible defect in the function of CD4+CD25^{high} cells has been reported in patients with RR-MS (1, 19–21). In this paper, we revisited this observation by analyzing the properties of subpopulations of CD4+CD25^{high} T cells, taking into consideration the recent findings that activated/memory cells expressing CD127 are also present

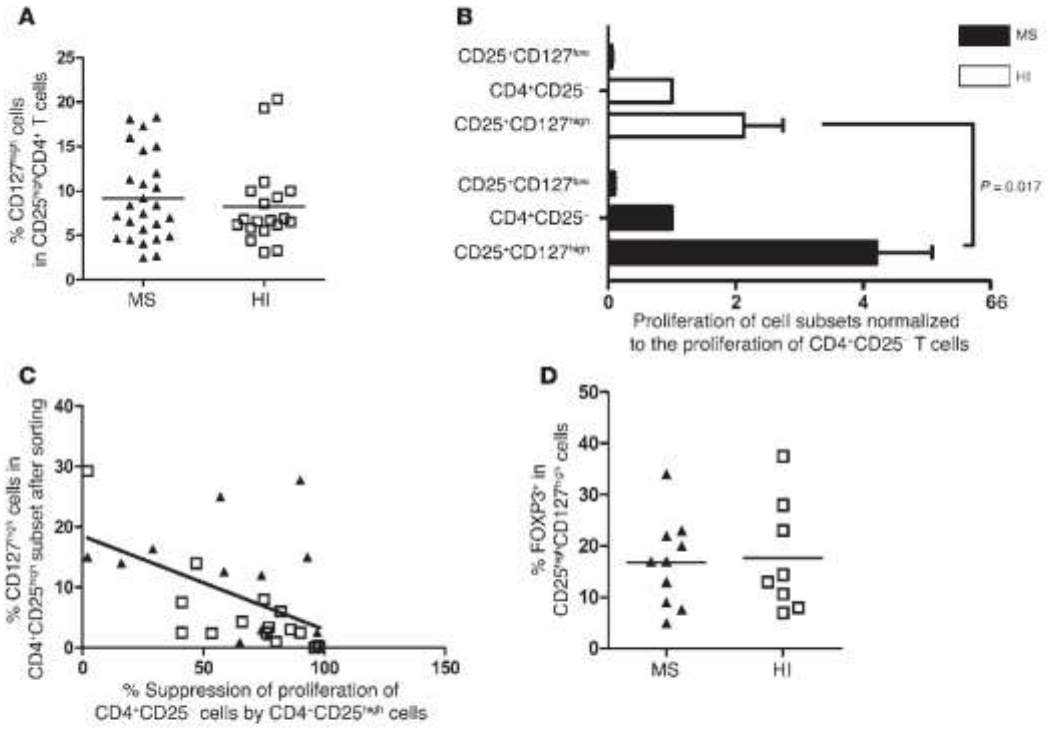


Figure 5

Comparison of the proliferation of CD4+CD25^{high}CD127^{low} T cells and CD4+CD25^{high}CD127^{high} T cells. (**A**) CD4+ lymphocytes obtained from the peripheral blood of MS patients and healthy controls were stained with Pe-Cy7–conjugated anti-CD3, FITC-conjugated anti-CD8, Alexa Fluor 647–conjugated anti-CD25, and PE-conjugated anti-CD127. No statistically significant difference was observed in the frequency of CD4+CD25^{high}CD127^{high} T cells between MS patients (n = 25) and healthy controls (n = 19). Mean value is indicated for each group. (**B**) CD4+CD25^{high}CD127^{low} T cells or CD4+CD25^{high}CD127^{high} T cells were cocultured with irradiated autologous PBMCs and stimulated with anti-CD3 antibody. CD25^{high}CD127^{low} cells were isolated from 25 patients and 23 HIs. CD25^{high}CD127^{high} cells were isolated from 20 patients and 20 HIs. A significant difference was observed in the proliferation of CD25^{high}CD127^{high} T cells between MS patients and HIs (P = 0.017, Mann-Whitney U test). Bar graphs indicate the mean ± SD. (**C**) Suppression of proliferation of CD4+CD25^{-} cells by CD4+CD25^{high} cells was calculated in 13 patients and 15 HIs. The percentage of CD127^{high} cells present in the sorted CD4+CD25^{high} T cells was estimated in the same manner. A correlation was found between this percentage and the regulatory properties of CD4+CD25^{high} cells with a Pearson coefficient of r = –0.50 (P = 0.006, linear regression test). Black triangles represent data obtained from MS patients, and white squares represent data from HIs. (**D**) Intracellular FOXP3 staining was performed on CD4+CD25^{high}CD127^{high} T cells in 10 patients and 9 HIs. In **B** and **D**, horizontal lines indicate the mean.

within the CD4+CD25+ T cell population and can potentially interfere in the classical functional assays for measuring CD4+CD25^{high} Treg suppressive properties (13, 14, 25). To our knowledge, this is the first study using CD127 to discriminate Tregs in MS patients and showing that this CD4+CD25^{high}CD127^{low} T cell subset actually has the same regulatory potency in patients and in age-matched control subjects. The frequency of the CD4+CD25^{high} T cells also appeared to be similar between MS patients and HIs, as reported previously (18). However, several studies have reported a defective suppressive function in CD4+CD25^{high} T cells from MS patients under polyclonal (21) or antigen-based stimulation (19, 20), suggesting that this defect might be involved in the physiopathology of MS (26, 27). At the time of these studies, CD127^{low} staining was not available for the CD4+CD25^{high} Tregs. Our investigations suggest that CD4+CD25^{high} Treg function may not be altered in MS, since CD4+CD25^{high}CD127^{low} T cells display a normal suppressive function. Rather, a discrete population of CD4+CD25^{high}CD127^{high} cells in patients is likely to interfere with the coculture assay by a trend for hyperproliferation and for producing more proinflammatory cytokines able to enhance CD25^{-} T cell proliferation. Our data shed new light on the heterogeneity of the CD4+CD25^{high} T cell population and suggest a possible role for CD4+CD25^{high}CD127^{high} cells in MS. These findings are indirectly supported by recent genomic studies in MS suggesting alterations in *IL2RA* and *IL7RA* genes (28–30).

As expected, we first confirmed a defective suppressive function of the CD4+CD25^{high} T cells in MS patients (39% in MS patients versus 69% in age-matched HIs, $P < 0.05$). However, because CD25 is not specific for Tregs but is also expressed by activated T cells (1, 31–33), it was important to take into consideration other markers that distinguish activated/memory cells not endowed with regulatory function. Another difficulty in assessing Treg function by studying CD4+CD25+ cells comes from the fact that the CD4+CD25^{high} population is difficult to distinguish from the CD4+CD25^{int} population (thought to contain activated T cells) because there is no clear and stereotyped cut-off between high and intermediate CD25 expression in humans (1, 2). In fact, our data show that changing the gat-

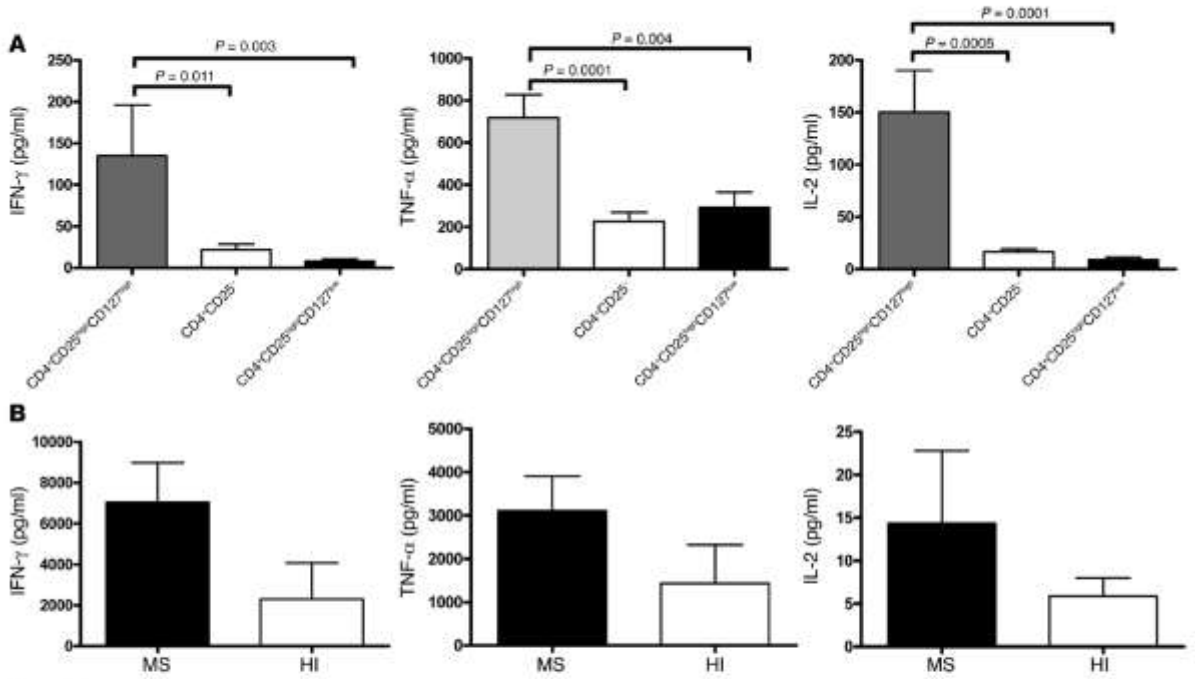


Figure 6

Cytokines secreted by the CD4+CD25^{high}CD127^{high}, CD4+CD25-, and CD4+CD25^{high}CD127^{low} populations in MS patients and HIs. (**A**) The supernatants from each well of the proliferation assays were removed 24 hours after the beginning of the incubation. The cytokines TNF-α, IFN-γ, and IL-2 were measured from 10 MS patients in the following 3 cell subsets: CD4+CD25^{high}CD127^{high}, CD4+CD25-, and CD4+CD25^{high}CD127^{low}. Mann-Whitney *U* tests were performed to compare the 3 cell subsets. (**B**) Comparison of cytokine production by CD4+CD25^{high}CD127^{high} T cells under CD3 polyclonal stimulation between MS patients (n = 4) and HIs (n = 4). Supernatants were removed 3 days after the beginning of the incubation. The results are the mean ± SD from 4 patients and 4 healthy controls.

ing stringency when sorting these cells dramatically affects the suppressive capacity of the CD4+CD25^{high} T cells in the proliferation assay, probably by introducing activated T cells in the coculture assay. Using too low a stringency sorting threshold may thus result in an apparent defect in regulatory function in the CD4+CD25^{high} T cell subset in MS patients as in HIs, but not in the same proportion (see Figure 1C and Figure 2B for the difference obtained in percentage of suppression when using a 2% or a 4% gating stringency both in patients or controls). Indeed, the same cells obtained from MS patients using a more stringent sorting threshold did not present abnormal regulatory function. It is thus difficult to compare the results obtained in different studies when Tregs are purified based solely on their expression of CD4 and CD25.

Recently, CD127 has been shown to be negatively correlated with FOXP3 expression in CD4+CD25^{high} T cells, enabling improved sorting of viable Tregs (13, 14). Thus, we used anti-CD127 to discriminate the properties of CD127^{high}-depleted cells among the CD4+CD25^{high} T cell subset in MS patients and HIs and found that the suppressive function of the CD4+CD25^{high}CD127^{low} cells was similar between the 2 groups. Furthermore, 94% of the CD4+CD25^{high}CD127^{low} T cells expressed FOXP3 protein compared with 82% in CD4+CD25^{high} cells ($P < 0.0001$, Mann-Whitney *U* test; Figure 7), indicating that the cells sorted using the CD127 marker are a purer population than those obtained using only CD25. The comparable high FOXP3+ score in the CD127-depleted CD25^{high} cells and the comparable regulatory function observed in MS patients and HIs also suggests that MS patients have no defect in their Tregs. Hence, when comparing the production of IFN-γ by CD4+CD25- cells under polyclonal stimulation in the presence or absence of CD4+CD25^{high}CD127^{low} cells, a similar suppressive property was found for this Treg subset, confirming the data observed with the proliferation assays. This observation of a similar regulatory function between MS patients and HIs suggests that contaminating CD127^{high} T cells interfere in the suppression assays. The presence of activated CD127^{high} T cells within the CD4+CD25^{high} T cells of patients with MS could explain the discrepancy observed between the 2 groups despite a similar frequency of CD4+CD25^{high}CD127^{high} cells in MS patients and HIs. An enhanced proliferation of this T cell subset was observed compared with the proliferation of CD25- cells, suggesting that this subpopulation may exhibit an abnormal activation state in MS patients. This would at least partly explain the defect in suppressive function observed with the CD4+CD25^{high} T cells. In addition, in our experiments the CD4+CD25^{high}CD127^{high} T cells exhibited a proinflammatory profile as measured by the levels of IL-2, IFN-γ, and TNF-α produced in the supernatants of the proliferation assays as compared with the responder CD4+CD25- cell subset. Hence, when compared with HIs, this subset of cells from MS patients seemed to produce more proinflammatory cytokines (IFN-γ, TNF-α, and IL-2). The extent to which this increased production of T cell mitogenic cytokines (such as IL-2 and IFN-γ; ref. 34) may play a role in the proliferation of the CD25- T cell subset within the coculture system

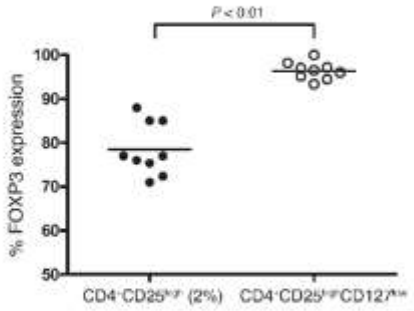


Figure 7
Comparison of FOXP3 expression in CD4+CD25^{high} T cells and CD4+CD25^{high}CD127^{low} cells. Intracellular FOXP3 staining was performed in 9 HIs. A significant difference was observed between the top 2% of sorted CD4+CD25^{high} and CD4+CD25^{high}CD127^{low} T cells ($P < 0.0001$, Mann-Whitney U test). The horizontal lines indicate the mean values for each group.

requires further investigation. Recently, CD4+CD25^{high}CD127^{high} cells have also been suggested to play a role in allograft rejection in humans. Grafts infiltrated by CD4+CD25^{high}CD127^{high} T cells contained allospecific CD4+ T cells and also secreted effector cytokines such as TNF-α and IFN-γ (35). Altogether, it is possible that this CD4+CD25^{high}CD127^{high} T cell subset plays a role in MS or other inflammatory processes. Our observations also indirectly corroborate the results of 3 recent studies on risk alleles associated with MS, which revealed an alteration of 2 genes, *IL2RA* and *IL7RA* (28–30). Recently, Venken and colleagues also used CD127 to discriminate memory and naive Tregs in MS patients (36). They still found a defective suppressive function of the Tregs in MS patients, even after a sorting strategy based on CD127^{low} cells. However, they also used the counterpart CD4+CD25-CD127^{high} cells as responders. Our data suggest that these cells are different between MS patients and HIs, since we show here that CD4+CD25^{high}CD127^{high} cells may be hyperproliferative and produce more proinflammatory cytokines, also resulting in an apparent defect in regulation.

To conclude, using 2 different parameters (suppression of proliferation and cytokine production), our work suggests that the inhibitory function of the regulatory CD4+CD25^{high} T cell subset may not actually be altered in MS when contaminating CD127^{high} cells are removed. In addition, the counterpart CD4+CD25^{high}CD127^{high} T cell subpopulation in MS patients may proliferate more or produce more mitogenic cytokines, at least partly explaining the previous observation of a decreased suppressive property of CD4+CD25^{high} cells in MS patients. This CD4+CD25^{high}CD127^{high} T cell subset will be the subject of future investigations, since it might play a role in the inflammatory process observed in MS.

Methods

Patients. Thirty-four patients with definite MS according to the McDonald criteria (37) were enrolled in the study. All patients presented RR-MS and did not receive disease-modifying therapy (including corticosteroid boluses) for at least 3 months prior to testing. The patients were between the ages of 19 and 60 (mean, 35.9 ± 9.5 years), and their Kurtzke Expanded Disability Status Scale (EDSS) scores (38) were between 0 and 6.5 (mean, 1.75 ± 1.6). The disease duration ranged from 6 months to 17 years (mean, 4.5 ± 4.9 years). MS patients 1–25 were used for suppression and proliferation experiments concerning the different T cell subsets studied, while MS patients 25–34 were used for experiments concerning cytokine production. The characteristics of all patients are available in Supplemental Table 1.

Twenty-five HIs, matched for age with MS patients, between the ages of 21 and 60 (mean, 34.5 ± 8.7 years) and with no history of autoimmune diseases or recent infection episodes, were also enrolled in this study. All patients and HIs gave written informed consent prior to the study.

Isolation of CD4+CD25-, CD4+CD25^{high}, CD4+CD25^{high}CD127^{low}, and CD4+CD25^{high}CD127^{high} T cell populations. PBMCs were isolated from 100 ml of EDTA whole blood by density centrifugation over Ficoll-Paque (Eurobio). Freshly-isolated cells (1.5×10^8) from each experiment were incubated for 20 minutes in PBS with 30 μl FITC-conjugated anti-CD8, 30 μl PE-conjugated anti-CD127, 20 μl PE-Cy7–conjugated anti-CD3 (BD Biosciences), and 5 μl Alexa Fluor 647–conjugated anti-CD25 (anti-CD25 from Immunotech coupled to the fluorochrome using a molecular probe kit from Invitrogen). Human CD4+CD25^{high}, CD4+CD25-, CD4+CD25^{high}CD127^{low}, and CD4+CD25^{high}CD127^{high} T cells were then separated from PBMCs using a high-speed cell sorter (FACSAria; BD Biosciences). Purity was systematically greater than 98% for CD4+CD25- T cells and greater than 95% for CD4+CD25^{high}CD127^{low} cells. For sorting, parameters were set once and automatic compensations were performed using FACSDiva software (BD Biosciences). Because we performed anti-CD3 staining for the sorting procedure, we checked that this staining had no influence on further experiments. We compared the proliferation of CD4+CD25- and CD4+CD25^{high}CD127^{low} cells as well as their coculture when the sorting was performed with or without anti-CD3. We did not find any difference in terms of proliferation or suppression (data not shown).

Proliferation assay. All experiments were performed on fresh peripheral blood lymphocytes. To assess the functional activity of the different subsets of cells (CD4+CD25^{high} and CD4+CD25^{high}CD127^{low}), 2×10^4 responder cells (CD4+CD25- T cells) were cocultured for 5 days with 2×10^4 CD4+CD25^{high} cells or CD4+CD25^{high}CD127^{low} cells (ratio 1:1) and 1×10^5 autologous irradiated PBMCs in complete RPMI 1640 medium supplemented with HEPES, L-glutamine, penicillin, streptomycin, sodium pyruvate, nonessential aminoacids, and 10% human AB serum. All experiments were run in duplicate in 96-well plates bound with anti-CD3 (Orthoclone OKT3; Janssen-Cilag) at 1 μg/ml as described previously (39). Anti-CD3 antibody was used as a polyclonal T cell stimulus. Cultures were incubated at 37° C in a humidified atmosphere. After 5 days of culture, the cells were pulsed with 1 μCi per well of ^{3}H-thymidine (Amersham Biosciences) for 16 hours. The cells were then harvested and counted in a scintillation counter. ^{3}H-thymidine incorporation was measured as cpm. The percentage of suppression of the responding cell proliferation was determined as 1 – (proliferation of coculture / proliferation of responder population alone) × 100, where proliferation was expressed as cpm.

In order to compare the proliferation of each cell subpopulation and among individuals, and because of variability in proliferation from one patient to another, the proliferation of the T cell subsets (CD4+CD25^{high}, CD4+CD25^{high}CD127^{low}, and CD4+CD25^{high}CD127^{high}) was normalized to the proliferation of the CD4+CD25- subset in each patient or control. In each case, baseline proliferation in absolute cpm was checked, and several representative examples are provided. All data concerning the proliferation of each subpopulation of T cells for each patient and HI are available in Supplemental Figures 1–4.

Measurement of cytokine production. Cytokines (IL-2, TNF-α, and IFN-γ) were measured in supernatants taken from each well 24 hours after the initiation of coculture using a multiplex fluorescent bead immunoassay (Lincoplex) together with a Luminex device. To assess the suppression of IL-2 and IFN-γ production by CD4+CD25- T cells, these cytokines were measured after 3 days in the cocultured supernatants of CD4+CD25^{high}CD127^{low} and

$CD4^+CD25^-$ responder cells (ratio 1:1) and 1×10^5 autologous irradiated PBMCs under the CD3 polyclonal stimulation as described above.

For comparison of cytokine production by $CD4^+CD25^{high}CD127^{high}$ T cells from patients and controls, $CD4^+CD25^{high}CD127^{high}$ T cells were cocultured with irradiated PBMCs as described above together with anti-CD3. Supernatants were removed 3 days after the beginning of the assay, and IFN-γ, TNF-α, and IL-2 were measured using the multiplex fluorescent bead immunoassay as above. For this experiment, 4 more patients and HIs were randomly included in the study according to the criteria set out in the patients section.

FACS analysis. PBMCs (10^6 per sample) were stained with PE-Cy7–conjugated anti-CD3, FITC-conjugated anti-CD8, PE-Cy5–conjugated anti-CD25, and PE-conjugated anti-CD127 (all from BD Biosciences), followed by intracellular staining with human FOXP3 (APC conjugate) staining assay kit (Imgenex).

Statistics. Statistical analyses were performed using GraphPad Prism 4.0. Parametric statistical analysis (mean and SEM) was performed using standard methods. Significant differences were calculated using the nonparametric Mann-Whitney *U* test, and linear regression analysis was performed using the Pearson correlation test. For all tests, *P* values of less than 0.05 were considered significant.

Acknowledgments

We would like to thank Joanna Ashton-Chess for her help in manuscript editing. This work was supported by a grant from the Collège des Enseignants de Neurologie et les Journées de Neurologie de Langue Française (to L. Michel) and a grant from the Association pour la Recherche sur la Sclérose en Plaques (to L. Berthelot).

Received for publication February 18, 2008, and accepted in revised form July 16, 2008.

Address correspondence to: Jean-Paul Soulillou, INSERM U643, 20 Bd J. Monnet, F-44093 Nantes Cedex, France. Phone: 33-2-40-08-74-10; Fax: 33-2-40-08-74-11; E-mail: Jean-Paul.Soulillou@univ-nantes.fr.

Sophie Brouard, Jean-Paul Soulillou, and David-Axel Laplaud are co-senior authors.

Laure Michel, Laureline Berthelot, and Ségolène Pettré contributed equally to this work.

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Forts de ces résultats, cette première étude sur les cellules CD4+CD25high exprimant la chaine α du récepteur à l'IL7 chez les patients SEP atteints de forme rémittente et non-traités a été complétée par la caractérisation phénotypique des deux sous-types cellulaires et par une étude fonctionnelle visant à étudier les capacités de régulation des lymphocytes T CD4+CD25highCD127- en présence de MBP ainsi que la réactivité des cellules CD4+CD25highCD127+ à cette protéine de la myéline.

A. Étude phénotypique des lymphocytes T CD4+CD25highCD127- et CD4+CD25highCD127+

a. Étude de phénotypiques des cellules régulatrices CD4+CD25highCD127-

i. Répartition des Treg au sein des fractions Fr I, Fr II et Fr III

Récemment, Miyara et ses collaborateurs se sont intéressés à l'étude de l'hétérogénéité des cellules régulatrices (nTreg) à la fois chez les individus sains mais aussi dans différentes pathologies auto-immunes. Cette équipe a montré que cette population cellulaire peut être divisée en trois sous-populations en fonction de l'expression des marqueurs de surface CD45RA et CD25. Les trois fractions ainsi obtenues sont définies de la façon suivante : la fraction I (Fr I), la fraction II (Fr II) et la fraction III (Fr III). Fr I correspond aux cellules CD25++CD45RA+Foxp3low non-activées possédant une fonction suppressive. Fr II correspond aux cellules CD25+++CD45RA-Foxp3high activées possédant elles aussi une fonction suppressive. La dernière fraction, Fr III, correspond aux cellules CD25++CD45RA-Foxp3low. Cette dernière sous-population n'a pas de fonction suppressive et présente un potentiel Th17 (Miyara et al. 2009).

Une étude phénotypique incluant ces deux marqueurs a été réalisée pour déterminer si les cellules nTreg des patients atteints de SEP de forme rémittente et non-traités, différaient dans la répartition des trois fractions définies, apportant indirectement de nouvelles informations sur les fonctions associées à ces cellules.

La figure 9A présente la répartition de la fraction Fr I au sein des lymphocytes T CD4+, celle-ci est similaire chez les patients atteints de SEP et chez les témoins appariés selon l'âge et le sexe (Fr I : MS 1,26±0,81% vs HV 1,16±0,57%). La fraction Fr II est elle aussi comparable entre les patients atteints de SEP et les témoins (Fr II : MS 0,36±0,24% vs HV

0,44±0,28%) (Figure 9B). Aucune différence n'est observée dans la répartition de la fraction Fr III (Fr III : MS 1,12±0,47% vs HV 1,28±0,36%) (Figure 9C).

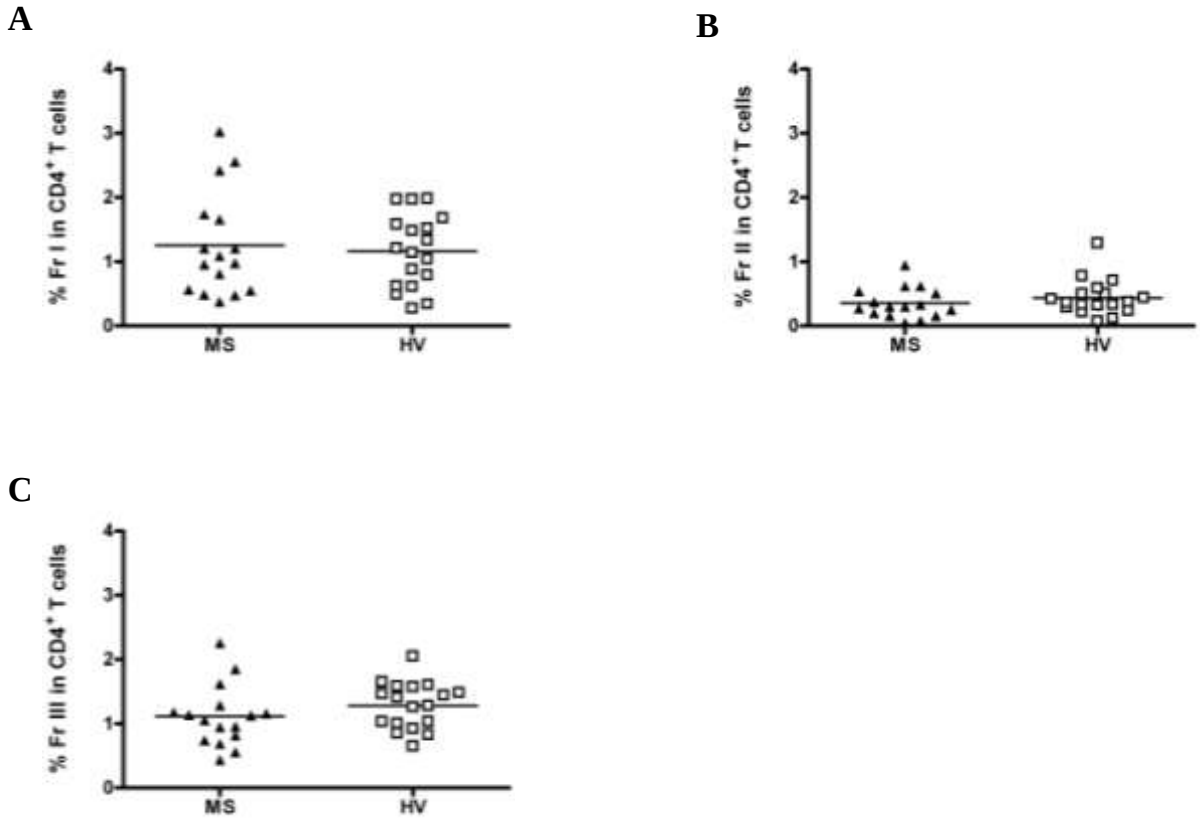

Figure 9: Répartition des cellules nTreg en trois sous-populations définies par l'expression des marqueurs CD45RA et CD25

Les PBMC sont marqués avec des anticorps anti-CD3-APC-Cy7, anti-CD4-PE-Cy7, anti-CD25-PE et anti-CD45RA-APC. Les cellules sont analysées par cytométrie en flux en positionnant une fenêtre de sélection sur les lymphocytes CD4+. **A.** La fraction I (Fr I) correspond à des cellules nTreg non-activées. **B.** La fraction II (Fr II) correspond à des cellules nTreg activées. **C.** La fraction III (Fr III) correspond à des cellules non-régulatrices possédant un potentiel Th17. Aucune différence significative n'est observée pour la fréquence de ces trois sous-populations entre les patients atteints de SEP non-traités (MS=16) et les témoins (HV=18) appariés selon l'âge et le sexe.

Les cellules non-activées possédant une capacité suppressive (Fr I), des cellules activées suppressives (Fr II) et des cellules non-suppressives ayant un potentiel Th17 (Fr III) composant la population régulatrice nTreg sont en proportions semblables dans le sang périphérique des patients atteints de SEP de forme rémittente, non-traités, et dans celui des témoins. Ces résultats suggèrent donc que les populations régulatrices nTreg possèdent les mêmes capacités fonctionnelles que ce soit chez les patients ou chez les témoins.

ii. Expression des marqueurs impliqués dans la SEP au sein des Treg

L'étude phénotypique des cellules régulatrices T CD4+CD25highCD127- a été complétée par la caractérisation de marqueurs de surface décrit comme éventuellement plus spécifiques de la pathologie SEP. Les récepteurs des chemiokines ont été impliqués dans la SEP en particulier CCR2 et CCR5 identifiés dans les tissus lésés et associés aux phases de poussées (Ransohoff et al. 1999 ; Huang et al. 2000). Dans les formes primaires progressives de SEP, CCR2 est surexprimé sur les lymphocytes tandis que CCR5 a une expression diminuée par rapport aux formes rémittente de SEP (Sørensen et al. 2001).

L'expression de molécules d'adhérence impliquées dans la transmigration au travers de la BHE comme VLA4, LFA1 et CD44 a été aussi étudiée. Il a été montré que les molécules d'adhérence VLA4 et CD44 étaient plus fortement exprimées au sein de la population T totale chez les patients atteints de SEP en poussée (Soilu-Hänninen et al. 2005).

L'analyse des PBMC de malades et de témoins ne m'a cependant pas permis de confirmer une différence d'expression des récepteurs de chemiokines intervenant aussi dans la migration des leucocytes dans le SNC : CCR2 (MS 16,25±14,52% vs HV 19,92±23,33%) et CCR5 (MS 17,19±10,67% vs HV 18,79±9,01%) (Figure 10A) ainsi que pour l'expression de molécules d'adhérence: VLA4 (intégrine α4 ou CD49d ; MS 46,59±16,08% vs HV 54,65±15,55%), LFA1 (CD11a ; MS 99,82±0,21% vs HV 99,68±0,55%) et CD44 (MS 99,86±0,17% vs HV 99,36±0,93%) (Figure 10B).

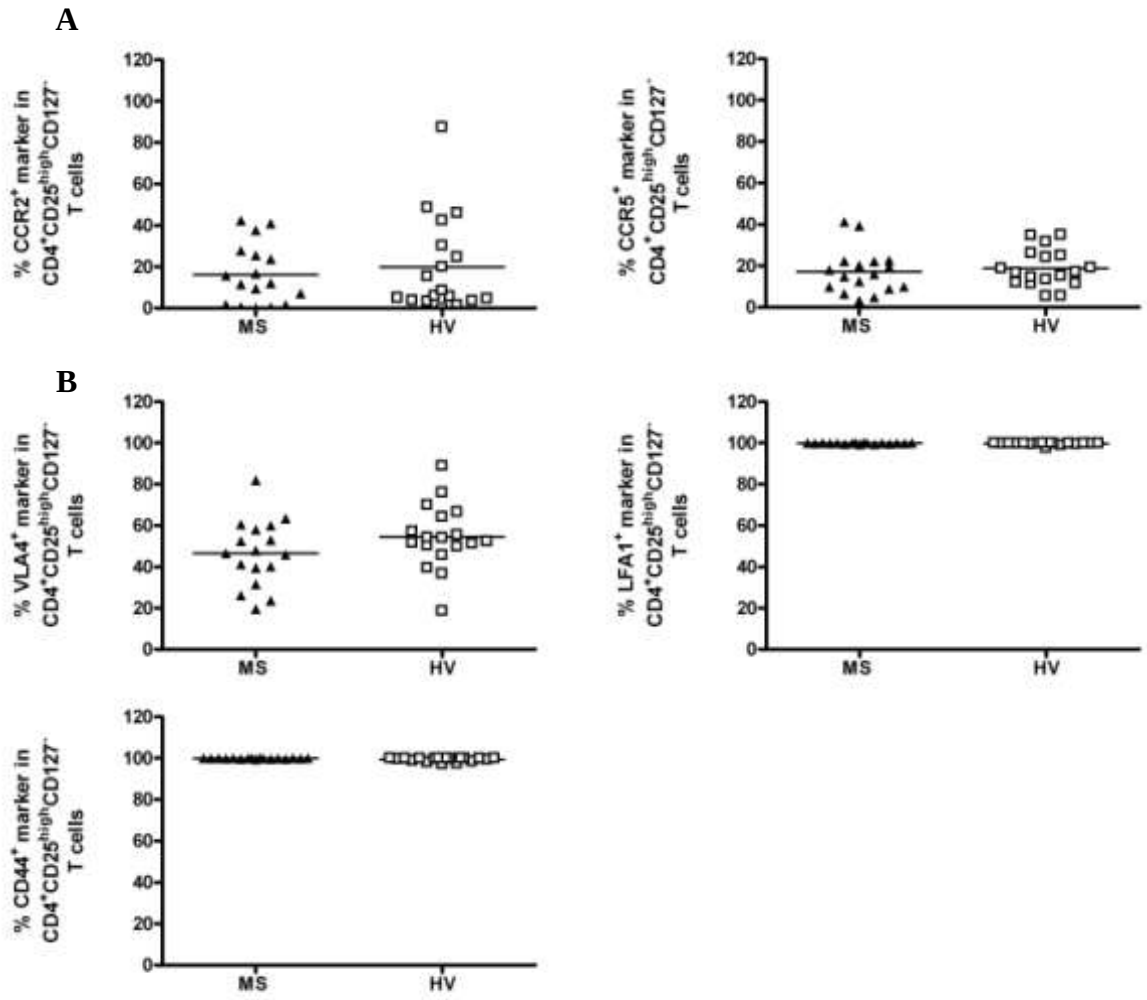

Figure 10: Fréquences des cellules exprimant CCR2, CCR5, VLA4, LFA1 et CD44 au sein des lymphocytes T CD4+CD25highCD127-

Les PBMC sont marqués avec des anticorps anti-CD3-APC-Cy7, anti-CD4-PE-Cy7, anti-CD25-PE et anti-CD45RA-APC. Les cellules sont analysées par cytométrie en flux en positionnant une fenêtre de sélection sur les lymphocytes T CD4+CD25highCD127-. **A.** Fréquence des cellules exprimant CCR2 et CCR5 au sein des lymphocytes T CD4+CD25highCD127-. **B.** Fréquence des cellules exprimant les molécules d'adhérence VLA4, LFA1 et CD44 au sein des lymphocytes T CD4+CD25highCD127-. Aucune différence significative n'est observée pour les 4 marqueurs étudiés entre les patients atteints de SEP non-traités (MS=17) et les témoins (HV=18) appariés selon l'âge et le sexe.

Ainsi, comme nous l'avons montré dans l'article II, les cellules régulatrices nTreg des patients atteints de SEP ne diffèrent ni par leur capacité suppressive, ni par leur profil de sécrétion de cytokines par rapport aux individus sains. Ces cellules ne diffèrent pas non plus par la répartition des trois fractions Fr I, Fr II et Fr III, ni par l'expression de surface des marqueurs CCR2, CCR5, VLA4, LFA1 et CD44.

b. Caractérisation phénotypique des cellules T CD4+CD25highCD127+

L'autre volet de l'étude phénotypique des PBMC fraichement isolés à partir du sang périphérique des patients atteints de SEP et des témoins est la caractérisation des cellules T CD4+CD25highCD127+ que ce soit pour des marqueurs exprimés par les cellules nTreg mais non-spécifiques de ces cellules, pour des marqueurs connus pour jouer un rôle dans l'immunité mais aussi impliqués dans la pathologie de la SEP.

i. Fréquence et MFI du CD127 chez les patients SEP

Dans un premier temps, la fréquence des cellules positives au CD127 au sein des cellules régulatrices T CD4+CD25high a été étudiée à la fois chez les patients atteints de SEP de forme rémittente-récurrente, non-traités, et chez les témoins appariés selon l'âge et le sexe. Comme mentionné précédemment dans l'article II, aucune différence de fréquence de ces cellules au sein de la population nTreg n'est constatée (MS 7,66±3,9% ; HV 8,05±3,25%) (Figure 11A). De plus, aucune différence en termes d'intensité moyenne de fluorescence n'a été observée pour le marqueur CD127 entre les patients atteints de SEP et les témoins (Figure 11B).

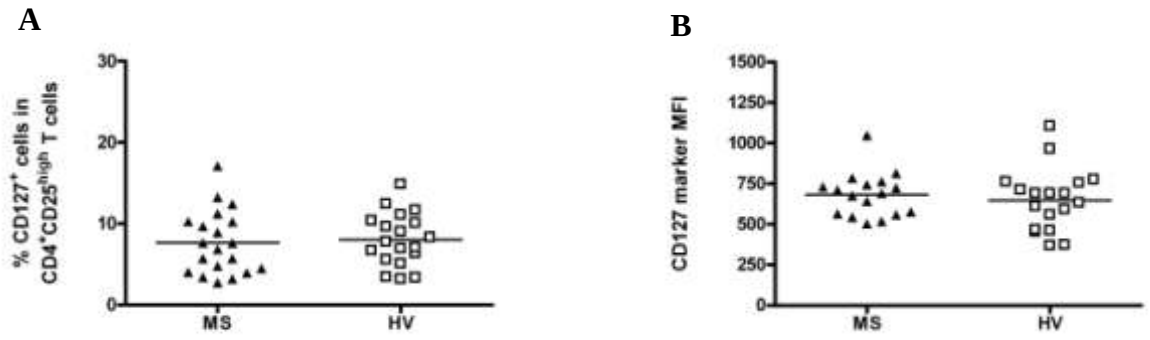

Figure 11: Étude de la fréquence et de l'intensité moyenne de fluorescence du marqueur CD127 au sein des lymphocytes T $CD4^+CD25^{high}$

Les PBMC sont marqués avec des anticorps anti-CD3-APC-Cy7, anti-CD4-PE-Cy7, anti-CD25-PE et un anticorps anti-CD127 biotinilé + streptavidine Alexa 405. L'analyse en cytométrie en flux ne met pas en évidence de différence significative entre les patients SEP de forme rémittente, non-traités (MS=17) et les témoins (HV=18) appariés selon l'âge et le sexe. **A.** La fréquence des cellules positives au marqueur CD127 est obtenue en plaçant une fenêtre de sélection sur les cellules T $CD4^+CD25^{high}$. **B.** Intensité moyenne de fluorescence du marqueur CD127.

ii. Expression des marqueurs non-spécifiques des Treg

Pour s'assurer que les cellules $CD4^+CD25^{high}CD127^+$ sont bien distinctes de la population $CD4^+CD25^{high}CD127^-$ possédant la fonction suppressive, une caractérisation phénotypique pour des marqueurs non-spécifiques des cellules nTreg a été réalisée (Figure 12). En effet, les cellules nTreg peuvent être distinguées des cellules effectrices activées par l'expression du marqueur CD62L. Plus de 95% des cellules T $CD4^+CD25^{high}$ sont $CD62L^+$. De plus, ces cellules isolées à partir du sang périphérique sont majoritairement $CD45RO^+$ et $CD45RA^-$ (Dieckmann et al. 2001). La grande majorité des nTreg présentent un phénotype de cellules mémoires centrales. Cette population cellulaire est aussi enrichie en marqueurs impliqués dans l'immunité comme CD95 (Fas), CTLA4 et CD103 mais n'exprime pas CD28. La Figure 12A montre l'expression prédominante de CD28 à la surface des cellules T $CD4^+CD25^{high}CD127^+$ (MS 99,31±2,67% vs HV 99,94±0,07%) tandis qu'il n'y a pas d'expression de CD28 à la surface des nTreg. L'expression de CTLA4 (MS 11,79±11,37% vs HV 19,60±24,17%) et de CD103 (MS 1,13±0,88% vs HV 2,5±1,14%) est très faible à la surface de ces cellules à l'inverse de ce qui est connu pour les nTreg (Figure 12B et 12C).

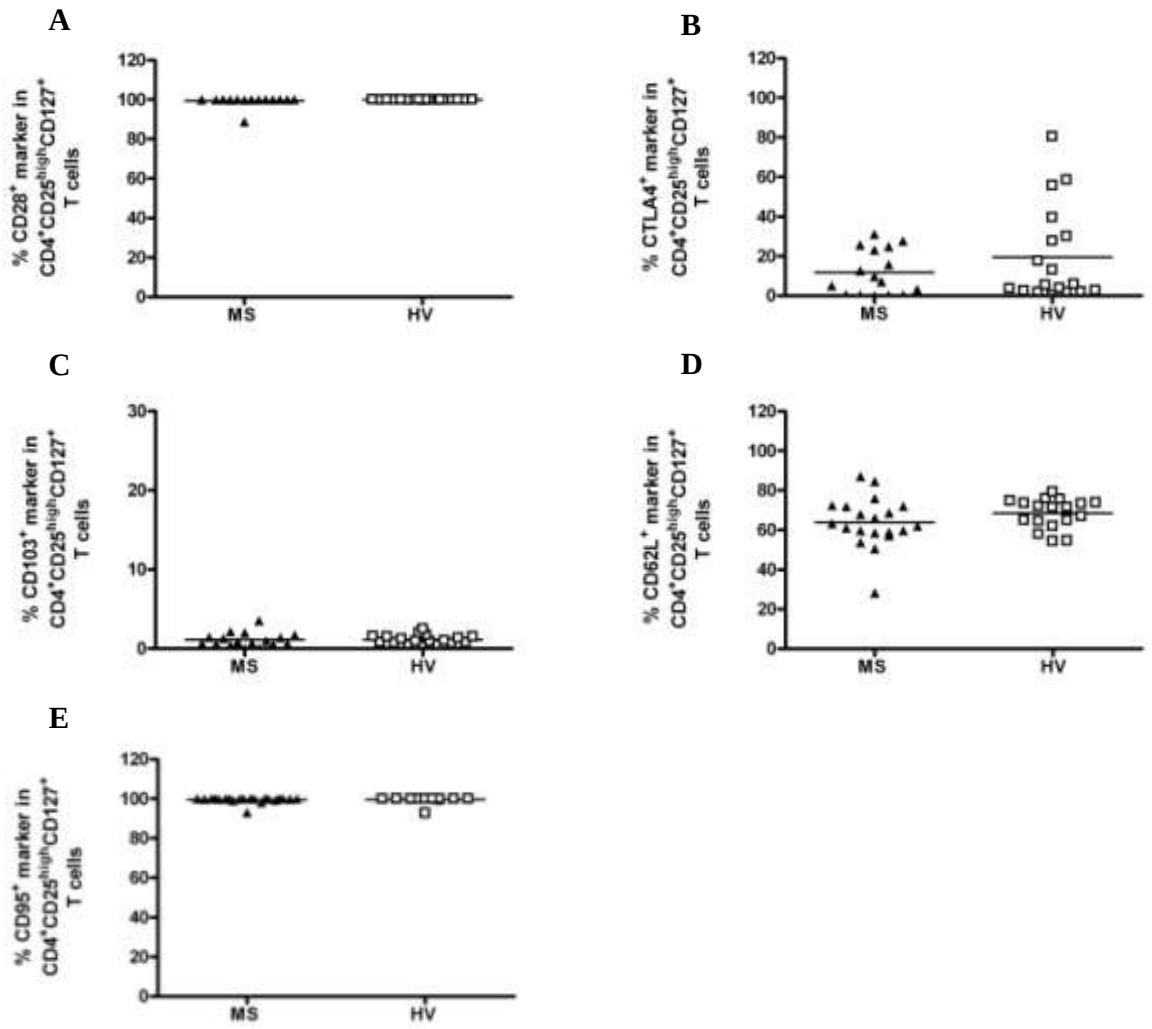

Figure 12: Fréquences des cellules exprimant CD28, CTLA4, CD103, CD62L et CD95 au sein des cellules T $CD4^{+}CD25^{high}CD127^{+}$

Les PBMC sont marqués avec des anticorps anti-CD3-APC-Cy7, anti-CD4-PE-Cy7, anti-CD25-PE et un anticorps anti-CD127 biotinilé + streptavidine Alexa 405. L'analyse en cytométrie en flux ne met pas en évidence de différence significative entre les patients SEP de forme rémittente, non-traités (MS=17) et les témoins (HV=18) appariés selon l'âge et le sexe. **A.** La fréquence des cellules exprimant CD28 au sein des cellules T $CD4^{+}CD25^{high}CD127^{+}$. **B.** La fréquence des cellules exprimant CTLA4 au sein des cellules T $CD4^{+}CD25^{high}CD127^{+}$. **C.** La fréquence des cellules exprimant CD103 au sein des lymphocytes T $CD4^{+}CD25^{high}CD127^{+}$. **D.** La fréquence des cellules exprimant CD62L au sein des lymphocytes T $CD4^{+}CD25^{high}CD127^{+}$. **E.** La fréquence des cellules exprimant CD95 au sein des lymphocytes T $CD4^{+}CD25^{high}CD127^{+}$.

CD62L semble être plus faiblement exprimé à la surface des cellules T $CD4^+CD25^{high}CD127^+$ mais aucune différence significative n'est observée (MS 63,99±12,66% vs HV 68,44±7,49%) (Figure 12D). L'expression de CD95 est, quant à elle, similaire dans les deux populations de cellules (MS 99,49±1,51% vs HV 99,55±1,70%).

De plus, pour l'ensemble de ces marqueurs connus pour être exprimés par les lymphocytes T $CD4^+CD25^{high}$ leur expression est similaire entre les patients atteints de SEP et les témoins.

iii. Expression de CD45RO, CD45RA et CCR7

La caractérisation des cellules T $CD4^+CD25^{high}CD127^+$ est approfondie par l'étude de l'expression de marqueur mémoire CD45RO, naïf CD45RA et CCR7. La co-expression de CD45RO et de CCR7 nous permet d'évaluer la proportion des cellules mémoires centrales ($CD45RO^+CCR7^+$) et des cellules mémoires effectrices ($CD45RO^+CCR7^-$) au sein de la population d'intérêt.

Chez l'Homme, la molécule CD127 est exprimée à un niveau relativement élevé sur les cellules T $CD4^+$ et $CD8^+$ naïves. L'expression de CD127 sur les cellules T $CD4^+$ naïves augmente avec une stimulation via l'IL7 (Swainson et al. 2006). Lozza et ses collaborateurs ont montré que quelque soit la stimulation des cellules T $CD4^+$ naïves, le CD127 est fortement exprimé et la force de la stimulation peut induire la mort cellulaire ou l'engagement de ces cellules vers un profil mémoire central ou mémoire effecteur (Lozza et al. 2008 ; Racape et al. 2009).

Comme le montre la Figure 13, l'expression des marqueurs mémoire/naïf à la surface des cellules T $CD4^+CD25^{high}CD127^+$ est comparable entre les patients atteints de SEP de forme rémittente et les témoins ($CD45RO^+$: MS 91,52±8,02% vs HV 96,32±3,29% ; $CD45RA^+$: MS 81,54±19,1% vs HV 82,26±24,92%). Ces cellules semblent présenter un phénotype mémoire pour la grande majorité et semblent exprimer à la fois le marqueur CD45RA. Elles sont en majorité du type mémoires centrales (CD45RO+CCR7+ : MS 69,38±16,33% vs HV 76,83±21,36%) avec une proportion plus faible de type mémoires effectrices (CD45RO+CCR7- : MS 21,80±18,21% vs HV 19,58±21,78%). Cette observation est retrouvée à la fois chez les patients atteints de SEP et les témoins appariés selon l'âge et le sexe (Figure 13 C et D).

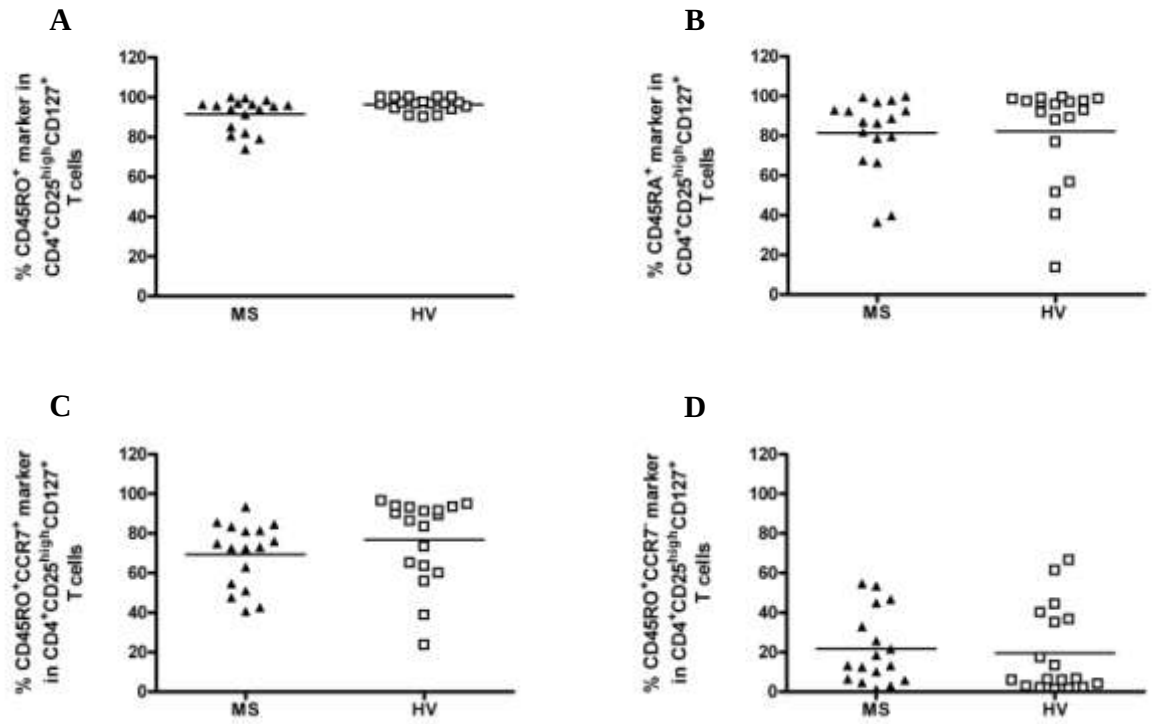

Figure 13: Fréquence des marqueurs CD45RO, CD45RA et CCR7 à la surface des cellules T $CD4^{+}CD25^{high}CD127^{+}$

Les PBMC sont marqués avec des anticorps anti-CD3-APC-Cy7, anti-CD4-PE-Cy7, anti-CD25-PE et un anticorps anti-CD127 biotinilé + streptavidine Alexa 405. L'analyse en cytométrie en flux ne met pas en évidence de différence significative entre les patients SEP de forme rémittente, non-traités (MS=17) et les témoins (HV=18) appariés selon l'âge et le sexe. **A.** La fréquence des cellules mémoires ($CD45RO^{+}$) au sein des cellules T $CD4^{+}CD25^{high}CD127^{+}$. **B.** La fréquence des cellules exprimant CD45RA au sein des cellules T $CD4^{+}CD25^{high}$CD127+. **C.** La fréquence des cellules mémoires ($CD45RO^{+}CCR7^{+}$) au sein des lymphocytes T $CD4^{+}CD25^{high}$CD127+. **D.** La fréquence des cellules mémoires ($CD45RO^{+}CCR7^{-}$) au sein des lymphocytes T $CD4^{+}CD25^{high}$CD127+.

iv. Expression des marqueurs impliqués dans la SEP

La dernière partie de mon étude phénotypique a inclus l'estimation de la proportion des cellules exprimant des marqueurs impliqués dans l'immunité mais aussi plus spécifiques de la pathologie au sein des lymphocytes T $CD4^{+}CD25^{high}CD127^{+}$.

La figure 14A ne montre pas de différence de fréquence des cellules positives au CCR2 (MS 19,18±16,06% vs HV 23,84±24,18%) et au CCR5 (MS 8,51±5,04% vs HV 12,94±6,25%). L'observation de l'expression de surface des intégrines lymphocytaires impliquées dans le recrutement sur le site de l'affection et favorisant l'infiltration dans le SNC ne révèle pas non plus de différence entre les malades et les témoins (VLA4 : MS 68,32±13,01% vs HV 76,32±12,67% ; LFA1 : MS 99,89±0,22% vs HV 99,90±0,17% ; CD44 : MS 99,86±0,26% vs HV 99,38±1,60%) (Figure 14B). L'expression d'IL23R, marqueur du « profil » Th17, ne diffère pas entre les patients atteints de SEP de forme rémittente et les témoins (MS 4,02±3,09% vs HV 3,58±3,63%) (Figure 14C).

La caractérisation phénotypique des cellules T $CD4^{+}CD25^{high}CD127^{+}$ a permis de mettre en évidence que cette sous-population des cellules régulatrices $CD4^{+}CD25^{high}$ se distinguaient de la vraie population régulatrice $CD4^{+}CD25^{high}CD127^{-}$ par l'expression et l'absence d'expression de certains marqueurs.

Cependant, aucune différence dans l'expression de l'ensemble des marqueurs testés n'a été observée entre les cellules des patients atteints de SEP de forme rémittente, non-traités et les témoins appariés selon l'âge et le sexe.

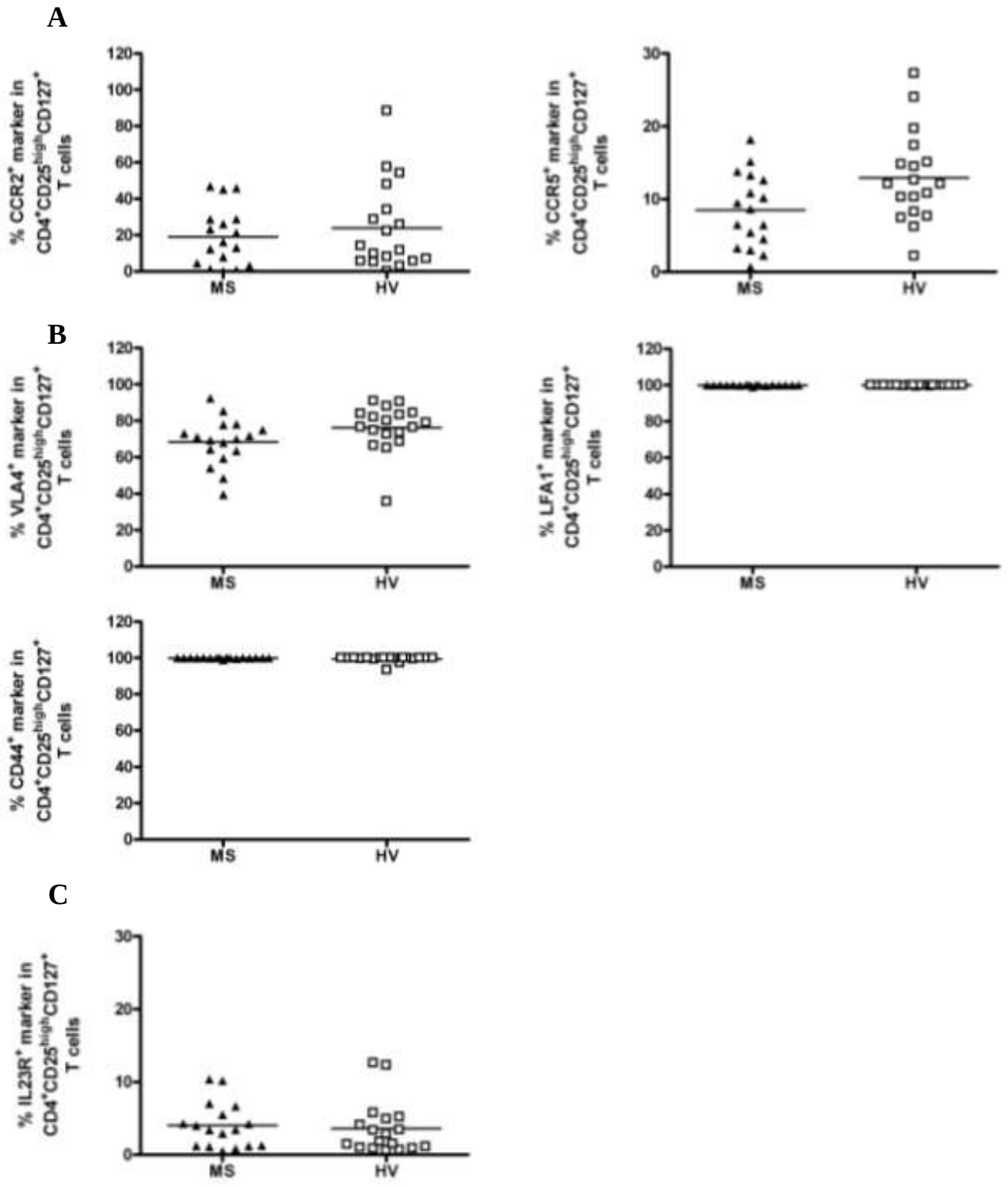

Figure 14: Étude phénotypique des cellules T $CD4^{+}CD25^{high}CD127^{+}$ à partir de PBMC

Les PBMC sont marqués avec des anticorps anti-CD3-APC-Cy7, anti-CD4-PE-Cy7, anti-CD25-PE et un anticorps anti-CD127 biotinilé + streptavidine Alexa 405. **A.** La fréquence de cellules exprimant les marqueurs CCR2 et au CCR5 au sein des lymphocytes T $CD4^{+}CD25^{high}CD127^{+}$. **B.** La fréquence des cellules exprimant les marqueurs VLA4, LFA1 et CD44 au sein des lymphocytes T $CD4^{+}CD25^{high}CD127^{+}$. **C.** La fréquence des cellules exprimant l'IL23R à la surface des lymphocytes T $CD4^{+}CD25^{high}CD127^{+}$. Ces résultats sont obtenus par analyse en cytométrie en flux, aucune différence significative n'est observée entre les groupes appariés selon l'âge et le sexe (MS=17 ; HV=18).

B. Étude fonctionnelle des deux sous-populations lymphocytaires $CD4^+CD25^{high}$ en présence de MBP

Dans la SEP, le processus inflammatoire aboutissant à la destruction de la myéline pourrait être enclenché par les cellules T auto-immunes reconnaissant les composants de la myéline. Nous avons donc cherché à tester, d'une part la réactivité, des cellules T $CD4^+CD25^{high}CD127^+$ en présence de MBP, l'une des protéines de la myéline. D'autre part, nous avons testé la capacité des cellules régulatrices T $CD4^+CD25^{high}CD127^-$ à réguler la réponse des cellules effectrices T $CD4^+CD25^-$ en présence de MBP.

a. Prolifération en présence de MBP

Dans le but d'étudier la réactivité des cellules T $CD4^+CD25^{high}CD127^+$ à la MBP, une culture de ces cellules en présence de PBMC autologues irradiés et stimulés par 1µg de MBP (concentration finale de 5µg/ml) pendant 5 jours a été réalisée. Les différentes populations cellulaires $CD4^+CD25^-$, $CD4^+CD25^{high}CD127^-$ et $CD4^+CD25^{high}CD127^+$ sont obtenues après tri cellulaire réalisé au FACSAria®. Cette sélection a été effectuée de la même façon que décrite précédemment dans l'article II. La figure 15A ne montre pas de différence de prolifération pour les trois types cellulaires stimulés par la MBP. Cependant, les cellules T $CD4^+CD25^{high}CD127^+$ des patients atteints de SEP de forme rémittente, non-traités, sembleraient avoir une tendance à proliférer davantage comparées aux cellules des témoins (MS 79450±126800 vs HV 17170±11320 ; p =0,56).

b. Régulation en présence de MBP

La fonction régulatrice des cellules T $CD4^+CD25^{high}CD127^-$ a ensuite été testée en présence de MBP. Pour cela, les cellules T $CD4^+CD25^{high}CD127^-$ sont co-cultivées en présence de MBP avec des cellules T $CD4^+CD25^-$ autologues ainsi que des PBMC irradiés autologues pendant 5 jours. Dans ces conditions, la figure 15B révèle une capacité régulatrice similaire entre les cellules T $CD4^+CD25^{high}CD127^-$ des patients atteints de SEP et celles des témoins (MS 61,43±33,67% vs HV 64,47±39,56%) sur la prolifération des cellules T $CD4^+CD25^-$ autologues.

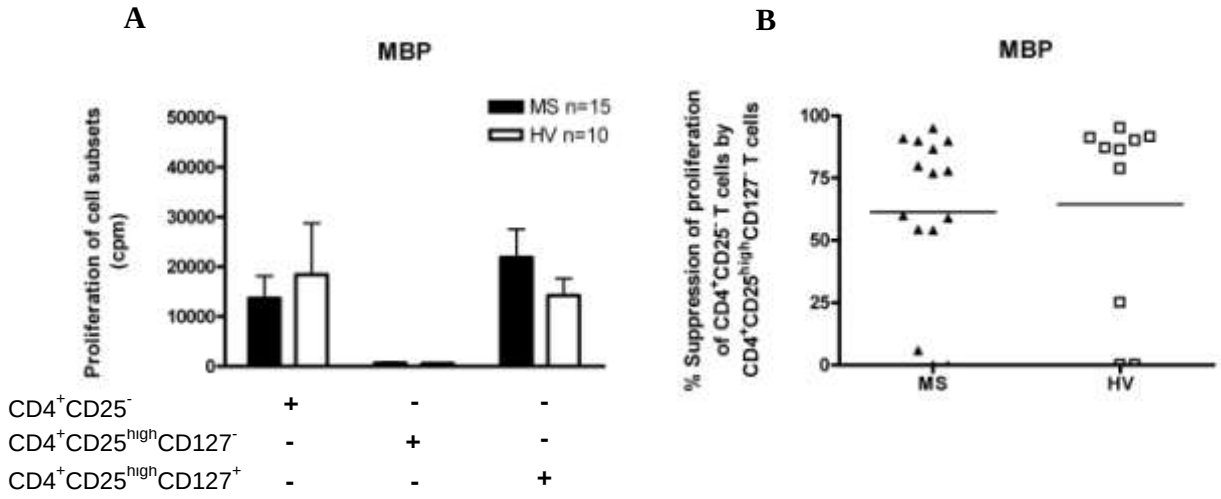

Figure 15: Comparaison de la prolifération des $CD4^+CD25^{high}CD127^+$ et de la fonction suppressive des $CD4^+CD25^{high}CD127^-$ en présence de MBP

Les PBMC sont marqués à l'aide d'anticorps anti-CD3-PE-Cy7, anti-CD4-FITC, anti-CD25-Alexa Fluor 647 et anti-CD127 PE. Les différentes populations cellulaires sont obtenues par tri au FACSAria®. Les cellules sont mises en culture pendant 5 jours en présence de PBMC autologues irradiés et stimulées avec 1µg de MBP par puits. Aucune différence significative n'a été observée entre les patients atteints de SEP, non-traités (MS=15) et les témoins (HV=10) appariés selon l'âge et le sexe. A. Prolifération des cellules T $CD4^+CD25^-$, $CD4^+CD25^{high}CD127^-$ et $CD4^+CD25^{high}CD127^+$ après 5jours de culture en présence de MBP. B. Pourcentage de suppression de la prolifération des cellules T $CD4^+CD25^-$ par les cellules T $CD4^+CD25^{high}CD127^-$ en présence de MBP.

Cette dernière observation va à l'encontre de travaux réalisés par d'autres équipes. En effet, Kumar et ses collaborateurs rapportent un défaut de régulation des cellules T $CD4^+CD25^{high}$ de patients atteints de SEP en présence de MBP (Kumar et al. 2006). Un défaut de régulation a aussi été observé chez les malades dans des tests fonctionnels impliquant des cellules T $CD4^+CD25^{high}$ en présence de MOG (Haas et al.2005).

ARTICLE III : Reconnaissance des cellules T par les CPA grâce au transfert de protéine détectant une forte fréquence des cellules T auto-réactives chez les patients non-traités atteints de Sclérose en Plaques de forme rémittente.

Par : Bahbouhi B, Pettré S, Berthelot L, Elong-Ngomo A, Michel L, Degauque N, Wiertlewski S, Lefrère F, Meyniel C, Delcroix C, Brouard S, Laplaud DA*, Soulillou JP*,

(*les auteurs ont contribués de manière égale à ce travail)

Article soumis dans : Journal of Clinical Investigation

T-cell recognition of self APC by protein transfer assay reveals a high frequency of anti-myelin T-cell autoreactivity in patients with multiple sclerosis

Bouchaib Bahbouhi[1], Ségolène Pettré[1], LaureLine Berthelot[1], Annie Elong Ngomo[1], Laure Michel[2], Nicolas Degauque[1], Sandrine Wiertlewski[2,3], Fabienne Lefrère[2], Claire Meyniel[3], Catherine Delcroix[4], Sophie Brouard[1], David-Axel Laplaud[1,2,3#], and Jean-Paul Soulillou[1#*]

[1] INSERM, UMR 643, Nantes, F44093 France; CHU Nantes, Institut de Transplantation et de Recherche en Transplantation (ITERT) Nantes, F44000 France; Université de Nantes, Faculté de Médecine, Nantes, F44000 France

[2] INSERM CIC004, University Hospital, Nantes, 44093

[3]Department of Neurology, CHU Nantes, F-44093 Nantes, France.

[4]Department of Hemodialysis, CHU Nantes, F-44093 Nantes, France.

# The two senior authors are listed by alphabetical order and have contributed equally.

*Corresponding author: Jean-Paul Soulillou. INSERM, UMR 643, Institut de Transplantation Et de Recherches en Transplantation, ITERT, 30 Boulevard Jean Monnet, Nantes, 44900 France.

Tel: (33) 240 084633. FAX: (33) 240 087411. Email: Jean-Paul.Soulillou@univ-nantes.fr

Key words: multiple sclerosis, trogocytosis, CD45RO, CD14, monocytes, myelin, TRAP, ELISPOT, CD40L, CD62L

ABSTRACT

Although peripheral-blood myelin-autoreactive T-cells (PB-MATC) are thought to play a key role in multiple sclerosis (MS), they are generally considered to have qualitative differences rather than quantitative ones as compared to those found in healthy controls. Here we revisited the assessment of PB-MATC by a new approach based on their combined ability to acquire membrane proteins from autologous APC (TRAP for T-cell Recognition of self APC by Protein transfer) and to respond to whole myelin extract as the stimulating autoantigen. Using this approach, the PB-MATC frequency in MS patients (n=22) was found to be unexpectedly high (median 2.07%, range 0-4.24) and to significantly exceed that of age/gender matched healthy individuals (median 0.1%, range 0-5.3%, n=18; $p < 0.0001$). These data correlated with whole myelin-induced IFNγ-ELISPOT patterns although the values obtained with ELISPOT were 58 times lower than with TRAP. PB-MATC were memory T cells expressing CD40L with a $CD62^{low}$ phenotype, suggesting their ability for homing to tissues. Collectively, these new data show a very high frequency of autoreactive T-cells during the relapsing phase of the disease lacking in age/gender-matched healthy individuals, and therefore firmly support an autoimmune etiology in MS.

INTRODUCTION

Immune system deregulation in multiple sclerosis (MS) has long been suggested by the presence of T-cells and activated monocytes/macrophages within lesions of *postmortem* brain tissues from MS patients. The current physio-pathological concept of MS autoimmune etiology lies in the presence of activated peripheral-blood myelin-autoreactive T-cells (PB-MATC) able to transmigrate the BBB endothelium (1-2). To date, however, the PB-MATC frequency has not been consistently shown to differ in MS patients from that of age-matched healthy individuals (3-8). An alternative hypothesis involves other abnormalities occurring in MS, such as a particular PB-MATC phenotype (8), a defect in T-cell regulation (9) or a dysfunction of the blood-brain barrier (BBB) (10). Our failure to confirm a defect in circulating $CD4^+CD25^+CD127^-$ T-regulatory cells in MS patients (11) and to discriminate MS patients from HI in terms of their frequency of myelin-autoreactive $CD8^+$ T-cells detected by ELISPOT (3) prompted us to revisit the frequency analysis of PB-MATC using a novel approach. Current PB-MATC frequency estimation is either based on the production of a limited number of cytokines (ELISPOT) or on stimulation by potentially autoimmune peptides or soluble proteins. Precisely assessing PB-MATC frequency in MS with a minimum of measurement bias and by taking into account all possible autoantigens is a prerequisite for establishing MS autoimmune etiology.

In this report, we took advantage of the ability of memory T-cells to exchange surface-membrane molecules with APC in an antigen-dependent manner by a mechanism called *trogocytosis* or *T-cell recognition of APC by protein transfer* (TRAP) occurring during dissociation of immune synapses. The TRAP was first described in rodents to measure a response against viral antigens or transgenic clones (12-13) and more recently was adapted to human in the context of cancer or viral diseases (14-15) although in more favorable experimental contexts than autoimmunity including homogeneous T-cell clones (13) or T-cell detection at the peak of an induced immune response (16). Despite its validation as a reliable method, the TRAP assay has not yet been used to analyze freshly isolated T-cells from patients

presenting a possible autoimmune disease particularly with MS. Current techniques do not measure the global pool of autoreactive T-cells because these cells might have different activation patterns and a wide-spectrum of activation-thresholds. In this respect, the TRAP assay offers a more global readout. Finally, we used whole brain myelin extract as a source of autoantigens instead of myelin-derived peptides or soluble proteins. We demonstrated that endogenous processing of whole myelin extract and its cross-presentation may enhance PB-MATC detection by increasing both the number and types of presented epitopes.

This new experimental approach clearly established a high level of autoreactivity against myelin on blood T-cells of MS patients and showed that MS patients presented an unexpectedly high frequency of memory T-cell reactivity against myelin determinants. This frequency significantly surpassed that of gender/age-matched normal individuals who only weakly responded to the whole myelin autoantigen. Collectively, our data uphold the concept of an expanded anti-myelin autoreactive T-cell pool in MS.

RESULTS

TRAP detects antigen-specific T-cell response in selected individuals: We first analyze the TRAP response in individuals with pre-established reactivity against CMV or MBP-derived MHC class I-restricted peptides.

CMV+ individuals: Supplementary Fig 1A-B show T-cell proliferation in CMV^+ donors with median 5% (range 5-8%) of their $CD45RO^+CD3^+$ T-cells becoming $CSFE^{low}$ in the presence of CMV-pp65 protein compared to only 0.5% in CMV^- control individuals (range 0.5-2%). Furthermore, only $CD45RO^+$ T-cells from CMV^+ individuals were IFNγ-$ELISPOT^+$ (inserts, suppl. Fig 1A) in the presence of CMV-pp65. Supplementary Fig 2A-B show that only $CD45RO^+$ lymphocytes from CMV^+ individuals (median 3.7% range 3-5.8%) displayed a higher $CD3^+$ TRAP signal in the presence of pp65 compared to CMV^- controls (median 0.2% range 0-0.2%). The ELISPOT assay also detected a greater response in CMV^+ individuals (median 86 IFNγ-spots range 38-385) compared to their CMV^- counterparts (median 57 IFNγ-spots range 13-60, $p < 0.007$, suppl. Fig 2C) although at a 94-fold lower frequency compared to the TRAP-based estimations. There was however a significant correlation between the TRAP and ELISPOT assays (suppl. Fig 2D, $r^2=0.82$, $p < 0.01$). Finally, pp65-tetramer sorted T-cells stimulated by pp65 peptide-pulsed ETB-LCL (used here as APC) did exhibit a 6.8 fold increase in TRAP signal (68%, suppl. Fig 3) compared to stimulation by non-pulsed ETB-LCL (10%).

Individuals with pre-established $CD8^+$ T cells reactive to MHC class I-restricted MBP peptides: We first looked whether biotinylated and fluorescently labeled myelin (myelin-F*) was internalized in the $CD14^+$ monocytes used for T-cell stimulation. Figure 1A shows that myelin-F* was phagocyted by monocytes (22%, $p < 0.01$) and myelin-F* internalization was abolished by the phagocytosis inhibitor APO. In addition, the percentage of HLA-DR^{bright} cells was three times higher in myelin-fed monocytes compared to non-fed controls (Figure 1B) (21±8% for MS patients and 28± 7.8% for HI *versus* 7.3±3% and 6.3±4% respectively, $p < 0.002$). We previously identified MS patients and HI with circulating $CD8^+$ T-cells

reactive to MHC class I restricted MBP peptides in the IFNγ-ELISPOT assay (3). Frozen PBMC samples from such preselected MS patients and two normal individuals were available and were tested for TRAP (n=4). Irrelevant peptide served to determine the background signal. A representative TRAP reactivity on separated T-cells is shown in Figure 2. MBP-derived peptides restricted by MHC class I induced a 6 fold increase in the % of $TRAP^+CD3^+CD4^-$ cells (1.2% *versus* 0.2% background) but, as expected, not in $CD3^+CD4^+$ T-cells. Whole myelin extract induced only a moderate increase in $TRAP^+CD3^+CD4^+$ T-cells (0.6% TRAP *versus* 0.3% background). However, an 8-fold increase in $CD3^+CD4^-TRAP^+$ T-cells (1.6% TRAP *versus* 0.2% background) was observed with whole myelin extract. CD40L, which is transiently expressed on activated T-cells consecutively to their antigenic stimulation (17-19) was also increased on $TRAP^+$ T-cells. Finally, two HI with pre-established $CD8^+$ autoreactivity against myelin-derived peptides in ELISPOT assay (3) were also analyzed with similar results (Figure 3A-B, 6% *versus* 0.1% and 3% *versus* 0.2%). These individuals were also positive in the IFNγ-ELISPOT assay in the presence of whole myelin (median 192.5 INFγ-spots, range 86-245 compared to background 22.5 INFγ–spots, range 8-23, $p < 0.0002$; Figure 3C). Collectively, these experiments show that monocytes internalized myelin and efficiently cross-presented myelin determinants to $CD8^+$ T-cells.

Whole myelin extract induces a strong TRAP signal in a cohort of MS patients with remitting relapsing disease.

Next the frequency of memory $CD3^+$ T-cells acquiring monocyte-derived fluorescence in the presence of the whole myelin extract was analyzed in a cohort of 22 untreated MS patients and 18 gender- and age-matched healthy individuals (HI).

The median background signal for MS patients (median 1%, range 0-4%) and HI (median 1.45%, range 0.1-4%) was similar (Figure 4A). In contrast, median TRAP signal in the presence of the whole myelin extract in MS patients (median 3.5%, range 1-10%) was increased and highly significantly different from related values in HI (median 1.45%, range 0.2-7.5%; $p < 0.0077$). If the background was subtracted from

the whole myelin-induced TRAP value in an attempt to measure myelin-specific TRAP signal, MS patients had 2.07% median (range 0-4.24%) myelin-autoreactive $TRAP^+$ T-cells, a value still highly significantly exceeding that of HI (median 0.1%, range 0-5.3%, $p < 0.0001$; Figure 4B). MS patients reacting to whole myelin extract did not exhibit a significantly higher TRAP signal to apoliporotein prepared using a similar procedure to that for whole myelin (1.2±0.45%), suggesting an absence of a T-cell response to lipid myelin components (not shown).

Finally, myelin-TRAP reactive T-cell frequency was considerably higher than that of IFNγ-producing cells in the ELISPOT with an approximately 58 fold increase. However, there was a significant correlation between ELISPOT and TRAP frequencies in samples tested simultaneously (Figure 4C, $r^2=0.82$, $p < 0.0001$). Collectively, the data indicate that untreated MS patients are characterized by a high frequency of myelin autoreactive T-cells and substantially differed from healthy individuals on the basis of the number of circulating memory T-cells reacting against myelin-pulsed autologous APC.

Whole myelin $TRAP^+$ cells are activated memory T-cells and anti-MHC class II blocking antibodies prevent TRAP induction in $CD4^+$ T-lymphocytes.

We have already mentioned that $TRAP^+$ T-cells predominantly express CD40L in MS patients and HI tested in preliminary experiments (Fig 2-3). We next extended the phenotypic characterization of $TRAP^+$ T-cells to the 22 MS patients of the study cohort in addition to other activation markers (CD62L, CD95, and HLA-DR). As suggested by the preliminary data, CD40L discriminated $TRAP^+$ from $TRAP^-$ memory T-cells reactive to whole myelin extract (Figure 4D, median 82.5%, range 60-96%, $CD40L^+TRAP^+$ *versus* median 12.5%, range 2-27%, $CD40L^+TRAP^-$; $p < 0.0002$). In addition, $TRAP^+$ cells were more frequently $CD62L^{low}$ (median 72%, range 62.3-95%) compared to the $TRAP^-$ counterparts (median 27%, range 26-50.8%, $p < 0.0006$; Figure 5A). In contrast, $TRAP^+$ T-cells had roughly similar median frequencies of $CD95^+$ (Figure 5B, median 33%, range 33-39%) and $HLA\text{-}DR^+$ (Figure 5C, median 18%, range 14-20%) compared to $TRAP^-$ T-cells (median 36%, range 36-40% for

CD95 and median 18%, range 10-51% for HLA-DR). Finally, whole myelin TRAP reactivity was detected at similar levels in both CD4 and CD8 T-cells in MS patients (Figure 6A, inserted representative dot blot). Figure 6B shows that whole myelin-induced $TRAP^+CD3^+CD4^+$ median value in MS patients (median 3.8%, range 1.5-5%) was decreased to the background levels (median 0.65%, range 0.3-2%, $p < 0.0006$) by the addition of anti-MHC class II blocking antibodies in the test, indicating that the myelin-induced $TRAP^+$ signal was TCR-dependent. It should be noted that the antibodies could not abolish the background signal, suggesting that some APC molecule transfer occurred in a TCR-independent manner.

Correlations of myelin-specific TRAP values with history of relapses and EDSS scores in MS patients

Further analysis of the data showed that myelin-specific response was significantly higher in patients tested at the time of a relapse (median 3.2%, range 2.6-6%) as compared to the remaining MS patients seen during a remission period (median 1.55%, range 0-4.24%, $p<0.05$, Mann-Whitney test) or to control individuals (median 0.1%, range 0-5.3, $p < 0.01$, Man-Whitney test; Figure 7A). Both MS subgroups still had median frequency of memory T-cells autoreactive to myelin significantly higher than controls ($p<0.001$, Kruskall-Wallis test). In addition, if MS patients are ranked according to EDSS scores, the data suggest that those with the highest EDSS scores also had the highest level of myelin autoreactive T-cell frequency (median 3%, range 2.6-6%) compared to those with relatively lower EDSS-Sc (median 1.6%, range 0-4.24%) or to controls (median 0.1%, range 0-5.3%; $p < 0.0001$; Figure 7B). TRAP values in all groups still significantly exceeded those in healthy individuals. No statistical significance could be found between age-matched MS female and male patients in terms of myelin-specific TRAP values.

DISCUSSION

The question of whether or not myelin-autoreactive T-cells (PB-MATC) are more numerous in peripheral blood of MS patients compared to healthy individuals (HI) is an important issue for the understanding of the disease process. Most studies have reported similar frequencies of autoreactive T-cells in groups of MS patients and HI (4-8, 20-21) including our own on $CD8^+$ T-cells (3). However, some reports have shown higher frequencies in MS patients (22-24) or a higher level of myelin autoreactivity in MS patients without a specific estimate of PB-MATC frequencies (25-26). Two important factors might underline these discrepancies: the type of autoantigens used and the PB-MATC detection method. In fact, none of these studies have used whole myelin extract as the autoantigen but myelin-derived peptides or molecules which likely do not represent all determinants that could be involved in the disease. In addition, the ELISPOT or LDA readouts only detect autoreactive T-cells secreting a limited number of cytokines (usually INFγ) or able to proliferate, but not cells secreting other cytokines or with a high activation threshold. For example Bieganowska et al (27) reported a frequency of blood T-cells expressing the MBPp85-99-associated TCR chain transcripts as high as 1/300, which far exceeded the frequencies they calculated by LDA (1×10^{-5}-10^{-6}).

In an attempt to better estimate myelin autoreactive T-cell frequency, we adopted in this paper two novel approaches including the use of whole myelin extract as the autoantigen and the transfer of plasma-membrane proteins from autologous APC to T-cells upon immune synapse formation, a cytokine and cell proliferation independent readout which measures T-cell engagement in an immune synapse, an obligatory step for a T-cell immune response (28) and allows an estimation of antigen-specific T-cell frequency whatever the restricting MHC allele (12-13,15-16, 29). Utilization of whole myelin extract was suggested by the *in vivo* observation where monocytes/macrophages contain myelin determinants in brain tissue during MS (2). Because the TRAP has been predominantly described in animal models (12-13, 16), our study conducted in humans included certain validation steps. We first tested TRAP using CMV-

pp65 antigen in individuals with positive CMV serology and by testing the response to whole myelin in individuals in whom the presence of circulating $CD8^+$ PB-MATC had been pre-established by ELISPOT (3). About 4% blood memory T-cells of CMV^+ donors were pp65-TRAP reactive whereas no response was observed in CMV^- subjects. These frequencies in subjects with positive serology at a distance from the acute disease are within the range of frequencies reported in human subjects using CMV-specific tetramers (30) or by flow cytometry intracellular cytokine staining (31). In addition, pp65-TRAP reactivity correlated to pp65 antigen ability to induce T-cell proliferation (CSFE) or IFNγ secretion (ELISPOT). However, pp65-$TRAP^+$ T-cell frequencies were 94 fold higher than those measured by the ELISPOT, suggesting that using IFNγ as the unique parameter to measure the T-cell response can result in a gross underestimation of the frequency of committed cells as also reported recently in animals where the authors (16) have also found comparable differences in the frequencies estimated in ELISPOT (0.01%) and TRAP assays (4.77%). Importantly, TRAP detected a CD8 response both in the presence of CMH class I restricted peptides and whole myelin extract in the test, indicating cross-presentation to CD8+ T-cells.

Using this experimental strategy in MS patients (n=22) and age/gender matched HI (n=18), we showed that 3.5% (1-10%) of MS patient T-cells acquired labeled plasma-membrane proteins from their myelin-pulsed autologous APC which strongly exceeded the background signal and what was observed in the HI group. The antigen-specificity of the TRAP phenomenon has also been suggested by linear correlation with tetramer reactivity, dependence upon MHC loading with the specific antigenic peptide (12-13, 15-16) and cytotoxic function of isolated $TRAP^+$ T-cells towards target cells pulsed with specific antigenic peptides (13). Despite these studies suggesting that TRAP is TCR-dependent, we found that MHC blocking did not eradicate the background signal, indicating that at least some memory T-cells interact with monocytes in a TCR-independent manner. This is also suggested by the relatively high background signal we observed in the CMV-tetramer-sorted T-cell line. It is thus unlikely that this background only reflects the physiological size of an endogenous T-cell memory pool against environmental antigens and

engaging TCR-mediated synapses with APC. Such antigen-independent background signals have also been observed in other TRAP studies (13, 16, 32-33). For these reasons our data and conclusions were only based on the net increase in TRAP signal observed in the absence and presence of whole myelin extract in the test. Interestingly, this antigen-mediated increase was abolished by blocking CMH-II in $CD4^+$ T-cells. In addition, the background levels were identical in MS patients and HI who thus *only* differed after the addition of myelin antigens. The importance of using total myelin extract as antigen was also suggested by the fact that total myelin extract-induced ELISPOT also discriminated MS patients from normal individuals.

The data also show that whereas the expression of some activation markers (CD95 and HLA-DR) did not differ between $TRAP^-$ and $TRAP^+$ T-cells, CD40L was expressed at higher level on $TRAP^+$ T-cells. CD40L has indeed been reported to be involved in immune synapse formation, APC maturation and their ability to prime T cell responses (34-35) suggesting that the antigen-driven TRAP signal was mediated by cognate interaction of T-cells with autologous APC. Finally, increased $CD62L^{low}$ cells of $TRAP^+$ phenotype suggests that these cells could gain access to the brain by crossing the BBB. This possibility is also suggested by the significant correlations between TRAP values and more recent relapses or EDSS scores in MS patients.

The median frequency of myelin-driven autoreactivity in memory T-cells of MS patients 2.07% (range 0-4.24%) belongs to another level of magnitude compared to previously estimated frequencies in multiple sclerosis, including LDA ($0.9\text{-}1.2\times10^{-6}$) or ELISPOT studies ($1\text{-}4\times10^{-5}$) (3, 5, 6, 8, 21). Thus, estimation of autoreactive T-cell frequency is highly dependent on methodology and most likely has been underestimated as shown by Tomaru et al (15) when measuring HTLV and CMV-specific $CD8^+$ T-cells by a modified TRAP assay. Lipids (70% of whole myelin) have been reported to possibly exert a co-stimulatory function (36-37). A lipid-bound native-like preparation of MBP has been associated with a higher T-cell proliferation response in MS patients than in HI compared to purified lipid-free MBP (38). For this reason we tested apoliprotein as an irrelevant antigen, prepared under conditions similar to our

whole myelin extract, and we did not find an increased TRAP response in MS patients or in HI, suggesting that the lipid associated with proteins was not sufficient to trigger TRAP. The myelin-TRAP reactive T-cell subpopulation likely contains multiple antigen specificities, which also partly explains the high frequency of T-cells responding to myelin extract and might be related in part to a molecular mimicry phenomenon as hypothesized in MS (39-40). High PB-MATC frequencies in MS patients could therefore reflect continuous stimulation by environmental antigens expanding the initial anti-myelin memory pool. Finally, and with the caution required to interpret relatively small (but also clinically homogeneous) cohorts of patients, our approach was able to discriminate MS patients according to the presence of recent relapses and severity of the disease (EDSS-SC), suggesting that the test could be useful in clinics either as a prognostic or a diagnostic tool.

Collectively, our data show a high frequency of anti-myelin memory $CD3^+$ T-cells in the blood of MS patients with an activated phenotype $CD40L^+CD62L^{low}$. In contrast to most of the previously published studies, this finding suggests an abnormal and strongly enlarged autoreactive memory T-cell pool in MS, therefore strongly supporting the autoimmune etiology of the disease. The measurement of surface membrane protein exchange in combination with the use of whole myelin extract as the triggering antigen would thus allow substantial progress in the characterization of the autoreactive T cells in MS and possibly other autoimmune diseases.

METHODS

Patients and control individuals. Untreated patients with relapsing-remitting MS (18 females and 4 males, median age 36 years, range 22-60 years) were enrolled in the study. Three patients were seen at the time of their first clinical event (Clinically Isolated Syndrome) but finally had MS according to the MacDonald's criteria. The clinical information relative to each enrolled MS patient is detailed in Table 1. MS was diagnosed according to the revised Mc Donald criteria (41) with EDSS scores ranging from 0 to 4.5, and patients were recruited at the Neurology Department of the Nantes University Hospital. Age- and gender-matched healthy donors (n=18) were selected for comparison (14 females and 4 males, median age 36 years, 24-62 years). In addition, the blood samples of 5 CMV^+ and 5 CMV^- patients under hemodialysis were used in preliminary TRAP assays. Blood samples of 2 MS patients and 2 healthy individuals with blood $CD8^+$ T cells reacting to MHC class I-restricted linear MBP peptides described in our previous study (3) were also included in the preliminary TRAP assays. The study design was approved by the University Hospital Ethical Committee and all the patients signed an informed consent

Blood sample processing. Blood samples from each enrolled individual were coded, and PBMC were isolated from fresh blood samples on a Ficoll gradient by gently overlaying 20 ml of 2 fold-diluted blood in PBS 1× on 10 ml Ficoll (lymphosep., Biowest, Nuaille, France) in a 50 ml tube. The tubes were centrifuged at 2,500 rpm/min for 20 min at 20°C, and the lymphocyte layer at the ficoll interface collected and washed twice in PBS 1× and counted in 0.2% Eosin (viability at least > 95%). $CD45RO^+$ memory lymphocytes and $CD14^+$ monocytes were positively selected with specific microbead-antibody conjugates in accordance with the manufacturer's instructions (Miltenyi Biotech, Paris, France). Briefly, $50\text{-}100\times10^6$ PBMC were first incubated in 400-800 µl staining buffer (0.5% bovine serum albumin (BSA) and 2 mM ethylene diamine tetraacetic acid (EDTA) in PBS 1× supplemented with 100-200 µl undiluted anti-CD45RO or anti-CD14 microbead-coupled antibodies (Miltenyi Biotech). The cells were incubated with gentle agitation for 15 min at $^+4$°C, washed, and resuspended in 5-10 ml staining buffer.

Cells were sorted through manual columns hung on magnets to allow binding of labeled cells and elimination of non-labeled cells. Labeled cells were finally recovered by removing the columns from the magnet and flushing with staining buffer to detach the cells. The purity yield was routinely > 95%.

In the preliminary TRAP validation experiment, frozen PBMC of MS patients previously tested for their myelin autoreactivity were included in the study. Frozen aliquots were thawed by partly melting them in a water bath at 37°C and the cells rapidly transferred to pre-warmed culture medium. The cells were then collected by centrifugation at 1000 rpm/min and counted. RPMI culture medium (Sigma, Saint-Quentin Fallavier, France) supplemented with 100 U/ml penicillin, 100 µg/ ml streptomycin, 2 mM L-glutamine, and with 2% fetal calf serum (Biowest), served as a culture medium throughout the study.

T-cell clones. Biotinylated pHLA-A2 monomers, $pp65_{(495-502)}$/A*0201 were synthesized as previously described (42) by the IFR26 protein core facility (Nantes, France).The HLA-A2 heavy chain carrying an Ala to Val substitution in the α3 domain at Position 245 was used to reduce the affinity for CD8 co-receptor. A tetramer was generated after incubation of biotinylated pMHC pp65/A*0201 monomer with PE-conjugated streptavidin. The $pp65_{(495-502)}$/A*0201-reactive sorted T-cell clone and EBV-transformed B-lymphoblastoid cell line (ETB-LCL) were kindly provided by Alexis Morice and Elisabeth Chalmeau (INSERM U892, Nantes, France). ETB-LCL was typed by HLA class I DNA sequencing.

Antigens. Cytomegalovirus (CMV) recombinant pp-65 protein (Miltenyi Biotech) was used at the dose recommended by the manufacturer. Brain-derived myelin basic protein (MBP) and proteo-lipid protein (PLP) were from ABD Serotec (Oxford, England), they were dissolved in RPMI culture medium and used at 30 µg/ml for T-cell stimulation. Whole human myelin extract (1 mg lyophilized product) was from AbD Serotec. Myelin was re-suspended in RPMI at 0.5 mg/ml, roughly vortexed/sonicated, and 10 µg was used for T-cell stimulation. Apolipoprotein (ABD Serotec) was treated as myelin and served as a control for cell reactivity to lipids. Myelin-protein derived linear peptides were previously described (3)

and were all synthesized (Mimotopes, Clayton Australia) and dissolved in 4% DMSO. All peptides were used at 10 µg/ml for cell stimulation.

Myelin labeling and endocytosis. Whole myelin extract (1 mg) was washed in PBS 1× by centrifugation at 2500 rpm for 5 min, then resuspended in biotin solution (5 mM in PBS 1×) (biotin purchased from Perbio Science France SAS, Brebieres, France), vortexed for 20-30 s, and incubated for 30 min at 37°C. Excess biotin was removed by washing. The biotinylated myelin pellet was then labeled with fluorochrome-streptavidin conjugates (Beckman Coulter) for 30 min at 37°C. The tubes were centrifuged to recover labeled-myelin pellet, washed in PBS 1× by centrifugation (2500 rpm for 1 min repeated three times) to eliminate free fluorochromes, and finally resuspended in 1 ml PBS 1×. Next, 10 µg of labeled myelin was added to 1×10^5 enriched $CD14^+$ monocytes in 100 µl RPMI overnight in the presence or absence of 10 µM phenylarsine oxide (APO) (Sigma). Non-labeled myelin or cells incubated alone served as negative controls. Myelin-fed monocytes were washed at least three times in PBS 1× containing 2 mM EDTA (Sigma) to eliminate free/surface-adsorbed myelin, permeabilized in PERMFIX 1× solution (BD Biosciences Le-Pont-de-Claix Cedex, France) and analyzed on a flow cytometer (LSRII, BD Biosciences). The background signal was defined by cells incubated alone or with unlabeled myelin. Monocytes phagocyting myelin were characterized by the intracellular presence of the marker initially used to label myelin. In other assays, monocytes were pre-incubated with whole myelin overnight, myelin excess was eliminated by repeated washing steps, and then monocytes were analyzed for the expression of HLA-DR.

TRAP assay. The TRAP or trogocytosis protocol was performed as previously described (12,13) but with modifications to optimize the direct *ex vivo* detection and quantification of myelin-autoreactive T-cells in human blood samples. Purified $CD14^+$ monocytes (3×10^5 from each MS patient and HI) were incubated with antigens overnight (for peptides) or 48 h (myelin and soluble proteins) in 2% SVF-RPMI in 96 round-well plates. Meanwhile, the autologous $CD45RO^+$ T-cells purified on the same day were maintained under resting conditions in culture medium while the monocytes were "fed" with myelin.

Antigen-sensitized monocytes were then washed twice at 2,500 rpm for 40s, biotinylated with 50 µl of 5 mM biotin for 30 min at 37°C, then washed and labeled with fluorochrome-strepdavidin conjugates for 30 min at 37°C. Labeled monocytes were mixed with 3×10^5 purified $CD45RO^+$ lymphocytes in the presence of replenished antigens, centrifuged at 2,500 rpm for 40s to promote cell-cell contacts, and incubated overnight. At the end of the incubation, co-cultures in the plates were centrifuged to discard culture medium, and the cell pellets were re-suspended in 2 mM EDTA in PBS 1× containing the labeling antibodies (200 µl volumes). The plate contents were then transferred to clean tubes for cytometer reading, vortexed 20-30s to disrupt conjugates, and antibodies left to bind for 20 min at $^+4$°C during shaking. The lymphocytes, identified on the basis of their FCS and SSC properties and by CD3 staining, were then gated and analyzed for the acquisition of monocyte-fluorescence expressed as [TRAP (%) = number of fluorescent T-cells / total number of gated T-cells × 100]. At least 20, 000 events were recorded for each sample tested.

For TRAP assay blocking experiments, fluorochrome-labeled monocytes were incubated with 500 ng/ml of mouse polyclonal anti-HLA-II (TU39, BD Biosciences) for 1 h at 37°C or purified mouse IgG. The $CD45RO^+$ enriched cell fraction was then added and the TRAP assay performed as described above.

In the TRAP experiments using the pp65-tetramer-sorted T-cells (responder) and ETB-LCL (APC), ETB-LCL were biotinylated with 1 mM biotin for 30 min, washed, labeled with streptavidin-fluorochrome conjugates for 30 min, and finally pulsed with 10 µM pp65-derived peptide at 37°C for 1 h. For the TRAP reaction, labeled and pp65-peptide loaded ETB-LCL were mixed with $pp65_{(495-502)}$/A*0201 T cells at a ratio of 1 to 4 for 1 hour. An anti-CD3 T-cell marker, together with pp65-tetramer, were used to identify $pp65_{(495-502)}$/A*0201 T cells and to measure TRAP frequency.

Flow cytometry immuno-staining. The human antibodies were conjugated to phycoerythrin (PE), fluorescein isothiocyanate (FITC), peridinin chlorophyll protein (PerCP), cy-chrome PE, or allophycocyanin (APC) including anti-CD4 (clone SK3), anti-CD8 (clone 53-1.7) anti-CD45RO (clone

UCHL1), anti-CD3 (clone HIT3a), anti-CD8 (clone SK-1), anti-CD25 (clone 2A3), anti-CD62L (clone DREG-56), anti-CD95 (DX2), anti-HLA-DR (clone L2-43 (G46-6)), and the unlabelled anti-HLA-II antibody (clone TU39). All these antibodies were purchased from BD Biosciences. Anti-CD40L-PE conjugate was from Beckman coulter (Villepinte, France).

Proliferation assay. Equivalent numbers of $CD45RO^+$ T-cells from CMV^+ and CMV^- normal donors (3×10^5) were loaded with CSFE (5 µM) for 15 min at 37°C, washed three times, and co-cultured with 10^5 syngeneic $CD14^+$ monocytes (1/3 ratio) in the presence or absence of CMV-pp65 recombinant protein for 3 days. Proliferation in collected cocultures was defined as the loss rate of the CSFE dye in gated $CD3^+$ T-cells by comparison to those cultured without the antigen (used to set the background quadrants). Cells were stained for the activation marker CD25 to ensure proliferating cells are activated (by measurement of $CD25^{bright}$ expression).

ELISPOT assay. Purified monocytes (10^5) were mixed with 3×10^5 $CD45RO^+$ lymphocytes under the conditions of the TRAP assay in each ELISPOT well plates (Thermo scientific, Rockford, USA), in the presence or absence of whole myelin, and incubated overnight (longer incubations resulted in a stronger background signal). As an additional control, $CD45RO^+$ lymphocytes were cultured separately in the presence or absence of myelin extract. The detection of IFNγ-producing cell spots was performed in accordance with the manufacturer's instructions. The spots were counted by the ELISPOT reader system (AID, Strassberg, Germany) as previously described[5]. The ELISPOT positive reactivity of the co-cultures against myelin was defined as a number of spots at least three times the global backgrounds from cocultures incubated alone and from myelin-stimulated $CD45RO^+$ lymphocytes cultured alone. In these assays, whole myelin extract added alone without the cells or $CD14^+$ monocytes cultured alone with myelin did not form characteristic IFNγ-spots after stringent washing of the ELISPOT filters.

Statistical analysis. The two-tailed non-parametric *Mann-Whitney test* was used for comparison between MS patients and healthy individuals. *Kruskal Wallis* test was used for comparison of more than 2 groups. Significant differences were set at $p < 0.05$ (*), $p < 0.001$ (**), $p < 0.0001$ (***) (GraphPad Prism 4).

ACKNOWLEDGMENT

This work was supported by the *Association pour la Recherche sur la Sclérose En Plaques* (ARSEP, Paris, France). We would like to thank Dr Joanna Chess-Ashton for the manuscript English editing and Dr Yohann Foucher for his helpful advices on statistical analyses.

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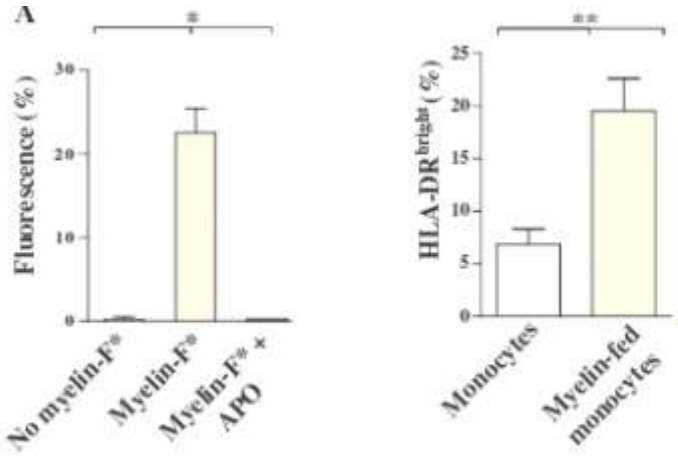


Figure 1. phagocytosis of whole myelin extract by CD14$^+$ monocytes. A: myelin that h biotinylated and labeled with fluorochromes (myelin-F*) was added to freshly-isolated m overnight. Permeabilized monocytes were tested for myelin fluorescence. Phagocytosis of n blocked by phenylarsine oxide (APO) (n=4, 2 MS patients and 2 HI). **B**: HLA-DR staining i monocytes displays HLA-DR^{dim} and HLA-DR^{bright} subpopulations (high surface expression significant increase in the HLA-DR^{bright} population when CD14$^+$ monocytes had phagocyted myc figure indicates average value ± SD from 4 individuals each tested separately.

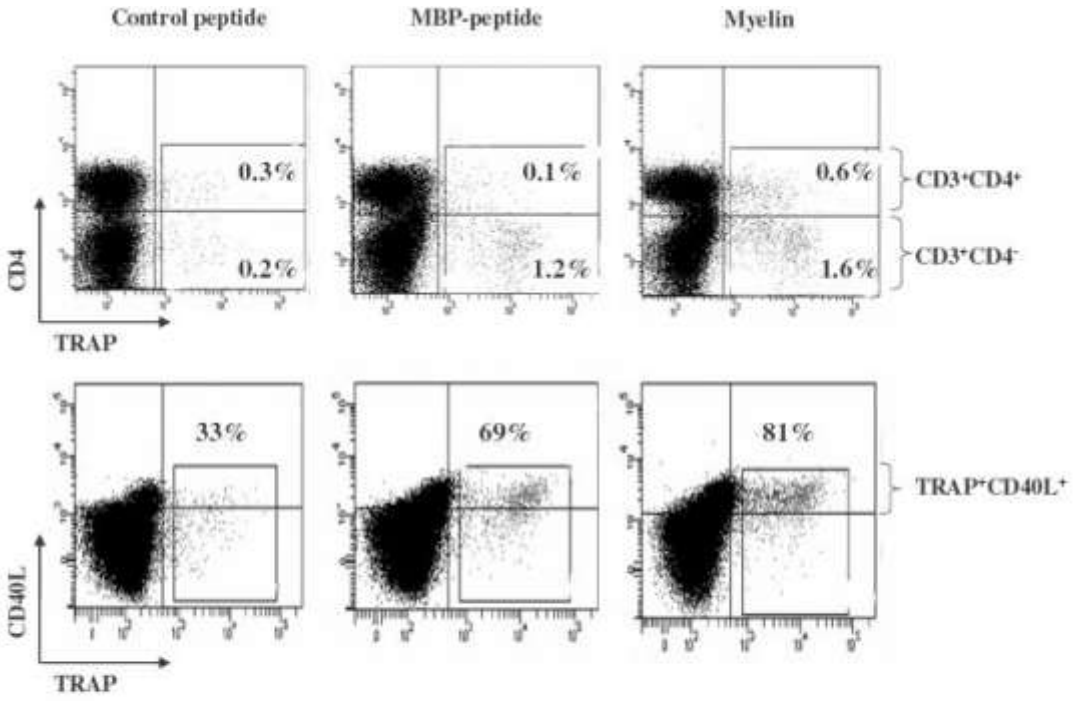


Figure 2. TRAP assay detects myelin reactivity in MS patients with myelin-specific CD8$^+$. TRAP assay was performed with monocytes pulsed with whole myelin extract or other myelin antigens as indicated. TRAP is displayed in the CD3$^+$ gate. The background was set using cocultures with monocytes pulsed with non-reactive peptide. The figure shows an experiment performed with one of the two selected MS patients. The top panels show the background, TRAP signal with MBP-derived peptide to which the patient had initially reacted, and TRAP with whole myelin extract. From left to right, bottom panels show CD40L expression as a function of the TRAP signal in the presence of irrelevant peptide, reactive MBP-derived peptide, and whole myelin extract.

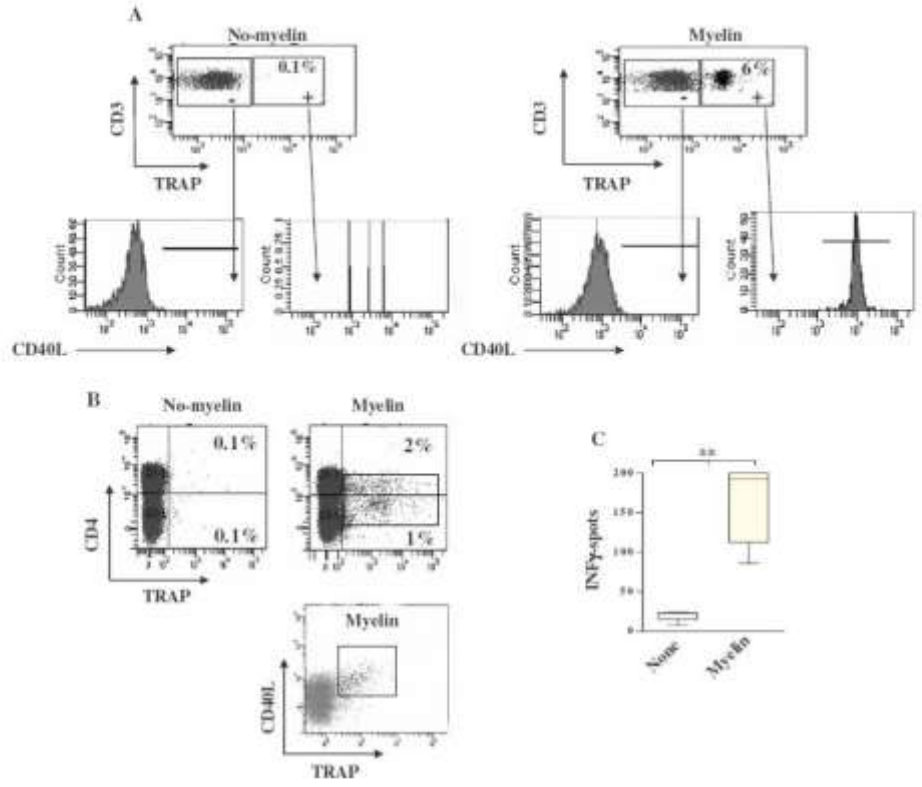


Figure 3. TRAP in healthy individuals with CD8$^+$ T-cells reactive to myelin-derived peptides. Fresh blood samples from the two healthy individuals were tested by the TRAP. **A**: one healthy donor with a positive TRAP signal (6% TRAP$^+$ *versus* 0.1% background). The histograms indicate CD40L staining in gated TRAP$^-$ and TRAP$^+$ subpopulations. Bars indicate CD40L positive signal. **B**: the TRAP signal is shown in gated CD3$^+$ and CD4$^{+/-}$ T-cells in the other healthy individual with CD40L expression in TRAP$^+$ T-cells. **C**: the same individuals were tested by ELISPOT by coculturing CD14$^+$ monocytes and CD45RO$^+$ lymphocytes in the presence of whole myelin extract overnight. The histogram shows average values from the four individuals tested separately in the presence of myelin (median 192.5 INFγ-spots, range 86-245) and in its absence (22.5 INFγ–spots, range 8-23, $p < 0.0002$, Mann-Whitney test).

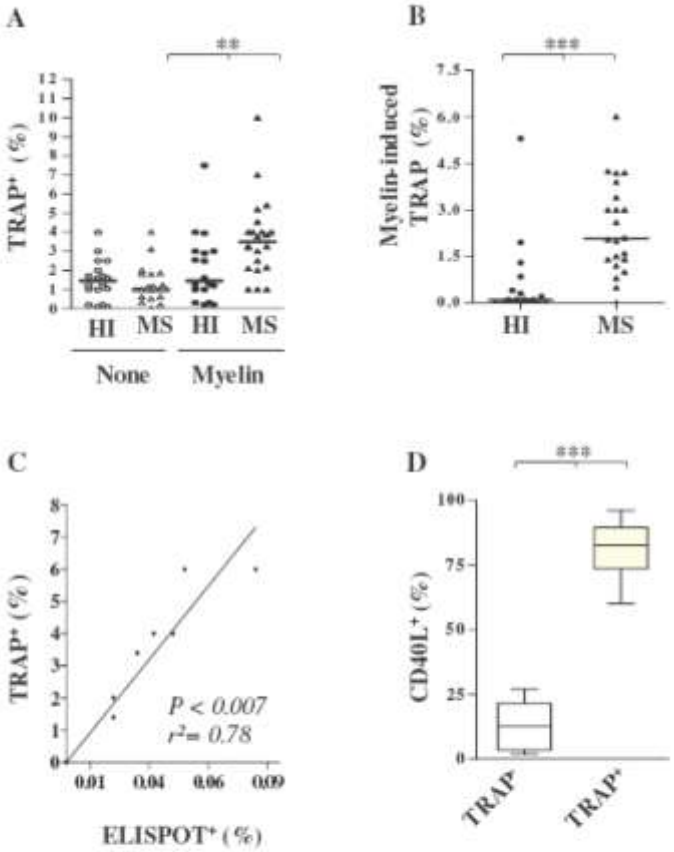


Figure 4. **Myelin-TRAP frequencies (%) are significantly higher in untreated MS patients at the onset of their disease by comparison to age-matched healthy individuals**. **A**: TRAP values in healthy controls (n=18) or MS patient group (n=19). The difference was significant between MS patients and HI in the presence of myelin (column C versus D, $p<0.01$, Mann-Whitney test) and within the group of MS patients in the presence or absence of myelin (column B versus D, $p<0.001$, Wilcoxon test). **B**: background was subtracted from each TRAP value measured in the presence of myelin in both groups then myelin-specific average TRAP values compared ($p<0.0001$, Mann-Whitney test). **C:** plotting of %TRAP *versus* ELISPOT in MS patients shows a significant correlation ($r^2=0.82$, $p<0.0001$, Linear Regression test). **D**: $TRAP^+$ T-cells reacting to the whole myelin extract expressed CD40L at higher levels than TRAP- ones (n=9, p<0.001, Mann-Whitney test).

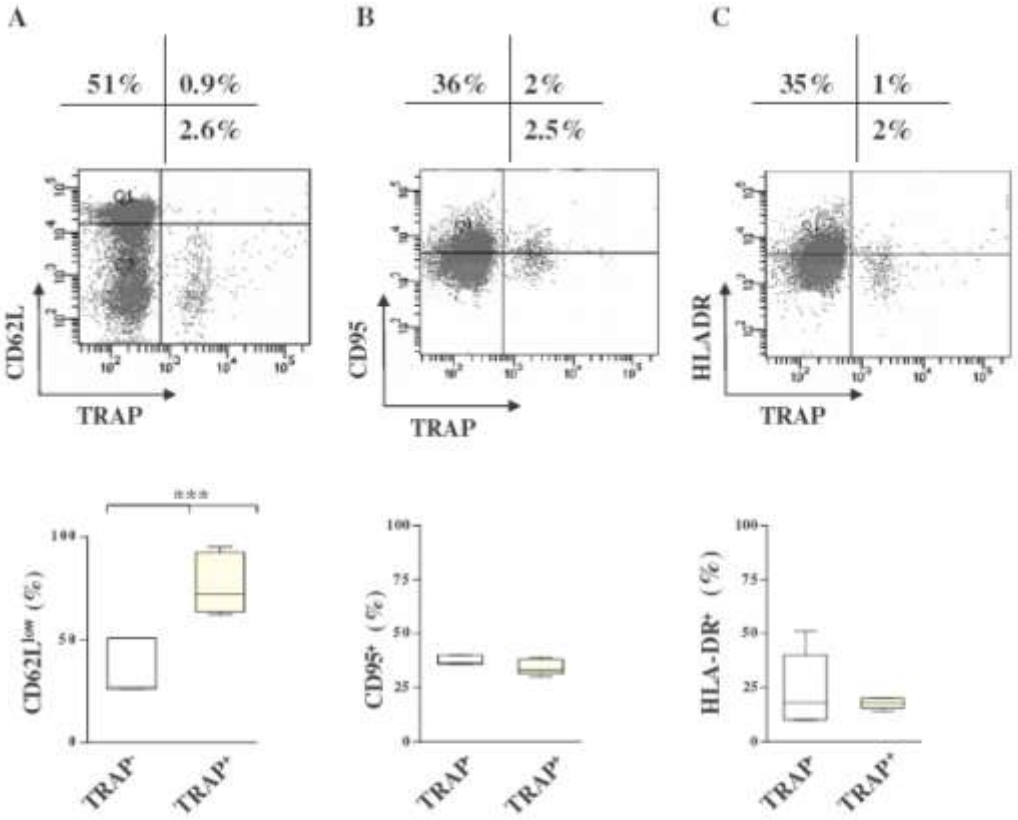


Figure 5. TRAP$^+$ cells are significantly more CD62L^{low} than TRAP$^-$ ones but do not differ in terms of CD95 or HLA-DR expression. A: CD3$^+$TRAP$^+$ T-cells display significantly more CD62L^{low} than TRAP$^-$ counterparts (one representative dot blot). **B**: TRAP$^+$ and TRAP$^-$ T-cells expressed CD95 at similar levels. **C**: TRAP$^+$ and TRAP$^-$ T-cells expressed HLA-DR at similar levels. Corresponding histograms below show data for each marker pooled from 5 MS patients.

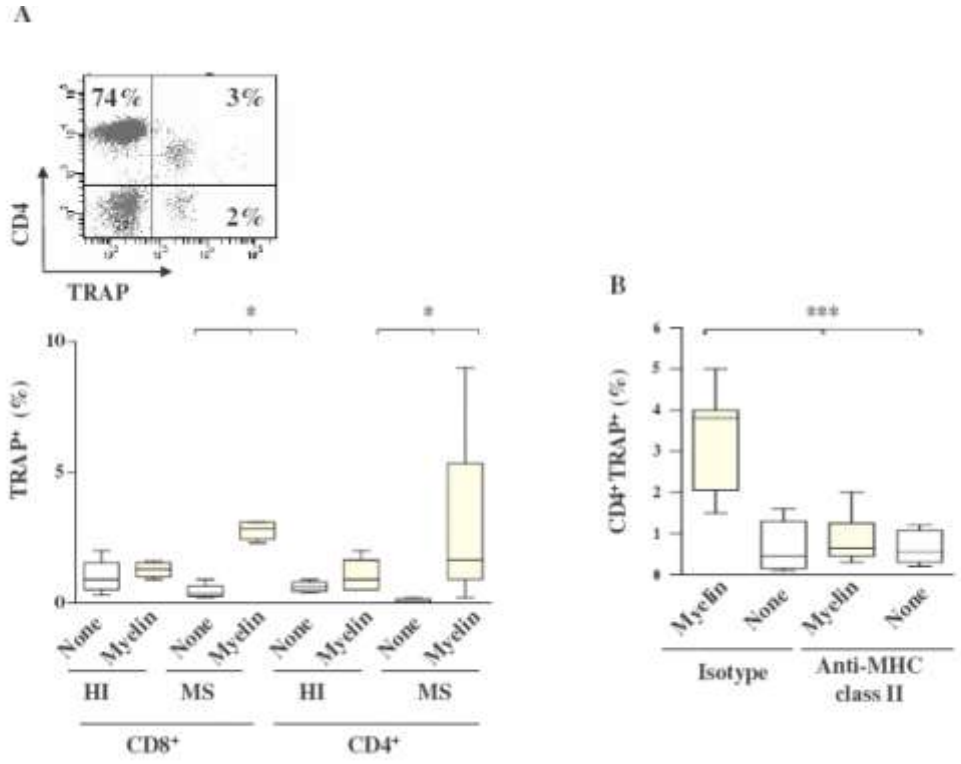


Figure 6. TRAP$^+$ cells are detected at roughly similar levels in CD4$^+$ and CD8$^+$ T-cells, and anti-MHC class II blocking antibody significantly inhibited TRAP reactivity in CD4$^+$ T-cells. A: TRAP signal in CD4$^+$ or CD8$^+$ T-cells (insert). The histogram shows median TRAP frequency in CD4$^+$ and CD8$^+$ T-cells. Data were pooled from 4 HI and 5 MS individuals. **B**: blocking of MHC class II at the monocyte surface during the TRAP assay results in a significant inhibition (n=8).

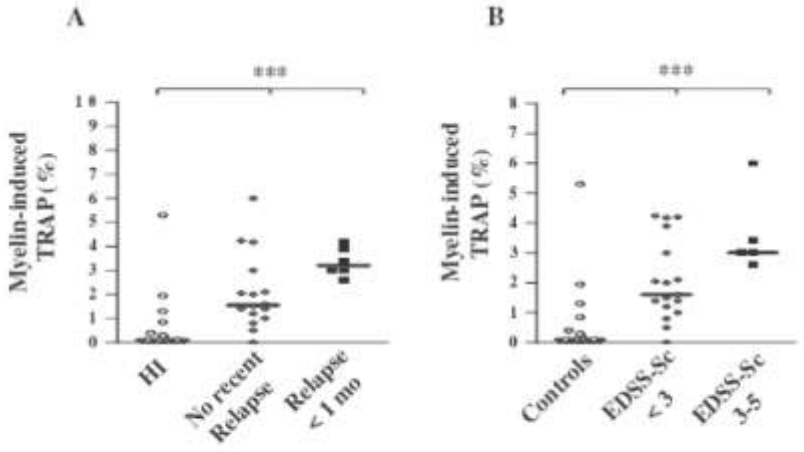
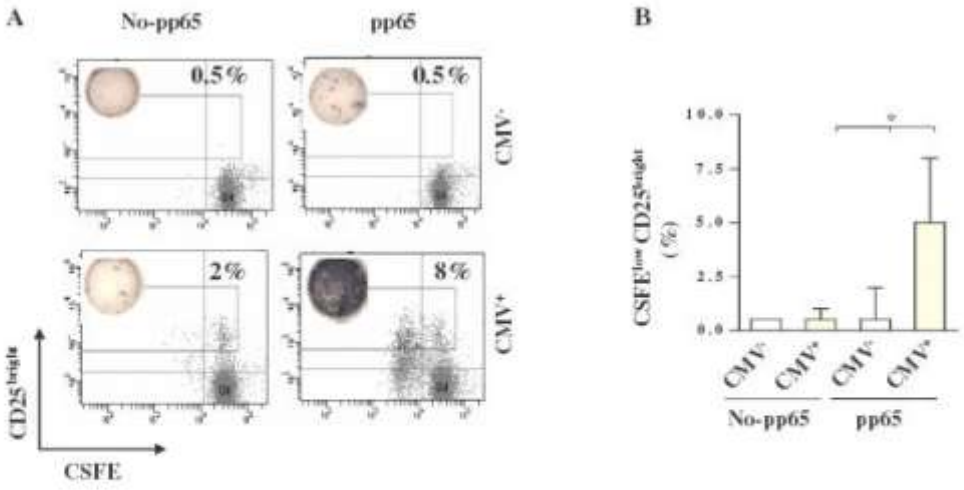


Fig 7. Myelin-specific TRAP values are significantly associated with history of relapses and EDSS scores in the studied MS patients. **A**: MS patients were subdivided into two subgroups based on their inclusion at the time of a relapse or a remission. Both groups are compared with the study healthy controls. **B**: MS patients were subdivided into 2 subgroups based on EDSS scores (< 3) and (3-5). The TRAP values were statistically different between the groups ($p<0.001$, Kruskall-Wallis test).

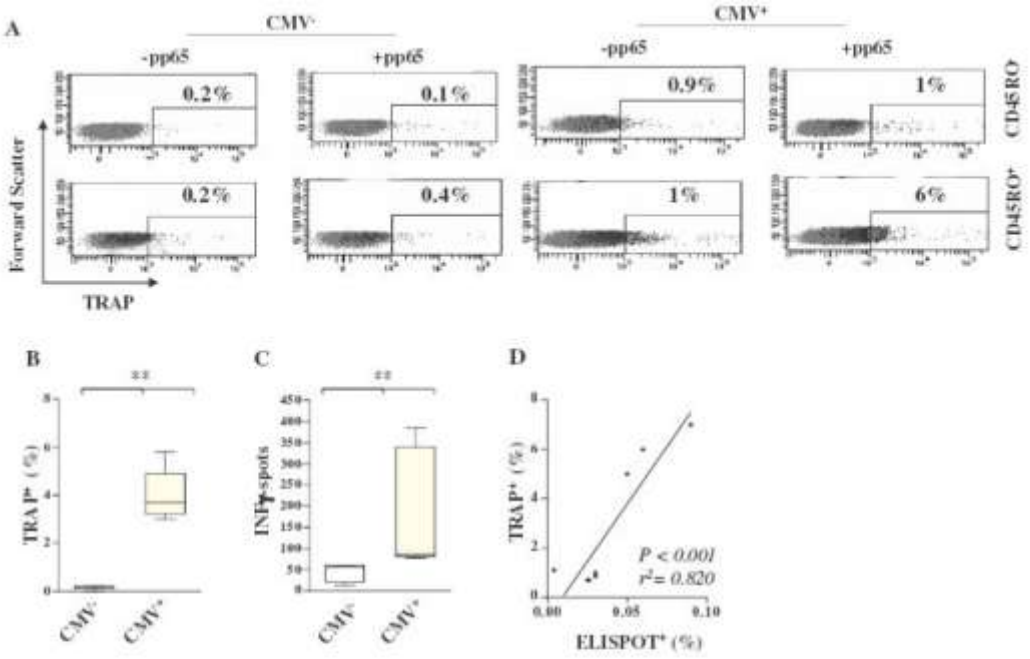
Table 1: Clinical characteristics of MS patients. The table displays all MS patients enrolled in the study at the time of blood collection.

Patient code	Age (year)	Gender	Disease duration (years)	Time until last relapse (months)	EDSS score
MS≠1	34	M	5	5	0
MS≠2	58	F	11	2	3
MS≠3	60	F	1	12	2
MS≠4	23	F	4	18	2.5
MS≠5	30	F	2	21	1
MS≠6	28	M	1▪	1	1
MS≠7	29	F	8	1	2
MS≠8	28	F	1	12	1
MS≠9	27	F	5	6	2.5
MS≠10	50	F	7	48	1.5
MS≠11	37	F	5	60	0
MS≠12	40	F	10	6	1.5
MS≠13	35	F	4	2	1.5
MS≠14	35	M	10	3	3
MS≠15	35	F	1▪	1	2
MS≠16	24	F	1	7▪▪	2
MS≠17	42	F	5	3	3
MS≠18	34	F	10	7▪▪	3
MS≠19	33	F	9	24	4.5
MS≠20	55	F	1.5	18	2
MS≠21	22	M	6°	4	1
MS≠22	38	F	0.5▪	15▪▪	2

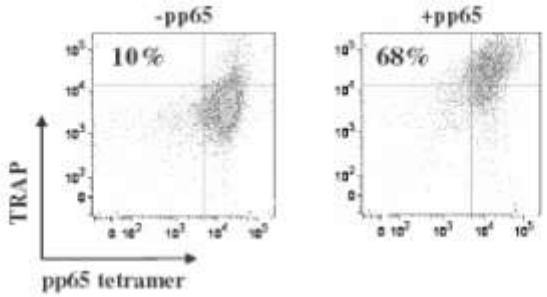
▪ indicates times in months; ▪▪ indicates times in days.



Supplementary Figure 1. Induction of proliferation and IFNγ-ELISPOT response of memory CD45RO$^+$ T-cells by autologous CD14$^+$ monocytes pulsed with CMV-pp65 protein. **A:** CD14$^+$ monoytes purified from blood samples of CMV$^-$ and CMV$^+$ individuals were pulsed or not with CMV pp65 antigen, and then cultured with autologous CSFE-labeled CD45RO$^+$ lymphocytes for 3 days (1/3 ratio). Cultures were stained with anti-CD3 and anti-CD25, and analyzed for proliferation. Proliferation is shown by CSFE dilution (shift to the left) and activation shown by CD25^{bright} expression. ELISPOT assays (round inserts) were also performed using pp65-pulsed monocytes cultured with CD45RO$^+$ cells overnight. The CMV$^+$ individual exhibits proliferation, and increased IFNγ production in ELISPOT. **B**: The histogram shows %CSFE-CD25^{bright} average values ± standard deviation (SD) in CMV$^-$ (n=3) and CMV$^+$ (n=3) individuals. The asterisk (throughout the figures) denotes statistical significance ($p < 0.05$; Kruskal Wallis test).



Supplementary Figure 2. TRAP induction in memory CD45RO$^+$ T-cells of CMV$^+$ individuals by CMV-pp65 pulsed autologous monocytes. A: CD14$^+$ monocytes from one CMV$^+$ and one CMV$^-$ individual were pulsed with CMV pp65 or left untreated for 2 days, then biotinylated and labeled with streptadividin-fluorochrome. Labeled monocytes were then cultured with CD45RO-enriched or CD45RO-depleted lymphocytes (1/3 ratio, $10^5/3\times10^5$) overnight. TRAP was calculated by determining the %CD3$^+$ T-cells that had acquired fluorescence from monocytes. The background was defined using cocultures with non-pulsed monoytes. Representative results in the CMV$^-$ and CMV$^+$ individuals are shown in both CD45RO$^-$ and CD45RO$^+$ fractions. TRAP$^+$ T-cells were not apoptotic cells and membrane exchange did not affect their size (forward scatter). **B**: significantly higher TRAP median values in CD45RO$^+$CD3$^+$ T-cells in CMV$^+$ (n=4) compared to CMV- individuals (n=4). **C**: median of IFNγ$^+$ spots detected by the ELISPOT was significantly higher in CMV$^+$ (n=5) compared to CMV$^-$ individuals (n=5). **D**: plotting of %TRAP *versus* ELISPOT values from 5 CMV$^-$ and 3 CMV$^+$ shows a significant correlation.



Supplementary Figure 3. TRAP values in a CMV$^+$ sorted T-cell line stimulated by pp65-stimulated APC (ETB-LCL). TRAP assay was performed with ETB-LCL as APC pulsed or not with pp-65-derived peptide, biotinylated and fluorochrome-labeled, and then mixed with CMV$^+$ T-cells sorted by a tetramer. Left panel indicates the TRAP background (10%) with non-pulsed ETB-LCL and the increased TRAP signal (68%) with pp65-pulsed ETB-LCL (right panel).

DISCUSSION ET PERSPECTIVES

La sclérose en plaques (SEP) est une maladie supposée auto-immune dans laquelle les lymphocytes T semblent jouer un rôle crucial.

Concernant la spécificité des lymphocytes T CD8+ dans la SEP, notre étude a permis de caractériser 69 nouveaux épitopes restreints pour les molécules de CMH de classe I chez l'Homme et peut permettre de développer de nouveaux outils d'exploration des réponses des lymphocytes T CD8+ auto-réactifs dans la SEP (Article I). Il n'y a cependant pas de différence de fréquences des lymphocytes T CD8+ auto-réactifs entre les patients atteints de SEP et les individus normaux, ni de différence de niveau de réponses. Chaque patient atteint de SEP présente de réponses particulières, propres à chaque individu et qui peuvent varier au cours du temps, rendant difficile et aléatoire dans ses conséquences la perspective d'un traitement basé sur les peptides altérés. Celui-ci implique en effet l'élaboration de peptides avec des séquences en acides aminés modifiés et induisant des réponses différentes de celles auto-réactives. L'absence de différence de fréquence des lymphocytes T CD8+ auto-réactifs contre des antigènes de la myéline chez les patients atteints de SEP et les individus normaux pourrait être due à une différence de régulation des réponses de ces études (Zhang et al. 1994 ; Hellings et al. 2001 ; Hollifield et al. 2003) ou un défaut de sensibilité à l'apoptose (Zang et al. 1999 ; Saresella et al. 2005). D'autre part, les lymphocytes T CD8+ des patients atteints de SEP présentent des capacités d'adhérence augmentées, permettant le passage de la BHE (Battistini et al. 2003). La migration de ces lymphocytes T dans le SNC pourrait diminuer leur nombre dans le sang des patients à la différence des individus sains. Par ailleurs, notre test a montré que les lymphocytes T CD8+ auto-réactifs produisent de l'IFNγ (une cytokine Th1) de façon équivalente chez les patients SEP et les témoins. Ce test n'est pas exhaustif et la production d'autres cytokines pourraient différencier les patients atteints de SEP des témoins, comme l'IL17 qui semble jouer un rôle important dans la pathologie.

Les travaux précédemment effectués s'intéressant à la capacité régulatrice des cellules T CD4+CD25high s'accordaient pour montrer un défaut de régulation chez les patients atteints de SEP (Viglietta et al. 2004 ; Haas et al. 2005 ; Kumar et al. 2006 ; Venken et al. Immunology 2008).

Dans l'article II, nous avons voulu explorer à nouveau cette fonction régulatrice tenant compte des données récentes concernant un nouveau marqueur, le CD127 (chaine α du récepteur à l'IL7), pour mieux discriminer les cellules régulatrices. Dans un premier temps, les cellules T CD4+CD25high sont obtenues à partir des PBMC par tri cellulaire réalisé au FACSAria® en plaçant une fenêtre de sélection triant jusqu'à 4% des cellules

CD4+CD25high au sein des lymphocytes T CD4+. Dans ces conditions, nous reproduisons les résultats obtenus par les autres équipes sur le défaut de régulation. Ensuite, nous avons modifié nos critères de tri en plaçant la fenêtre de sélection de sorte que les cellules régulatrices ne représentent qu'au plus 2% des cellules au sein des lymphocytes T CD4+. Les cellules CD4+CD25high ainsi obtenues avec cette fenêtre de tri plus sélective ne présentent plus de défaut de régulation suggérant que lors de la sélection avec une fenêtre de tri plus large des cellules activées ont été isolées en même temps que la population régulatrice d'intérêt.

Dans la suite de l'article II, nous avons utilisé le marqueur CD127 pour mieux discriminer les cellules régulatrices au sein des CD4+CD25high. Les cellules CD4+CD25highCD127- des patients atteints de SEP possèdent une capacité régulatrice similaire aux cellules des témoins appariés selon l'âge et le sexe. À cela s'ajoute les résultats obtenus pour les tests fonctionnels réalisés en présence de MBP. En effet, dans la suite de l'étude, nous avons observé que la capacité de régulation des cellules T CD4+CD25highCD127- sur la prolifération des CD4+CD25- autologues étaient semblables entre les patients atteints de SEP et les témoins en présence de MBP. Nos résultats ne confirment pas ceux précédemment publiés. Dans le cas de tests réalisés avec une stimulation polyclonale, les cellules CD4+CD25high des patients ont présenté un défaut de régulation des cellules CD4+CD25high (Viglietta et al. 2004 ; Haas et al. 2005 ; Venken et al. 2008). Lors des tests fonctionnels avec stimulation par les protéines de la myéline, que ce soit avec de la MOG ou de la MBP, un défaut de régulation des CD4+CD25high est aussi constaté (Haas et al. 2005, Kumar et al. 2006).

Une seule autre étude à notre connaissance a utilisé le marqueur CD127 pour sélectionner les cellules régulatrices dans le contexte de la SEP. Ces cellules ont aussi été choisies en intégrant les marqueurs mémoires et naïfs à la sélection. Les tests de régulation réalisés par Venken et ses collaborateurs ont retrouvé un défaut de régulation. Cependant, les cellules effectrices utilisées dans leur test fonctionnel n'étaient pas des CD4+CD25- mais des CD4+CD25-CD127high (Venken et al. JI 2008). Le défaut de régulation observé peut donc être dû non pas à une régulation défaillante mais à un excès de prolifération des cellules répondeuses.

D'autres études ne rapportent pas de défaut de régulation des CD4+CD25high. En effet, Fransson et ses collaborateurs ont montré que les cellules T CD4+CD25high des patients atteints de SEP ne présentaient pas de fonction régulatrice perturbée aussi bien dans le groupe de patients en rémission que dans celui des patients en poussée par rapport aux individus

sains. Cette étude met en évidence un niveau plus faible de la proportion des cellules T CD4+Foxp3+CD25+CD127- au sein des PBMC des patients (Fransson et al. 2009). Il semble important de noter que les cohortes de patients et de témoins choisies pour ce travail sont composées d'un nombre restreint d'individus. Celle des patients est constituée à la fois des patients en phase de rémission et en phase de poussée, traités ou non par des immunomodulateurs (IFN ou acétate de glatiramère). Venken et ses collaborateurs, eux aussi, n'ont pas montré de fonction régulatrice diminuée des CD4+CD25high chez les patients atteints de SEP de forme secondaire progressive (Venken et al. 2006). Des résultats similaires ont été obtenus par l'équipe de Bluestone chez les patients atteints de diabète de type 1 où l'utilisation du CD127 pour discriminer les Treg ne met pas en évidence de fonction régulatrice perturbée (Brusko et al. 2008).

Pour l'essentiel de ces études, la sélection des cellules régulatrices ne s'est faite uniquement que par l'expression du CD25 et comme nous l'avons montré dans l'article II, la position de la fenêtre de sélection des CD4+CD25high est primordiale. Si celle-ci est moins sélective le défaut de régulation apparaît, ceci suggérant que la sélection des cellules régulatrices en utilisant uniquement le marqueur CD25 peut comprendre des cellules activées perturbant à terme les tests de régulation.

Concernant la fréquence des cellules régulatrices CD4+CD25highCD127-, nous avons obtenu une proportion similaire entre les patients atteints de SEP de forme rémittente et les témoins. Ces résultats sont en accord avec ceux d'autres travaux (Putheti et al. 2004 ; Haas et al. 2005 ; Venken et al. 2006 ; Feger et al. 2007). Néanmoins, d'autres équipes ont montré une fréquence diminuée des cellules régulatrices au sein des PBMC chez les patients atteints SEP (Huan et al. 2005 ; Kumar et al. 2006 ; Venken et al. 2006 ; Venken et al. 2008 ; Fransson et al. 2009). Dans ces études, la fréquence peut être évaluée uniquement en fonction du marqueur CD25 ou en fonction de FoxP3 et de CD25 permettant pour la dernière combinaison une meilleure sélection des Treg.

Miyara et de ses collaborateurs ont subdivisés les Treg en trois sous-populations Fr I, Fr II et Fr III en fonction des marqueurs CD25, CD45 et FoxP3. Ces trois sous-populations sont en proportion différentes dans certaines maladies auto-immunes (Miyara et al. 2009). Basés sur cette subdivision, nos travaux n'ont pas trouvé de différence de fréquence pour les trois fractions étudiées chez les patients atteints de SEP comparées aux témoins. De plus, l'étude de l'expression de molécule d'adhérence à la surface des cellules T CD4+CD25+CD127- n'a pas montré de différence entre les malades et les individus sains. Récemment, il a été suggéré que la localisation du récepteur PD1 (programmed death receptor 1), permettait de distinguer

deux populations au sein des Treg : l'une naïve (localisation intracellulaire de PD1) et la deuxième activée (localisation extracellulaire de PD1) (Raimondi et al. 2006). Saresella et ses collaborateurs ont montré que la proportion des cellules PD1- et PD1+ Treg au sein des PBMC étaient similaires chez les patients atteints de SEP et les témoins (Saresella et al. 2008).

La définition phénotypique des Treg semble donc très complexe. Cette population cellulaire peut être divisée en plusieurs sous-groupes selon les marqueurs utilisés. Ces subdivisions phénotypiques peuvent être liées à des subdivisions fonctionnelles comme il est le cas pour les fractions Fr I, Fr II et Fr III.

La diversité des différentes cohortes de patients atteints de SEP et des témoins étudiées dans les différents travaux exposés précédemment pourrait elle aussi jouer un rôle sur les observations obtenues. En effet, ces cohortes ne sont pas totalement équivalentes sur les critères de l'âge, du handicap et de la durée de la maladie. Haas et ses collaborateurs ont récemment observé que la fonction suppressive des CD4+CD25high est diminuée sous stimulation polyclonale. Les cellules régulatrices T CD4+CD25+CD45RA+CD45RO-Foxp3+ exprimant CD31 (PECAM1) diminuent au sein des PBMC avec l'âge et sont moins importantes chez les patients atteints de SEP, âgés de moins de 30 ans. La fonction régulatrice des CD4+CD25high est altérée chez les patients atteints de SEP âgés de moins de 30 ans. Ce défaut de régulation n'est pas retrouvé dans les deux autres groupes dont l'âge est supérieur à 30 et à 45 ans (Haas et al. 2007). Venken et ses collaborateurs ont observé que la fréquence des cellules Treg naïves ainsi que celles des cellules Treg mémoires diminuent chez les patients atteints de SEP dont l'âge est compris entre 20 et 40 ans. La même observation est faite pour les cellules mémoires chez les patients atteints de SEP dont l'âge est compris entre 40 et 60 ans tandis que pour ce groupe de patients la fréquence des Treg naïves est semblable à celle des témoins (Venken et al. JI 2008).

McKay et ses collaborateurs ont montré que le marqueur CD127 était faiblement exprimé à la surface des différentes sous-populations de cellules régulatrices (CD4+CD25high, CD8+CD28-, CD3+CD56+ (NKT)) chez les patients atteints de SEP de forme primaire progressive ainsi que chez les témoins (McKay et al. 2008). Il a aussi été montré que l'expression de l'ARNm du CD127 est diminuée dans le sang des patients atteints de SEP de forme primaire progressive (Booth et al. 2005).

Les cellules régulatrices T CD4+CD25high ne sont pas uniquement incriminées dans la pathologie de la SEP. Ces cellules ont été aussi étudiées, en particulier, dans le lupus érythémateux systémique (SLE). Les résultats sur les fréquences des CD4+CD25high (Liu et al. 2004 ; Crispin et al. 2003 ; Fathy et al. 2005 ; Yates et al. 2008) et sur leur capacité de régulation (Bonelli et al. 2008 ; Lyssuk et al. 2007 ; Valencia et al. 2007 ; Venigalla et al. 2008) sont eux aussi contradictoires. Deux groupes ont inséré le CD127 à leurs critères de sélection des Treg et la proportion de Treg observée n'est plus diminuée (Venigalla et al. 2008 ; Yates et al. 2008). Horwitz propose la même hypothèse que la notre, suggérant que la sélection des Treg uniquement sur le CD25 ne permet d'obtenir qu'une population hétérogène contaminée par des cellules activées (Horwitz et al. 2008). Néanmoins, aucun test de régulation n'a été réalisé pour le SLE en incluant le CD127 ceci ne permettant donc pas de savoir si le défaut apparent de régulation résulte d'une résistance des cellules répondeuses ou d'une capacité altérée des cellules régulatrices.

Notre hypothèse expliquant le défaut de régulation observé dans la pathologie SEP est une contamination de la population CD4+CD25high par des cellules activées. Dans l'article II, nous montrons que lorsque nous utilisons une fenêtre de tri plus sélective ou lorsque l'on ajoute le marqueur CD127 permettant d'obtenir une population de cellules régulatrices plus pure, la capacité de régulation est semblable entre les patients atteints de SEP et les témoins appariés sur l'âge et le sexe. Les cellules suggérées comme contaminantes seraient donc les lymphocytes T CD4+CD25highCD127+. Dans l'article II, nous montrons que les cellules de patients atteints de SEP de forme rémittente semblent hyperprolifératives sous stimulation polyclonale et présenteraient un profil pro-inflammatoire avec sécrétion d'IL2, d'IFNγ et de TNFα. Lors des tests de prolifération en présence de MBP, la réactivité des cellules T CD4+CD25highCD127+ des malades semblait plus importante à celle des témoins mais pas significativement différente. À notre connaissance, aucun travail n'a étudié les caractéristiques de ces cellules. Notre étude phénotypique n'a pas révélée de différence de fréquence entre les patients SEP et les témoins pour l'expression de marqueurs impliqués dans l'immunité, pour l'expression des récepteurs aux chemiokines mais aussi pour l'expression de molécules d'adhérence impliquées dans la migration des cellules au sein du SNC.

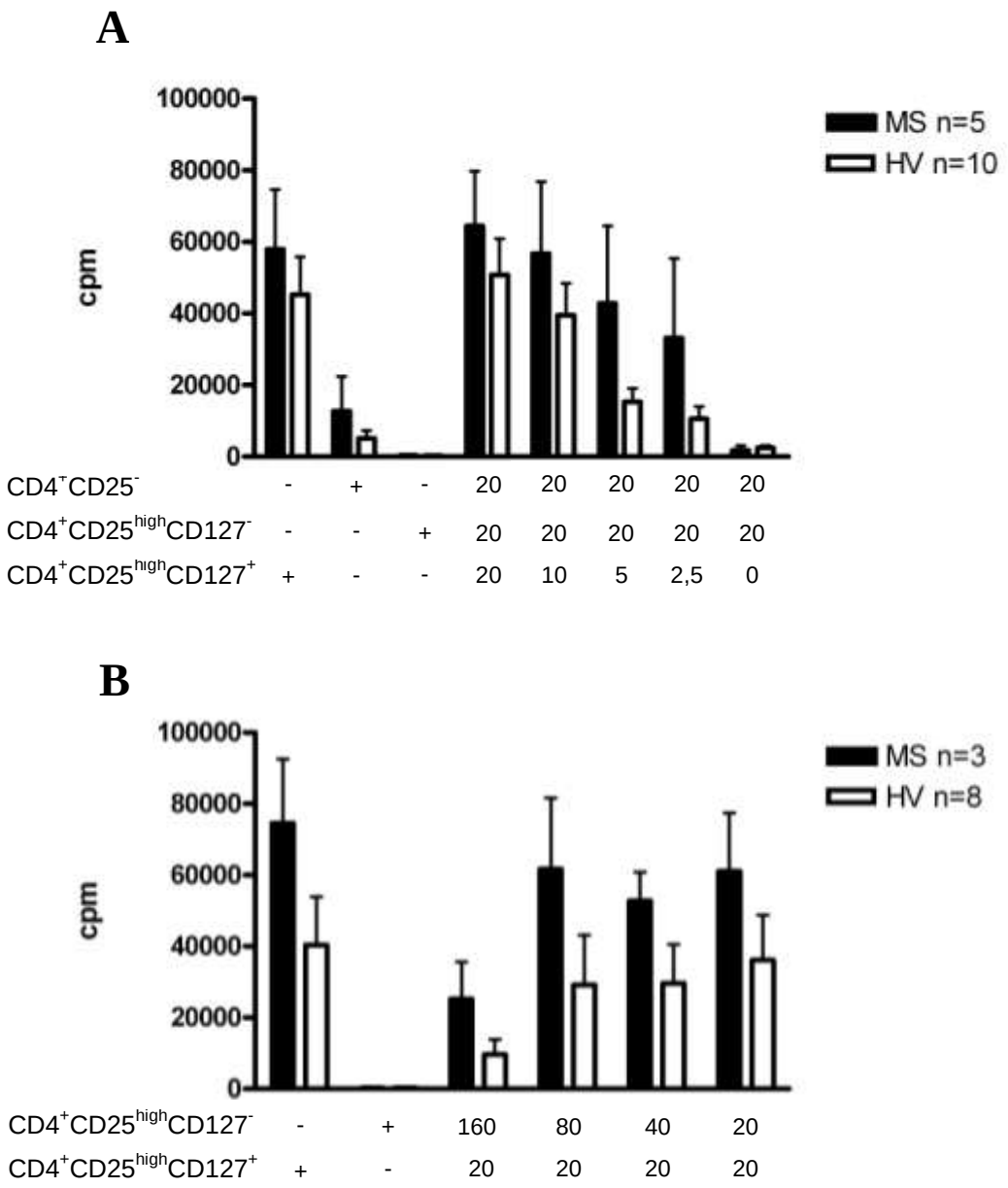


Figure 16 : Étude de la fonction suppressive des $CD4^+CD25^{high}CD127^-$ en présence de $CD4^+CD25^{high}CD127^+$

Les trois sous-populations cellulaires sont obtenues après tri au FACSAria® avec les mêmes critères de sélection utilisés dans l'Article I. Les cellules sont mises en culture, pendant 5 jours et stimulées par un anticorps anti-CD3 (1µg/ml) et un anticorps anti-CD28 (5µg/ml). Aucune différence significative n'a été observée entre les patients atteints de SEP, non-traités et les témoins. **A.** Prolifération des co-cultures de cellules T $CD4^+CD25^-$, de cellules $CD4^+CD25^{high}CD127^-$ et de cellules $CD4^+CD25^{high}CD127^+$ autologues. Les cellules $CD4^+CD25^-$ et $CD4^+CD25^{high}CD127^-$ sont incubées à 20.10^3 cellules par puits chacune tandis que la quantité des cellules $CD4^+CD25^{high}CD127^+$ ajoutées varient de 20.10^3, 10.10^3, 5.10^3 à $2,5.10^3$ cellules par puits. **B.** Prolifération des co-cultures de cellules $CD4^+CD25^{high}CD127^+$ et de cellules $CD4^+CD25^{high}CD127^-$ autologues. Les cellules $CD4^+CD25^{high}CD127^+$ sont incubées à 20.10^3 cellules par puits tandis que la quantité des $CD4^+CD25^{high}CD127^-$ varie de 160.10^3, 80.10^3, 40.10^3 à 20.10^3 cellules par puits.

Une étude est en cours pour évaluer le rôle que peut jouer cette sous-population cellulaire dans la perturbation de la régulation. Dans un premier temps, nous avons montré dans l'article II que lorsque les cellules CD4+CD25high étaient sélectionnées en enlevant les cellules CD4+CD25highCD127+, la capacité de régulation était similaire entre les patients atteints de SEP et les témoins. Nous cherchons à étudier si l'ajout de cellules CD4+CD25highCD127+ autologues, à différent ratio, perturbe la régulation des CD4+CD25highCD127- sur les CD4+CD25- autologues. Les résultats préliminaires présentés dans la figure 16A suggèrent un potentiel « perturbateur » des CD4+CD25highCD127+ lorsque ces cellules sont ajoutées au test de régulation classique et ceci quelque soit le ratio. Cependant, aucune différence significative n'est observée et les cohortes des patients et des individus sains est à compléter.

Nous avons montré dans l'article II, lors des tests de régulation allogéniques, que les cellules T CD4+CD25highCD127+ des patients atteints de SEP possédaient des fonctions régulatrices comparables à celles des témoins. Nous avons cherché à déterminer si les cellules CD4+CD25highCD127- pouvaient inhiber la prolifération des CD4+CD25highCD127+ autologues. Des tests de régulation ont été réalisés avec les cellules régulatrices CD4+CD25highCD127- mais, dans notre cas, les cellules répondeuses utilisées ont été les lymphocytes CD4+CD25highCD127+ autologues au lieu des CD4+CD25- classiquement utilisées. La figure 16B suggère que la prolifération des cellules CD4+CD25highCD127+ pourrait être inhibée par les lymphocytes CD4+CD25highCD127-. Cette régulation semblerait être effective chez les témoins mais pas chez les patients atteints de SEP. Ces résultats préliminaires devront être complétés en augmentant le nombre d'individus dans chaque cohorte.

La caractérisation phénotypique n'a pas mis en évidence de différence d'expression du récepteur à l'IL23, l'un des marqueurs du profil Th17, à la surface des lymphocytes T CD4+CD25highCD127+ entre les patients atteints de SEP et les témoins. Néanmoins, les résultats préliminaires obtenus sur la production d'IL17 dans les surnageants de culture semblent indiquer que les cellules des patients atteints de SEP sécrètent plus d'IL17 que les cellules des témoins (Figure 17). Dans un premier temps, d'autres tests fonctionnels seront réalisés et l'IL17 ainsi que d'autres cytokines comme l'IL9, l'IL21 et l'IL22, cytokines impliquées dans les profils Th récemment décrits, seront dosées dans les surnageants congelés avec un kit de dosage Luminex. Ceci nous apportera des informations complémentaires et

permettront de diriger nos investigations futures vers tel ou tel autre profil Th auquel semblera appartenir les lymphocytes T CD4+CD25highCD127+.

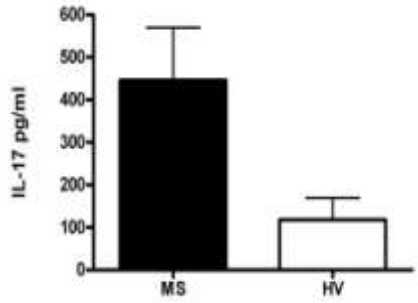


Figure 17 : Étude de la sécrétion d'IL17 par les lymphocytes $CD4^{+}CD25^{high}CD127^{+}$
Les surnageants de chaque puits des expériences de prolifération ont été prélevé au bout de 3 jours de culture sous stimulation polyclonale. La production d'IL17 par les cellules $CD4^{+}CD25^{high}CD127^{+}$ a été mesurée pour quatre patients atteints de SEP et quatre témoins. Aucune différence significative n'a été observée.

Cette nouvelle technique qu'est le TRAP (Article III) nous permet de détecter et de dénombrer les lymphocytes mémoires CD4+ et CD8+ indépendamment du contexte HLA, des peptides présentés et de la production de cytokine(s). En utilisant la méthode TRAP, nous pourrons directement caractériser les cellules T vivantes spécifiques de la myéline mais aussi les isoler, grâce au FACSAria®, pour les manipuler *ex vivo*.

Dans un premier temps, l'investigation qui sera entreprise, visera à caractériser plus précisément les cellules TRAP+ en regardant l'expression des marqueurs de surface tels que des marqueurs de migration comme PSGL1, LFA1 et VLA4, des marqueurs de co-stimulation comme CD28 et CTLA4 mais également le marqueur de homing vers les organes lymphoïdes secondaires qu'est le CD62L. Cette étude phénotypique permettra aussi d'évaluer d'une part la fréquence des cellules T CD4+CD25highCD127+ auto-réactives mais également l'expression de nouveaux marqueurs à la surface de ces cellules en utilisant le protocole mis au point au sein du laboratoire (Article III).

Un autre volet de l'étude des cellules TRAP+ sera la caractérisation de leur capacité de transmigration au travers d'une barrière de cellules endothéliales immortalisées de la BHE en se basant sur le travail préalablement réalisé au sein de notre laboratoire (Annexe II). Nous nous intéresserons à la transmigration des lymphocytes T CD4+ et CD8+ auto-réactifs mis en évidence par la technique TRAP mais aussi des cellules T CD4+CD25highCD127+. Pour

cela, les cellules TRAP+ seront isolées par cytométrie en flux puis mis en présence de notre modèle de BHE. Nous avons montré précédemment que les lymphocytes T CD4+CD25highCD127+ expriment à leur surface des molécules d'adhérence importantes pour la migration dans le SNC comme LFA1 et VLA4 (partie résultats). L'expression d'autres marqueurs tels que PSGL1 chez les patients atteints de SEP sera elle aussi étudiée et comparée à celle des cellules circulantes provenant des individus sains.

L'ensemble de ces expériences a pour but d'apporter un éclaircissement sur certains points comme : **1.** les lymphocytes T spécifiques de la myéline ont-ils une capacité de transmigration à travers le modèle de BHE supérieure par rapport aux autres lymphocytes ? **2.** leur migration est-elle conditionnée par l'ajout d'antigène provenant du SNC ? **3.** ces cellules sont-elles plus activées, plus cytotoxiques après avoir migré dans le SNC ? **4.** Quelle(s) est la molécule(s) d'adhérence prépondérante(s) impliquée(s) dans leur transmigration ?

Le profil transcriptionnel des cellules TRAP+ sera, lui aussi, étudié en utilisant la technique des microarrays. Les résultats obtenus seront ensuite validés par qPCR.

Pour étudier l'implication clinique des cellules TRAP+ observées dans le sang périphériques des patients atteints de SEP, nous déterminerons la fréquence de ces cellules au sein du LCR des patients comparée à celle obtenue dans le sang. De plus, un groupe de patients atteints d'autres maladies neurologiques sera inclus comme groupe témoin.

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ANNEXES

ANNEXE I : Incapacité de l'acétate de glatiramère à modifier le répertoire des cellules T périphérique chez les patients atteints de Sclérose en Plaques de forme rémittente

Failure of Glatiramer acetate to modify the peripheral T cell repertoire of relapsing-remitting multiple sclerosis patients.

Laureline Berthelot[1], Patrick Miqueu[1,4], Ségolène Pettré[1], Julien Moynard[1], Sandrine Wiertlewski[2], Fabienne Lefrère[3], Sophie Brouard[1], Jean-Paul Soulillou[1], David-Axel Laplaud[1,2,3].

[1] : INSERM, U643, et ITERT, Nantes, F-44093 France

[2]: CHU de Nantes, Service de Neurologie, Nantes, F-44093 France

[3] : INSERM, CIC004, CHU de Nantes, F-44093 France

[4] : TcLand Expression, 21 rue de la Noue Bras de Fer 44200 Nantes France

Corresponding author: David-Axel Laplaud, INSERM U643, 30 Bd J. Monnet, 44093, Nantes Cedex, France

David.Laplaud@univ-nantes.fr, fax : +33 2 40 08 74 11

Abbreviations:

MS: multiple sclerosis

GA: Glatiramer Acetate

TCR: T-cell receptor

CDR3-LD: Complementary determining region 3- length distribution

Abstract

Glatiramer acetate (GA) is a random copolymer used as an immunomodulatory treatment in relapsing-remitting multiple sclerosis (RR-MS). Its mechanisms of action are poorly understood and several hypotheses have been put forward, the majority of which rely on in-vitro studies. It has been hypothesised that further to processing by APC, GA could provide a large number of different epitopes with a possible sequence similarity to auto-antigens, which are able to stimulate a large proportion of T cells. Given that in a previous study we showed that the circulating T cells of MS patients present more alterations of the Vβ T-cell receptor (TCR) usage than normal individuals, we explored the possible effects of GA on ex vivo T cell repertoire of MS patients. Here we used Quantitative-PCR and electrophoresis to longitudinally analyse (and without any ex vivo stimulation), the CDR3 length distribution (LD) and the amount of Vβ TCR, as well as various cytokines, in the blood T cells of 10 RR-MS patients before and after 3 months and 2 years of GA treatment. In addition, we also determined the status of responder and non-responder patients after 24 months of GA treatment based on clinical and radiological criteria. We found no significant modification of cytokine production, Vβ TCR mRNA accumulation or CDR3-LD in the patients after short-term and long-term treatment. In addition, we did not observe any difference in CDR3-LD in the GA responder patients (n=6) compared to non-responder patients (n=4). Focusing our study on responder patients, we performed TCR repertoire analysis in the CD4+ and CD8+ compartment. Alterations of CDR3-LD were predominantly found in the CD8+ compartment, without any significant influence of GA treatment. Finally, the T cell repertoire variations in MS patients treated with GA and healthy controls were equivalent. Collectively, our data suggest that GA therapy does not induce significant variations in cytokine production or TCR usage in MS patients.

Introduction

Multiple Sclerosis (MS) is thought to be a T cell driven disease characterized by the presence of inflammatory and demyelinating patches throughout the white matter of the central nervous system [1]. One of the approved drugs used as a disease modifying therapy for relapsing-remitting MS is glatiramer acetate (GA, Copaxone®), a random copolymer of the following four amino-acids: glutamic acid, leucine, alanine and tyrosine, with various molecular weights ranging from 40 to 100 residues.

The mechanism of action of GA has still not been completely understood and different hypotheses have been formulated [2; 3]. Firstly, as the presence of GA-reactive CD4+ T cells with a Th2 polarisation has been shown in the blood of MS patients, a shift in cytokine production has thus been suggested, with a bystander suppression induced by the GA-reactive CD4+ T cells [4; 5; 6; 7]. This modification in the cytokine profile could also be due to a complex action of GA on antigen presenting cells (APCs), as recently suggested [8; 9; 10; 11]. In addition, GA could also act as an altered-ligand peptide and induce the apoptosis or the anergy of myelin-peptide reactive CD4+ T cells, as suggested by Aharoni et al. [12; 13]. Finally, the production of CD4+CD25high regulatory T cells could also contribute to the therapy of GA [14; 15; 16]. In parallel to its immune properties, another potential action of GA is the production of the neurotrophic factor BDNF from GA-selected T cells, suggesting a possible role in remyelination processes [17; 18]. Nevertheless, the majority of studies focusing on the mechanisms of action of GA have been conducted in vitro after cell culture. Direct ex-vivo analyses of fresh T cells are rather few and far between so as to provide a fuller description of these mechanisms.

As GA is a random copolymer of variable sequences and lengths, its processing by APCs could theoretically generate a high diversity of peptides with different sequences. As a result, it could be hypothesized that GA would modify the peripheral T cell repertoire by selecting various T cell clones. In fact, GA stimulation has been suggested to induce a focused oligoclonal CD8+ T cell repertoire [19]. In previous studies, using the TcLandscape methodology [20], we demonstrated that the T cell repertoire of MS patients is significantly more altered than that of healthy individuals, regardless of the disease stage of the patients (from the clinically isolated syndrome to the clinically confirmed and worsening forms)[21]. In addition, in a longitudinal study, the appearance of new altered Vβ families in the blood of MS patients correlated with new lesions in the brain of MS patients [22].

In this study, we compared the ex-vivo peripheral T cell repertoire of GA-treated MS patients before and after GA treatment, to test whether GA could result in the selection of T cell clones. In addition, because not all of the MS patients had a clinical response to GA treatment, after a 24-month follow-up the GA responders and non responders were selected in accordance with the criteria of Rio et al [23] and the peripheral T cell repertoire was compared at the CDR3 level for the two groups as well as a group of healthy individuals.

Materials and methods

Patients and healthy individuals

Ten patients with relapsing-remitting multiple sclerosis (RR-MS) as defined by the McDonald's criteria [24] and 7 age-matched healthy individuals (HI) were included in the study and followed for a two year period. Blood samples were collected at the outset of the treatment and after three and 24 to 34 months of Glatiramer Acetate (GA) treatment. Informed consent for participation in this study was obtained from MS patients and healthy individuals in compliance with our local university hospital ethical committee. The clinical characteristics of the patients are summarized in Table 1. The patients were considered as responders or non-responders to the therapy in accordance with Rio et al [23]. Briefly, after a follow-up of at least two years, patients were considered as responders to GA treatment if they had neither a relapse nor an exacerbation of the disease (no new lesions or gadolinium-enhancing lesions detected by MRI at the end of follow-up) and displayed no disease progression in terms of disability (assessed by the EDSS score).

Isolation of cell populations

Peripheral blood mononuclear cells (PBMC) were extracted from blood using Ficoll (Eurobio, Les Ulis, France). Some of the PBMC ($10x10^6$) were directly frozen in trizol® at -80°C and the rest were frozen in autologous plasma/20% DMSO. Cells were later thawed for cell sorting and phenotype analysis. PBMC were labelled with anti-CD3 coupled with PE-Cy7 and anti-CD8 coupled with APC (all antibodies were purchased from BD Biosciences) prior to CD4+ and CD8+ T cell isolation in a high-speed cell sorter (ARIA, BD Biosciences). DAPI solution was used to exclude the dying cells. Sort purity was >98% for each population. CD4/CD8 ratios were not modified by cryopreservation.

T cell repertoire analysis

PBMC and CD4+ and CD8+ T cells were frozen in trizol® reagent for RNA extraction. RNA from PBMC was extracted in accordance with the manufacturer's instructions. The concentration and quality of RNA for each sample was accurately determined using nanoRNA Chips (Agilent®, United Kingdom). Next, 2 μg of RNA were reverse transcribed using an Invitrogen cDNA synthesis kit (Boeringher Mannheim, Indianapolis, IN) and diluted to a final volume of 100μl. Complementary determining regions (CDR3) were amplified by quantitative PCR using 26 different Vβ primers and a Cβ primer in a 9700 thermocycler (Applied Biosystems). The PCR fragments were migrated in an acrylamide gel and the distribution of the different CDR3 lengths was analysed using Immunoscope® software [25; 26; 27]. The percentage of CDR3 length distribution (CDR3-LD) alteration for each Vβ family and the global percentage of CDR3-LD alteration for each individual or each group was calculated as described [28]. Briefly, the percentage of alteration was defined as the difference between the frequency of each CDR3 length in the distribution profile of the Vβ family being studied and the mean control distribution, calculated from 14 healthy individuals. Data from quantitative PCR and percentages of CDR3-LD alterations were represented in a global three-dimensional diagram known as the TcLandscape®. In this diagram the X axis displays the 26 human Vβ families; the Y axis shows the CDR3 lengths and the Z axis the quantitative ratio Vβ/HPRT. The colour code from deep blue (≤−30%) to dark red (≥+30%) represents the percentage of CDR3 alterations.

Quantitative PCR of cytokine and neurotrophic factor transcripts

We performed quantitative PCR in order to assess GA-induced Th2 bias and the production of neurotrophic factors, as described previously [17; 18]. In short, 11μL of cDNA from PBMC, CD4+ or CD8+ T cells were added to 12.5μL Master Mix (Applied Biosystems) and 1.5μL of each primer mix (Applied Biosystems). The transcripts analysed included Th1 cytokines (IFN-γ, TNF-α, IL-2, IL-6, IL-8 and IL-12) Th2 cytokines (IL-4, IL-5, IL-10, IL-13 and TGF-β), cytotoxic factors (Perforin and Granzyme B) and neurotrophic factors (BNDF, NGF, NT3 and GNDF).

Statistical analysis

A two-tailed Mann-Whitney test was used to compare MS patient and healthy control group values, i.e., percentages of CDR3-LD alterations and quantitative PCR values. A non parametric paired Wilcoxon test was performed for time variation studies in each group. A

two-way ANOVA was used to study the variation of the two groups for the required duration A p-value <0.05 was considered significant.

RESULTS

Repertoire analysis of non-separated T cells after GA treatment

GA stimulation has been reported to induce TCR biases in vitro [19]. To assess the potential modifications of the T cell repertoire induced by GA treatment in vivo, we performed quantitative PCR to measure Vβ family mRNA accumulation and to analyze the distribution of the different lengths of CDR3 for each Vβ family from fresh blood samples collected from MS patients before and after short-term (three months) and long-term (24 to 34 months) treatment with GA. Figure 1 shows three representative Tclandscapes® (see Materials and Methods for details), the graphic representation combining the Vβ family mRNA accumulation (shown as the Vβ/HPRT ratio) and the CDR3 length distribution (CDR3-LD). The colour code indicates the degree of CDR3-LD alterations (the percentages of alterations exceeding 30% are shown in red). ***Vβ mRNA accumulation.*** To determine the specific modification induced by GA treatment in terms of the level of Vβ family mRNA accumulation, we first studied the Vβ/HPRT ratio for the 26 different Vβ families obtained by quantitative PCR. There was no global trend towards either an increase or a decrease in Vβ mRNA accumulation after GA treatment for any of the ten MS patients or between the responders and non responders. When analysing each Vβ/HPRT ratio for each Vβ family, we observed modifications characterized by only a low level of variation (Fig 2 gives two representative patterns of the Vβ/HPRT ratio before and after three months of GA treatment). Each individual presented certain Vβ families with an increased Vβ/HPRT ratio and other Vβ families with a decreased ratio after GA treatment, regardless of whether or not they responded to treatment. Collectively, no significant increase or decrease in mRNA levels was observed after GA treatment. ***Analysis of CDR3 length distribution*** We then studied the CDR3 length distribution (CDR3-LD) of the ten MS patients before and three months after GA treatment. The global percentages of CDR3-LD alterations (compared to the Gaussian profile, see Materials and Methods) did not change with GA treatment (16.18±2.85% before GA treatment, 16.79±2.74% after three months of GA treatment and 15.96±2.49% after long-term treatment, p = ns). Analysis of each Vβ family, for each patient before and after GA treatment, showed minor variations of alteration, specific to individual and not specific to the

GA treatment. Repertoire alterations of responders and non responders also remained unchanged (Fig 3; for responders: 15.13±1.95% before GA treatment, 16.30±2.76% after three months of GA treatment and 15.77±3.03 after long-term treatment, p = ns, and for non responders: 17.76±3.53% before, 17.52±2.94 after three months of GA treatment and 16.33±1.27 after long-term treatment). Non responders presented a slightly more altered CDR3-LD than responders but the difference did not attain statistical significance. Variations in CDR3-LD (a decrease or increase in the percentage of alterations) were observed for several Vβ families after GA treatment (Fig 4). However, these variations did not concern a unique Vβ family and were equally distributed between the two groups of MS patients (responders and non responders).

T cell repertoire analysis in the CD4+ and CD8+ compartments

We previously described that CDR3-LD alterations were predominantly found in the CD8+ compartment in MS patients [21]. We thus studied the T cell repertoire in the CD4+ and CD8+ compartments in 5 responding MS patients before and after GA treatment. The CD8+ compartment appeared more altered in terms of the CDR3-LD (19.04±4.04%) than the CD4+ T cell compartment (15.2±1.66%) before GA treatment ($p<0.05$). However, three months of GA therapy did not induce significant modifications in the percentages of alterations (22.1±2.51% for the CD8+ compartment and 16.1±1.91%. for the CD4+ T cell compartment after GA treatment; Fig 3). There is also no difference for the Vβ/HPRT ratio after GA therapy (data not shown). As for total T cells, there was no major modifications of the T cell repertoire in the CD4+ or CD8+ compartments in the blood of MS patients after GA treatment.

T cell repertoire modification in GA-treated MS patients compared to healthy controls

To assess whether the small variations observed after three months of GA treatment could be due to the GA therapy, we analysed the T cell repertoire of seven healthy controls without treatment after an interval of three months. As shown on a representative example in Figure 5, roughly similar variations in Vβ/HPRT ratio for the 26 different Vβ families and in CDR3-LD alterations also appeared in healthy control samples. Collectively, there was no significant difference between the variations of the T cell repertoire observed in MS patients after GA treatment and the variations observed in healthy controls.

Expression of Cytokine and neurotrophic factor transcripts after GA treatment

Because a Th2 shift and a production of the neurotrophic factor BDNF were described in MS patients treated with GA, we performed quantitative PCR in order to measure the level of mRNA of several cytokines and neurotrophic factors. There was no significant difference in quantity of mRNA for any of the tested cytokines (IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IFN-γ, TNF-α, TGF-β, perforin and granzyme B) or for the neurotrophic factors (BDNF, NT3, NGF and GDNF) measured before and after GA treatment. Figure 6 shows the quantity of IFN-γ, TNF-α, TGF-β, IL-2, IL-4 and IL10 mRNA. Neurotrophic factor transcripts were not detectable in the PBMC of MS patients, before or after GA treatment. There was also no difference between responders and non responders. However, compared to healthy controls, MS patient PBMC significantly accumulated more TNF-α and IL-10 and less TGF-β transcripts ($p<0.05$).

DISCUSSION

In comparison with healthy individuals, MS patients present more CDR3 length distribution alterations in their blood T cell repertoire [21]. It has been shown that GA treatment acts on T cells and induces a Th2 shift in cytokine production [2]. In this paper, we revisited the effects of GA treatment on the T cell repertoire in the blood of MS patients. We used a global method taking into account both alterations in TCR Vβ CDR3 length distribution and the magnitude of clonal selection based on the quantity of Vβ transcripts [20]. With this method, modifications of CDR3 length distribution were detected after vaccination in tumor-bearing patients [29]. Despite the data of in vitro GA stimulation of T cells [19], we showed that treatment with GA for three months or longer does not induce significant TCR biases in MS patients. In addition, TCR changes during GA treatment in MS patients were similar to those observed in healthy individuals assessed during the same period of time. We were also unable to detect differences between responding and non responding MS patients despite the fact that our method is sensitive enough to detect variations in blood TCR alteration over time [30]. Collectively, these data suggest that GA treatment does not affect the peripheral blood T cell repertoire. The difference between the in vitro and in vivo effects of GA could be explained by the high amount of GA during in vitro stimulation or by the specific culture conditions. However, we cannot vouch that GA did not create clonal expansion of T cells in tissue compartments such as the brain, which were not explored in this study. In fact, it has been demonstrated in animals that GA-specific T cells can migrate into the brain [31].

Nevertheless, GA treatment has positive effects in MS patients and helps decrease relapse events (see [32] for review). Our data suggest that the mechanism of action of GA may not be T cell-mediated and may involve other cell types. In fact, GA treatment seems to induce type 2 monocytes [8; 9; 10; 11]. Antigen presenting cells (APC) could also be the major targets in GA treatment. Along these lines, it has been shown in animals that GA-primed DC can induce neuroprotection in a T cell-independent manner, and could act directly on neurons [33]. In addition, MBP liberated from the myelin sheath, presents a particular conformation and can bind aMb2 integrin expressed by phagocytes, an interaction that could be inhibited by GA [34] and could thus prevent the presentation of auto-antigens by APC to T cells.

Concerning B cells and antibody response to GA, polyclonal and not monoclonal antibodies against GA crossreact with MBP (Myelin Basic Protein) (Teitelbaum et al. 1991). GA has no effect on antibody response against MOG protein (Myelin and Oligodendrocyte Glycoprotein) (Khalil M et al. 2006). Specific anti-GA seem to switch from IgG1 to IgG4 in MS patients treated with GA and could drive the Th2 response (Brenner T et al. 2001, Farina C et al. 2002, Basile E et al. 2006). In an animal model of demyelination (Theiler's virus), the antibodies against GA seem to induce a remyelination (Ure DR et al. 2002). The effect of GA on B cells could partially explain the benefits of this treatment in MS patients.

We have shown that MS patients present an increased production of TNF-α regardless of whether or not they were treated with GA, confirming an activated state of immune cells from MS patients, as described previously [35; 36; 37]. GA treatment seems to induce the production of TGF-β and IL-10 in MS patients, suggesting the induction of regulatory mechanisms. Nevertheless, we were unable to detect any differences in the quantity of mRNA of several cytokines and neurotrophic factors before or after three months or longer of GA therapy. Other studies reported increased IL-5, IL-13, IL-4 and TGF-β in the blood of MS patients treated with GA compared to non treated patients [6; 38] and these studies examined MS patients treated long-term (more than fifteen months of GA therapy). Wiesemann et al stimulated PBMC with GA in vitro, which may cause a major bias and explain the discrepancy with our study. In the paper by Miller et al, PBMC were collected from 10 patients treated with GA for one year. The follow-up of the patients was shorter than in our study and no control group was analysed in parallel. These differences in methodology may explain the differences observed between the two studies.

Finally, our results suggest that the clinical effect of GA in multiple sclerosis probably does not rely on the direct modification of T cell properties (clonal selection or cytokine production) but rather on its actions on other cell type(s).

Legends

Table 1: Clinical data of MS patients

GA= Glatiramer acetate. Patient status was evaluated in accordance with Rio criteria [23] after 24 months of follow-up. NR= non responding patients and R= responding patients. Briefly, MS patients were considered NR if they presented relapse or increased number of lesions at MRI. during the 24 months of follow-up

Figure 1: Three Tclanscapes® of patient MS-8 before and after GA treatment

Data from quantitative PCR and percentages of CDR3-LD alterations are represented in a global three-dimensional representation named TcLandscape®. The X axis displays the 26 human Vβ families, the Y axis shows the CDR3 lengths and the Z axis the quantitative ratio Vβ/HPRT. The colour code from deep blue (≤−30%) to dark red (≥+30%) represents the percentage of CDR3 alterations.

Figure 2: Vβ/HPRT ratio for Vβ2 and Vβ6.4

mRNA were extracted from PBMC of MS patients before and after three months of GA treatment and quantitative PCR was performed using Cβ and Vβ primers. Here two representative examples of Vβ/HPRT ratios are featured for the ten MS patients and for the responding and non responding MS patients.

Figure 3: Percentages of CDR3-LD alterations

To analyse CDR3-LD, the percentage of CDR3-LD alterations was calculated for blood samples obtained before, after three months of GA treatment and after long-term treatment with GA (M20-34). Percentage of alterations of the ten MS patients (**A**), of the four non responding MS patients (**B**), of six responding MS patients (**C**), of the CD4+ T cell compartment of 5 responding MS patients (**D**) and of the CD8+ T cell compartment of 5 responding MS patients (**E**).

Figure 4: Profiles of CDR3-LD distribution

Most of the CDR3-LD profiles of MS patients before and after three months of GA treatment were stable. However, some Vβ families presented an increased or decreased percentage of alteration after GA treatment.

Figure 5: Two Tclanscapes® of one healthy control at Month 0 and Month 3.

Seven healthy volunteers were monitored for 23 months. They received no treatment during this period. On this figure is shown a representative example for one healthy control. For this individual, the profile of Vβ 9 CDR3-LD became more altered after three months and the profile of Vβ 18 CDR3-LD stayed stable, it was not observed for all healthy individuals.

Figure 6: mRNA amount of cytokines before and after GA treatment

The relative quantification ($2^{-\Delta\Delta Ct}$) are calculated from quantitative PCR raw data andrepresented for each cytokine.

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Table 1

Patients	Age	Sex	EDSS Score Before GA treatment	EDSS Score After 24 months of GA treatment	Increase in T2 Lesions after two years	Number of relapses during the 24 months of study	Status
MS1	**26**	**F**	**1.5**	**1.5**	**+**	**0**	**NR**
MS2	**45**	**F**	**4**	**4**	**+**	**2**	**NR**
MS3	**36**	**F**	**2**	**2**	**+**	**2**	**NR**
MS4	**32**	**M**	**1.5**	**1.5**	**+**	**1**	**NR**
MS5	**49**	**F**	**4.5**	**4.5**	**-**	**0**	**R**
MS6	**57**	**F**	**2**	**2**	**-**	**0**	**R**
MS7	**42**	**F**	**2.5**	**2.5**	**-**	**0**	**R**
MS8	**49**	**F**	**3**	**2.5**	**-**	**0**	**R**
MS9	**41**	**F**	**2.5**	**2**	**-**	**0**	**R**
MS10	**29**	**F**	**1.5**	**0**	**-**	**0**	**R**

Figure 1

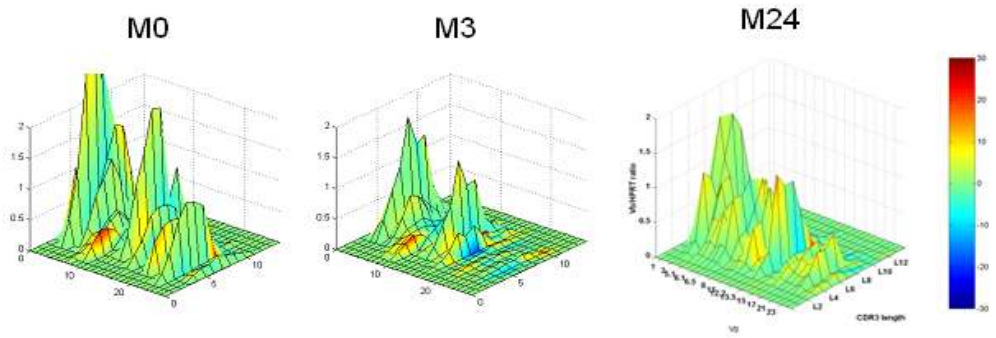


Figure 2

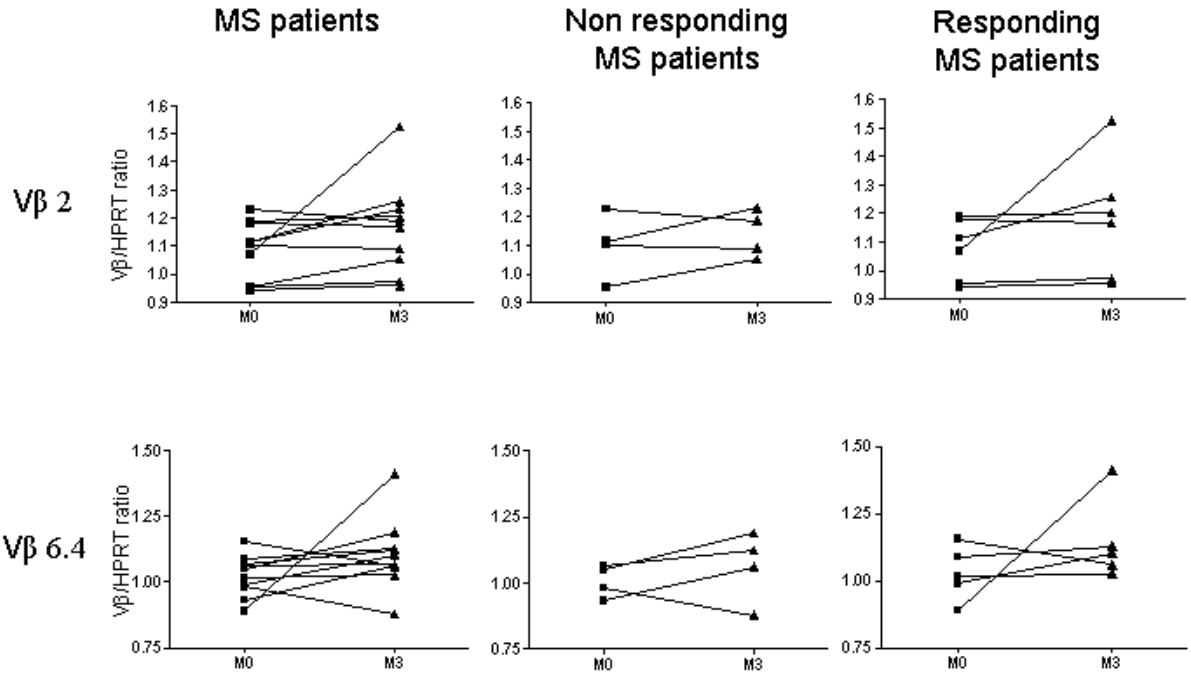

MS patients
Non responding MS patients
Responding MS patients
Vβ 2
Vβ 6.4
Vβ/HPRT ratio
M0
M3

Figure 3: Percentages of global alterations

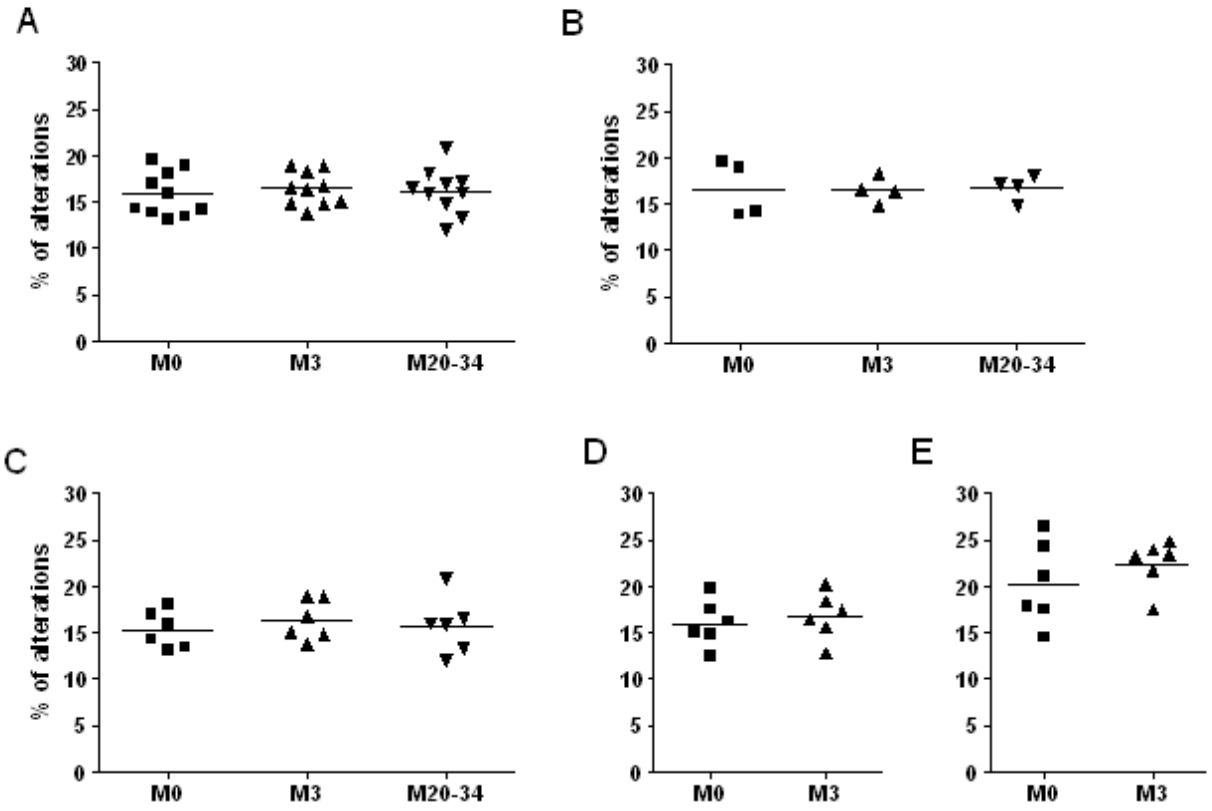


Figure 4

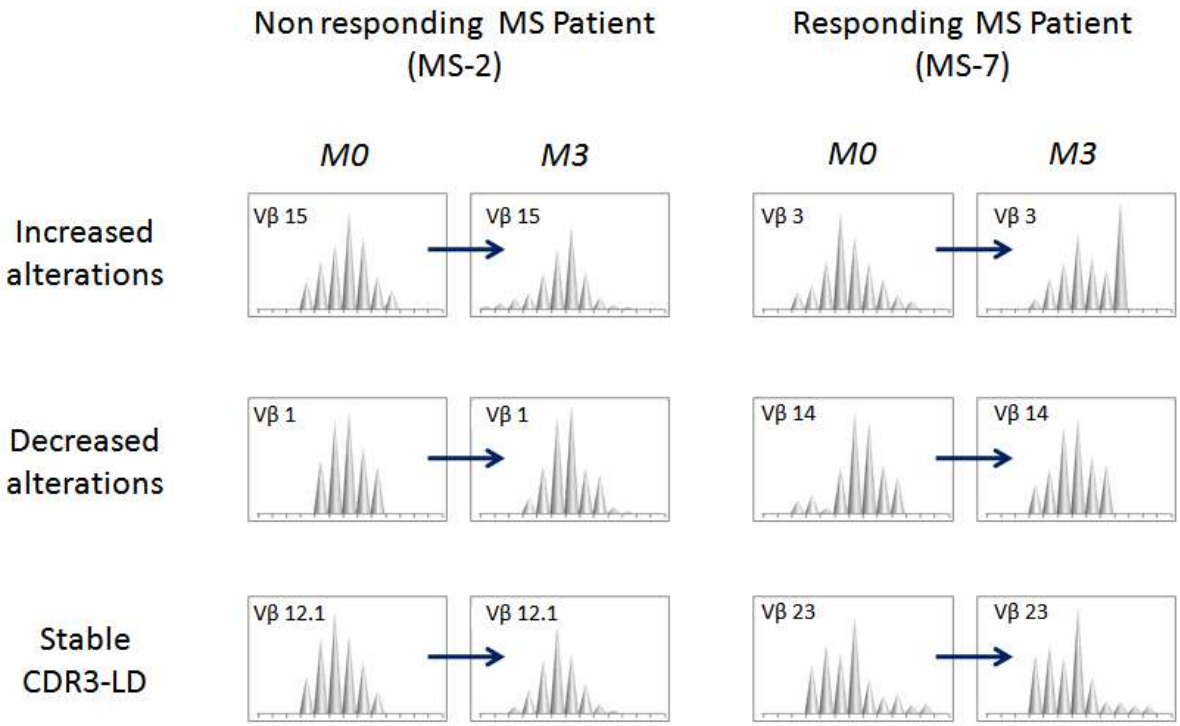


Figure 5

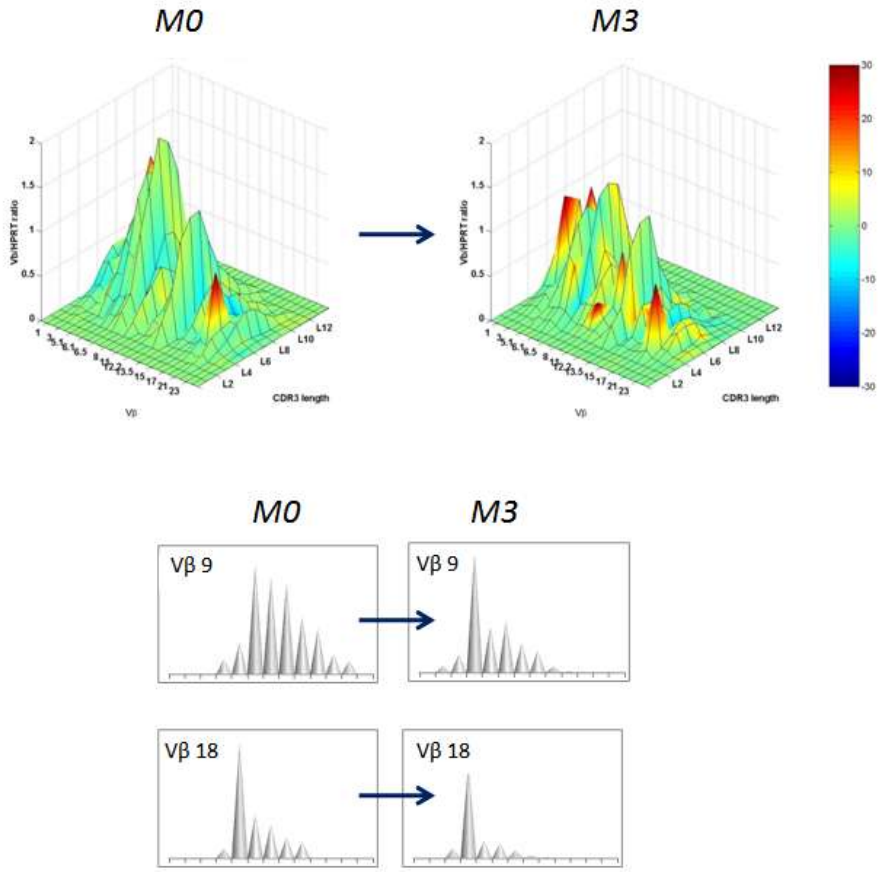
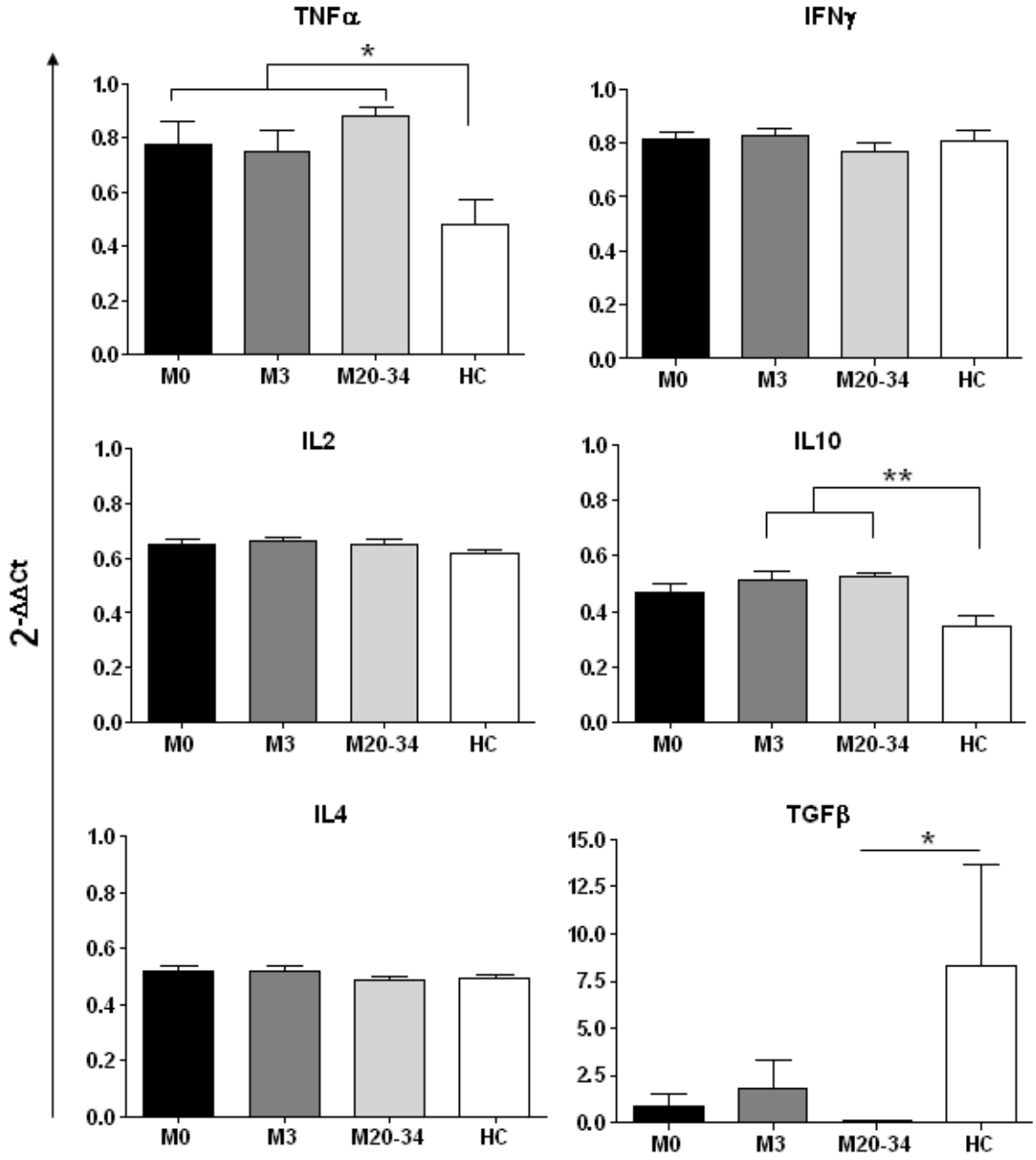


Figure 6 :



ANNEXE II : Les lymphocytes T CD4+ du sang périphérique des patients atteints de Sclérose en Plaques sont caractérisés par une forte expression de PSGL1 et d'une capacité de transmigration à travers la barrière hémato-encéphalique

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Article

Peripheral blood CD4+ T lymphocytes from multiple sclerosis patients are characterized by higher PSGL-1 expression and transmigration capacity across a human blood-brain barrier-derived endothelial cell line

Bouchaib Bahbouhi, Laureline Berthelot,* Ségolène Pettré,* Laure Michel,* Sandrine Wiertlewski,† Babette Weksler,‡ Ignacio-Andres Romero,§ Florence Miller,‖ Pierre-Olivier Couraud,‖ Sophie Brouard,* David-Axel Laplaud,*,†,1 and Jean-Paul Soulillou*,1,2*

*INSERM, UMR 643, CHU Nantes, Institut de Transplantation et de Recherche en Transplantation, ITERT, Université de Nantes, Faculté de Médecine, and CHU Hôtel Dieu, Nantes, France; †Service de Neurologie, Hôpital Laennec, CHU de Nantes, Nantes, France; ‡Weill Cornell Medical College, Division of Hematology-Oncology, New York, New York, USA; §Faculty of Science Walton Hall, Milton Keynes, United Kingdom; and ‖Institut Cochin, Université Paris Descartes, INSERM, U567, and CNRS, UMR 8104, Paris, France

RECEIVED OCTOBER 28, 2008; REVISED APRIL 20, 2009; ACCEPTED MAY 27, 2009. DOI: 10.1189/jlb.1008660

ABSTRACT

Mechanisms of T lymphocyte trafficking in the brain remain unclear in MS. We hypothesized that MS is associated with increased CD4+ and CD8+ T lymphocyte trafficking across the BBB. To test this hypothesis, we calculated the frequency of PSGL-1+/CD4+ and PSGL-1+CD8+ or LFA-1+/CD4+/CD8+ T cells in the PBMC of 27 patients with a RR-MS (21 untreated and six IFN-β-treated) and 18 HI. Next, we measured their ex vivo TR across resting and TNF-α-activated human BBB-derived hCMEC/D3 endothelial layers under static conditions. The frequency of PSGL-1+CD4+ T lymphocytes was significantly higher in treated or untreated MS patients than HI. Furthermore, resting hCMEC/D3 TR of CD4+ lymphocytes (purified or in PBMC) from treated or untreated MS patients were significantly higher than those of HI and associated with significant enrichments of CD4+PSGL+ or CD4+PSGL-1+CD45RO+ T cells in their transmigrating fractions. The TR of CD4+ and CD8+ from MS patients across TNF-α-activated hCMEC/D3 were also significantly higher than that observed in HI. Resting hCMEC/D3 transmigration was blocked significantly by anti-PSGL-1/anti-LFA-1 in all groups, and anti-VLA-4 inhibited transmigration of MS T cells specifically. Purified PSGL-1-negative CD4+ lymphocytes transmigrated resting hCMEC/D3 with <10% of transmigrating cells re-expressing PSGL-1, suggesting PSGL-1-independent transmigration mechanisms. The frequency of PSGL-1 was unchanged in CD8+ cells from MS patients, whereas CD8+LFA-1^{high} were reduced significantly in IFN-β-treated patients specifically. Collectively, MS is associated with an expanding pool of PSGL-1+CD4+ T lymphocytes able to transmigrate the BBB endothelium in vitro and possibly contributing to brain pathology. *J. Leukoc. Biol.* **86: 000–000; 2009.**

Introduction

There is compelling evidence for an autoimmune process in MS [1–3]. Qualitative changes in the expression of surface receptors or in trafficking properties of immune cells may play a major role in MS pathology. The capacity to transmigrate across the BBB is likely to be a key factor in MS pathology if peripheral effectors are involved [1–3]. In fact, inhibition of leukocyte adhesion to BBB cells by the peripheral administration of antibodies inhibiting α4-integrin reduces the number of gadolinium-enhanced lesions in the CNS of patients with MS [4–9].

PBMC transmigrate across the endothelium following a sequential and coordinated process involving selectin, integrins, and other adhesion molecules [10, 11]. The immune cells first tether and roll on the endothelium by binding to endothelial selectins in a PSGL-1-dependent manner and then adhere

Abbreviations: APC=allophycocyanin, BBB=blood-brain barrier, CSF=cerebrospinal fluid, EAE=experimental autoimmune encephalomyelitis, EGM-2=endothelial growth media-2, HI=healthy individual(s), MS=multiple sclerosis, PSGL-1=P-selectin glycoprotein ligand-1 (CD162), PR=relapsing-remitting, TR=transmigration rate, vwf=von Willebrand factor

1. These authors contributed equally to this work.
2. Correspondence: INSERM, UMR 643, Institut de Transplantation et de Recherches en Transplantation, ITERT, 30 Boulevard Jean Monnet, Nantes, 44000 France. E-mail: jean-paul.soulillou@univ-nantes.fr

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firmly to the endothelium by proteins of the integrin family and cross adjacent endothelial cells [10, 11]. Several types of activated leukocytes, including T and B lymphocytes, dendritic cells, and monocytes, can transmigrate across BBB-derived endothelial cells ex vivo [12–14].

An increased number of PBMC with adhesion and trafficking markers and their increased trafficking across BBB endothelium can potentially initiate the pathological inflammatory response in the brain in the course of MS disease [15–20]. Previous ex vivo transmigration studies have in fact shown an increased transmigration potency of PBMC isolated from MS patients across BBB-derived endothelial cells [15, 17, 20]. More specifically, some reports have demonstrated that the CD4+ T cells of MS patients have a higher transmigration ability than those of HI [18], and others have shown that CD4+ and CD8+ T lymphocytes from MS patients use different receptors, VLA-4 and PSGL-1, respectively, to bind the brain endothelium in animal studies [16]. To date, only a few studies have evaluated the transmigration pattern of blood T lymphocytes from MS patients relative to those from HI [15–20]. Despite this flow of information, there is also a debate on which type of T lymphocyte transmigrates more efficiently in patients with MS.

To provide more information about T lymphocyte trafficking in MS, we measured the ex vivo capacity of PBMC to transmigrate across the hCMEC/D3 cell line, previously isolated from human BBB [21, 22]. PBMC transmigration studies were combined with the measurements of the frequencies of PSGL-1+ and LFA-1+ T lymphocytes present in PBMC from patients with MS and from sex- and age-matched HI. PSGL-1 and LFA-1 were selected because of their sequential and complementary roles in the transmigration process, whereby PSGL-1 mediates the first steps of leukocyte capture on the endothelium, and LFA-1 intervenes in later phases of leukocyte adhesion to the endothelium [10, 11].

We describe a complex immunological alteration in MS involving an increase in the number of PSGL-1+CD4+ T cells in the peripheral blood being able to transmigrate BBB-derived endothelial monolayers to levels significantly higher than the equivalent population isolated from HI.

PATIENTS, MATERIALS, AND METHODS

Study populations

Twenty-seven patients RR-MS and 18 age-matched HI were enrolled in this study. RR-MS was clinically defined using the McDonald criteria [23]. There were 18 females and 9 males (median age of 37 years; range 19–57 years) with a median Expanded Disability Status Scale score of 2 (0–5). Six patients were treated with immunomodulatory drugs (IFN-β) for at least 3 months, and the remaining 21 patients had no treatment at the time of blood sampling. The HI included 10 females and eight males (median age of 36.5 years; range 22–62 years) who were interviewed to rule out recent inflammatory or infectious diseases. All individuals gave their informed consent before participating in the study. The University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks approved the study design.

PBMC preparation and phenotyping

PBMC were isolated from blood samples by Ficoll gradient (Lymphosep™, Biowest, Nuaille, France), counted, and frozen until use in liquid nitrogen in 10% DMSO (v/v in the donor plasma). When thawed, the cells were suspended in prewarmed RPMI culture medium (Sigma, Saint-Quentin Fallavier, France), supplemented with antibiotics and L-glutamine and containing 10% FCS (Biowest). Frozen PBMC aliquots (20×10^6/aliquot) from MS patients and HI were analyzed directly or used for purification of CD4+ and CD8+ lymphocytes. Viable cells in thawed PBMC samples were counted using 2% eosin (Sigma). PBMC samples with >30% mortality were discarded. CD3+, CD4+, and CD8+ lymphocytes were obtained from bulk PBMC by magnetic selection using antibody-coated magnetic beads, according to the manufacturer's recommendations (Miltenyi Biotech, Paris, France). For lymphocyte purification, PBMC were first incubated in staining buffer made with 0.5% BSA and 2 mM EDTA in PBS and containing microbead-coupled anti-CD3, anti-CD4, or anti-CD8 antibodies diluted to 1/10. The cells were incubated under gentle agitation for 15 min at +4°C. After washing, the cells were positively selected using an Automacs (Miltenyi Biotech). The purity yield was routinely >90%.

PBMC or purified CD3+, CD4+, and CD8+ lymphocytes were analyzed by flow cytometry (LSRII, BD Biosciences, Le-Pont-de-Claix Cedex, France) using a panel of mAb conjugated to PE, FITC, PerCP, cy-chrome-PE, or APC (BD Biosciences). The antibodies used included anti-CD11a (LFA-1; clone HT111), anti-LFA-1-blocking antibody (clone HT111), anti-CD4 (clone SK3), anti-PSGL-1 (clone KPL-1), anti-PSGL-1-blocking antibody (clone KPL-1), anti-CD45RO (clone UCHL1), anti-CD3 (clone HIT3a), anti-P-selectin (clone AK4), anti-CD49d (clone 9F10), anti-VLA-4-blocking antibody (HP2/1, Immunotech, Marseille, France), and blocking anti-VCAM-1 (CD106; BD Biosciences). Antibody isotypes were selected to match the specific antibodies described. Cells were incubated with the antibodies at optimal concentrations in the staining buffer (0.5% BSA in PBS) for 30 min at room temperature in the dark and then fixed with 2% formaldehyde. A gate was drawn on mononuclear cells on the basis of forward- and side-scatter parameters and analyzed for the frequency of the indicated markers by combining two markers (dot plots) or one marker (histograms). Unstained cells or cells stained with isotype-matched antibodies were used to subtract nonspecific signals. Unstained cells and cells stained with antibodies alone were also included in all of the experiments to compensate for spectral overlaps. Each sample included in the study was tested in duplicate or triplicate.

BBB-derived hCMEC/D3 cell line

This endothelial cell line was derived by sequential limiting dilution cloning of normal human brain microvascular endothelial cells that had been transduced by lentiviral vectors encoding human telomerase and SV40 T antigen. This human brain endothelial cell line has been demonstrated to mimic the BBB closely in vivo [21, 22]. hCMEC/D3 cells were cultured in EGM-2 (Cambrex Bio Sciences, Vervier, Belgium), supplemented with FCS and growth factors provided by the manufacturers at 37°C in a humidified atmosphere with 5% CO_2. The cells stably express normal endothelial markers, including CD31, vascular endothelial-cadherin, and vwf, as well as BBB markers, such as claudin-5 and zona occludens 1. Preliminary phenotype assays have shown that the hCMEC/D3 cell line used constitutively expresses ICAM-1 but not VCAM-1 under resting conditions. hCMEC/D3 cells were also treated with 100 U/ml TNF-α (eBiosciences, Montrouge, France) to study the expression of P-selectin and to induce the expression of VCAM-1.

To analyze PBMC adhesion, $2–5\times10^5$ PBMC were allowed to adhere to confluent hCMEC/D3 cells grown on collagen-I-coated plastic plates for 8 h under resting conditions in the absence of exogenous proinflammatory cytokines. Next, the nonadherent cells were removed, and hCMEC/D3 monolayers were washed and treated with EDTA (0.1 M) for 10 min to remove all cells from the wells. The mixed cell suspension was washed with PBS, stained for leukocyte-specific antibodies, and analyzed by flow cytom-

etry upon gating of the mononuclear cell population. The bystander endothelial cell gate identified by specific markers, mainly vwf, was analyzed for the expression of adhesion molecules including P-selectin, ICAM-1, and VCAM-1. In blocking-adhesion assays, PBMC were incubated for 2 h with blocking anti-PSGL-1 or anti-LFA-1 at 100 ng/ml, washed to remove antibody excess, and then used in the adhesion assays as described. Isotype-matched IgGs were used as negative controls. Blocking antibodies were replenished once after 4 h of incubation.

Transmigration assay

BBB-derived hCMEC/D3 endothelial cells (2×10^6) were grown on collagen-I-pretreated, 8 μm pore filter inserts in Transwell plates (Millipore, St Quentin en Yveline, France) under resting conditions in the absence of proinflammatory cytokines. The same numbers of hCMEC/D3 loaded on the filters were also used for growth on collagen-precoated plastic plates to monitor confluence, and transmigration assays with filter inserts were performed 1–2 days after complete confluence on control plastic wells. hCMEC/D3 cells could be detached from filters or plastic wells by tryspin and EDTA. When hCMEC/D3 reached confluence, the inserts were washed three times with fresh media to discard dead and nonadherent cells, and the adherent cells were transferred to new plates. A 50/50 (v/v) mixture of complete RPMI and EGM-2 was then added to the top and bottom compartments at a final volume in accordance with the manufacturer's recommendations. Equivalent numbers of PBMC ($2–5\times10^6$) or purified lymphocytes (10^5) from HI or MS patients were added gently to the top chamber on hCMEC/D3 layers grown on the insert filters and then left to transmigrate to the bottom chamber for 8 h (the optimal time for maximum recovery of the transmigrating cells, as defined in preliminary assays). The entire transmigrating cell populations present in the bottom chamber were collected by centrifugation at 1500 rpm/min, counted, and analyzed by flow cytometry. The bottom sides of the insert filters were also rinsed with 0.1 M EDTA to detach any adherent PBMC.

The T lymphocyte TR was determined by calculating (the number of T lymphocytes or PBMC in transmigrating fractions/the number of input T lymphocytes or PBMC) × 100 and by calculating the frequency of a marker in the transmigrating and nontransmigrating fractions. Blocking transmigration assays were performed using anti-PSGL-1 and anti-LFA-1 antibody concentrations as described above for the blocking-adhesion assays.

VLA-4 (CD49d)-blocking transmigration assay

CD3+ T lymphocytes were purified with anti-CD3 microbeads as described from PMBC samples of MS patients and HI, and then 10^4 purified cells were left to transmigrate for 8 h across TNF-α-stimulated hCMEC/D3 layers. In parallel, resting transmigration assays were performed under similar

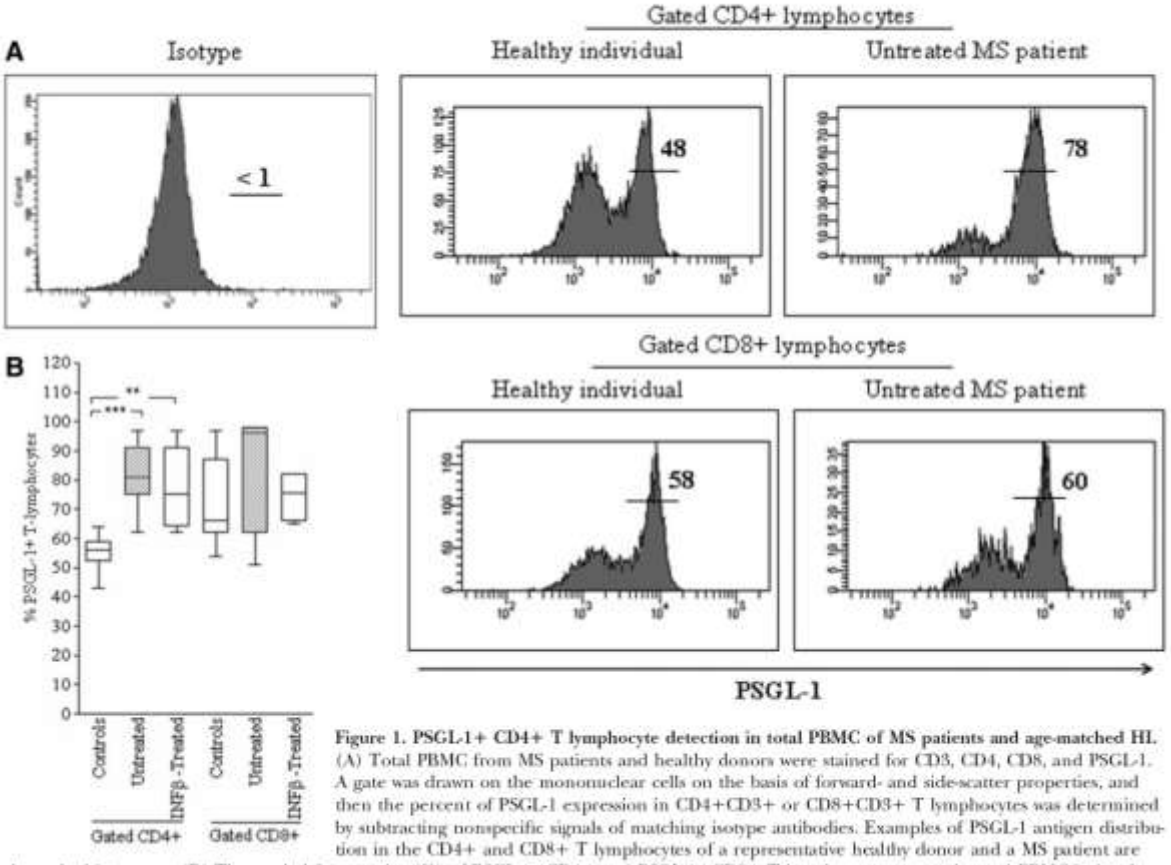

Figure 1. PSGL-1+ CD4+ T lymphocyte detection in total PBMC of MS patients and age-matched HI. (A) Total PBMC from MS patients and healthy donors were stained for CD3, CD4, CD8, and PSGL-1. A gate was drawn on the mononuclear cells on the basis of forward- and side-scatter properties, and then the percent of PSGL-1 expression in CD4+CD3+ or CD8+CD3+ T lymphocytes was determined by subtracting nonspecific signals of matching isotype antibodies. Examples of PSGL-1 antigen distribution in the CD4+ and CD8+ T lymphocytes of a representative healthy donor and a MS patient are shown by histograms. (B) The pooled frequencies (%) of PSGL-1+CD4+ and PSGL-1+CD8+ T lymphocytes present in total PBMC isolated from age-matched HI (Controls; n=11), untreated MS patients (n=14), and IFN-β (INFβ)-treated patients (n=6). Data are expressed as the median percentage of PSGL-1+ in CD4+ and CD8+ T cells. **, $P < 0.001$; ***, $P < 0.0001$.

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conditions for comparison. The number of transmigrating cells was counted in the transmigrating fractions, and then transmigrating ratios were calculated. Blocking transmigration assays were performed with activated hCMEC/D3 by using CD3+ T cells pretreated with 100 ng/ml specific anti-VLA-4 or anti-PSGL-1 isotypes or with anti-VLA-4 or anti-PSGL-1 antibodies for 2 h. The antibodies were replenished 4 h later during the assay to ensure the receptors were blocked continuously. For inhibition of VCAM-1 (CD106), resting or activated hCMEC/D3 layers were overlaid with anti-CD106-blocking antibody solution for 2 h and then used for the transmigration assay.

Indirect isolation of PSGL-1-negative CD4+ lymphocytes

Untouched CD4+ lymphocytes were first purified by a negative selection using an Automacs and a commercial kit (Miltenyi Biotech). Purified CD4+ cells (5×10^6) were incubated with 500 ng/ml mouse anti-human PSGL-1 (clone KPL-1) in 200 μl staining buffer for 20 min at +4°C under gentle shaking. The labeled cells were washed three times by centrifuging at 1500 rpm/min for 5 min and resuspended in 200 μl staining buffer (starting fractions). PSGL-1 labeling was verified in an aliquot of the starting fraction by staining with a secondary anti-mouse APC and analyzing on a flow cytometer. The remaining starting fraction was incubated with anti-mouse microbeads for 15 min at +4°C. After washing, PSGL-1+ cells were removed by a positive selection. The PSGL-1-negative fraction was collected and passed a second time through a magnetic column to remove any remaining labeled cells. Aliquots of the PSGL-negative fraction were stained with anti-mouse APC or with anti-PSGL-1-PE and a matched isotype. PSGL-1-negative CD4+ lymphocytes (10^6) were used for the transmigration assay, and then transmigrating and nontransmigrating fractions were stained for the CD4, PSGL-1, and CD45RO markers.

Statistical analysis

The nonparametric Mann-Whitney test was used for comparison between two groups, and the nonparametric Kruskal Wallis test was used for comparison of more than two groups. Differences were defined as *, $P < 0.01$; **, $P < 0.001$; and ***, $P < 0.0001$.

RESULTS

Significantly more PSGL-1+CD4+ T lymphocytes are detectable in the peripheral blood of patients with MS compared with HI

PBMC from MS patients and HI were screened for the expression of trafficking markers under similar experimental conditions. The prevalence of CD4+ or CD8+ T lymphocytes and CD19+ B lymphocytes did not statistically differ between frozen PBMC aliquots of MS patients and age-matched HI (not shown). PBMC were first tested by flow cytometry for the expression of PSGL-1 by antibody staining for CD3 (pan T cell marker), CD4, CD8, and PSGL-1. The mononuclear leukocytes were gated on the basis of forward- and side-scatter properties, and the percent PSGL-1 was assessed in the CD4+ and CD8+ T cell subsets. The percent PSGL-1+CD4+ T lymphocytes was higher in MS patients in comparison with HI (**Fig. 1A**, 78% vs. 48% in the illustrative MS patient and HI shown). In contrast, no significant difference could be found with CD8+ T cells (Fig. 1A, 58% vs. 60% in the illustrative MS patient and HI shown). By pooling all of the frequencies calculated in MS and HI, the median percent PSGL-1 in gated CD4+ T lymphocytes was 81% ± 11 in the untreated MS patients versus 58% ± 7 in HI (Fig. 1B, $P<0.0001$). This higher percentage of PSGL-1+CD4+ T lymphocytes was insensitive to IFN-β treatment, as no difference could be found between IFN-β-treated (75%±14) and untreated MS patients (81%±11), and by contrast, both MS subgroups had the percent of PSGL-1+CD4+ T cell significantly higher than HI (untreated $P<0.0001$; treated $P<0.001$; Fig. 1B). Finally, the median frequency of CD8+PSGL-1+ T lymphocytes did not differ between MS patients and HI nor between MS patient subgroups (Fig. 1B). The next step was that CD4+ lymphocytes

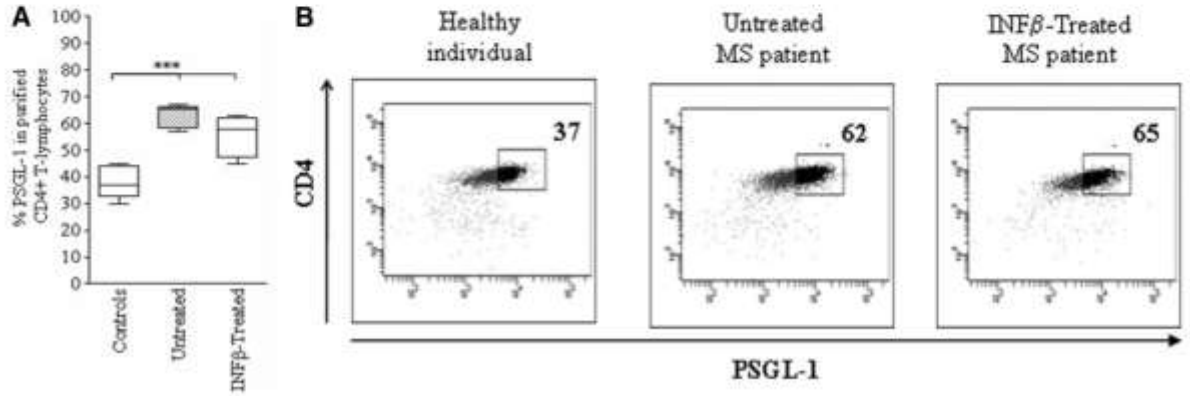

Figure 2. PSGL-1 frequency in purified CD4+ T lymphocytes. (A) CD4+ lymphocytes were isolated from a total PBMC of HI ($n=10$), untreated MS ($n=9$), and IFN-β-treated patients ($n=6$) and then stained for PSGL-1, CD4, and CD3. Data are expressed as the median percentage of PSGL-1 expression in purified CD4+ lymphocytes from all groups. ***, $P < 0.0001$. (B) Representative examples of CD4+PSGL+ frequency in MS patients compared with those of HI showing equivalent purity levels of CD4+ cells.

purified from PBMC of all groups were assessed for PSGL-1 expression. PSGL-1 frequency remained significantly higher in purified CD4+ lymphocytes from untreated (66%±4) and IFN-β-treated MS patients (58%±8) compared with HI (37%±6; **Fig. 2A**, $P<0.0001$). The isolated CD4+ lymphocytes were of equivalent purity levels in MS patients and HI (>90% purity) but clearly differed in percent PSGL-1 (Fig. 2B). On the whole, PBMC from MS patients contained a larger pool of circulating PSGL-1+CD4+ T cells.

The CD8+ T lymphocytes of IFN-β-treated MS patients display significantly fewer LFA-1^{high} cells as compared with age-matched HI

We next analyzed another trafficking marker, LFA-1, expressed on almost all of the CD3+ T cells. The LFA-1+ population could be separated into LFA-1^{low} and LFA-1^{high} subpopulations based on the position of the two peaks using the same scale in PBMC of MS patients and HI (**Fig. 3A**). To compare LFA-1 expression in MS patients and HI, percent LFA-1^{high} cells were calculated in the mononuclear gate using the same experimental procedure applied for PSGL-1. A significant difference was only observed when patients were separated into those treated with IFN-β and those without. The percent of CD8+LFA-1^{high} T lymphocytes was indeed down-regulated significantly in IFN-β-treated MS patients (18%±11) compared with untreated patients (39%±11) or HI (49%±15; Fig. 3B, $P<0.05$), therefore suggesting LFA-1 modulation by the regulatory effects of IFN-β. Contrarily, no significant changes in percent LFA-1^{high} were observed in CD4+ T lymphocytes. CD8+ lymphocytes were then purified from PBMC of IFN-β-treated patients and HI to reassess the percent LFA-1^{high} cells, which in purified CD8+ lymphocytes, was again significantly lower in IFN-β-treated MS patients (20%±4) relative to HI (37%±10; Fig. 3C, $P<0.001$). CD8+ cell purification did not alter the classical pattern of LFA-1 partition into a low and a high subpopulation, but the latter was decreased significantly in the IFN-β-treated MS patients (Fig. 3D). At least two key trafficking

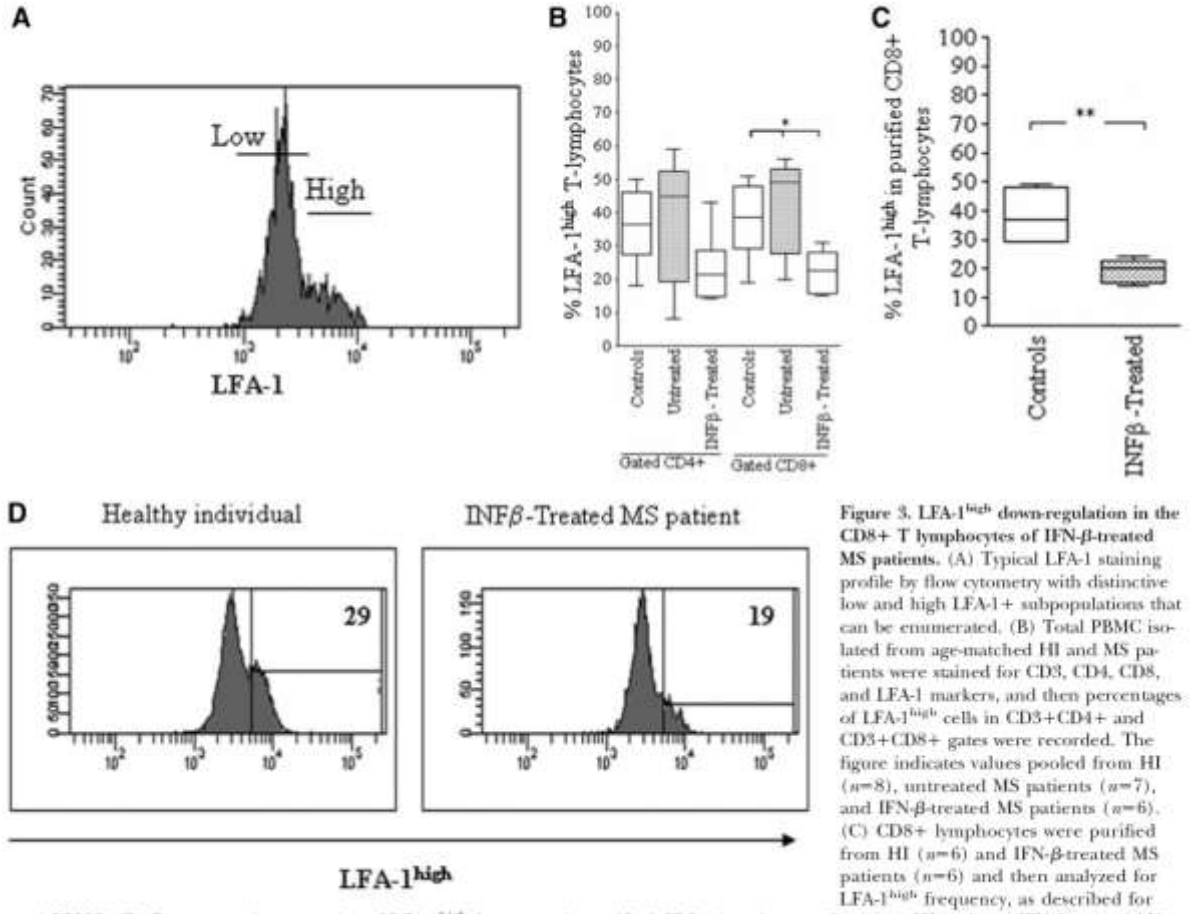

Figure 3. LFA-1^{high} down-regulation in the CD8+ T lymphocytes of IFN-β-treated MS patients. (A) Typical LFA-1 staining profile by flow cytometry with distinctive low and high LFA-1+ subpopulations that can be enumerated. (B) Total PBMC isolated from age-matched HI and MS patients were stained for CD3, CD4, CD8, and LFA-1 markers, and then percentages of LFA-1^{high} cells in CD3+CD4+ and CD3+CD8+ gates were recorded. The figure indicates values pooled from HI ($n=8$), untreated MS patients ($n=7$), and IFN-β-treated MS patients ($n=6$). (C) CD8+ lymphocytes were purified from HI ($n=6$) and IFN-β-treated MS patients ($n=6$) and then analyzed for LFA-1^{high} frequency, as described for total PBMC. (D) Representative examples of LFA-1^{high} frequency in purified CD8+ lymphocytes from one HI and one IFN-β-treated MS patient. *, $P < 0.01$; **, $P < 0.001$.

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markers (PSGL-1 and LFA-1) were therefore differentially regulated in MS patients.

CD4+ T lymphocytes from MS patients transmigrate more efficiently across resting human BBB-derived hCMEC/D3 monolayers in comparison with those from HI

The variations in percent PSGL-1+CD4+ and percent LFA-1^{high} CD8+ T lymphocytes found in PBMC of MS patients suggest a possible modulation of their transendothelial migration in MS disease. TRs were 2% ± 0.5 for MS PBMC and 1% ± 0.5 for HI PBMC ($P \approx 0.07$). In the absence of endothelial cells and in the sole presence of collagen on the filters, at least 95% of input PBMC was recovered from the bottom chambers. To determine whether trafficking of CD4+ and CD8+ T lymphocytes differed in MS patients and HI, percent CD4 and percent CD8 markers were first calculated in the transmigrating and nontransmigrating cells of MS patients and HI. The data show that percent CD4+ T lymphocytes were significantly higher in the transmigrating fractions of untreated (65%±5) and IFN-β-treated MS patients (60%±9) compared with HI (40%±7; **Fig. 4A**, $P<0.0001$). However, percent CD8+ T lymphocytes in transmigrating fractions were similar between untreated MS patients (25%±5) and HI (21%±5) but significantly reduced in the IFN-β-treated patients (13%±1; Fig. 4A). In a good accordance, percent CD4+ T cells in the nontransmigration fractions of HI (71%±7) became significantly higher than those of MS patients (64%±10; Fig. 4B, $P<0.01$), suggesting that distinct PBMC subsets may transmigrate in the MS and healthy groups and that MS CD4+ T cells are more prone to resting hCMEC/D3 transmigration.

To demonstrate that the CD4+ T cell transmigration capacity was not dependent on other cells in total PBMC, CD4+ and CD8+ lymphocytes were purified from the study groups and analyzed for transmigration (10^5 input cells). Purified CD4+ cells of untreated (2400±491) or IFN-β-treated MS patients (2794±1411) still transmigrated to a greater extent than CD4+ cells from age-matched HI (1230± 803; Fig. 4C, $P<0.001$ and $P<0.01$, respectively), therefore confirming the higher inherent capacity of MS CD4+ T cells to transmigrate BBB-derived endothelial cells.

We then determined whether LFA-1 down-regulation, initially found on CD8+ T cells of IFN-β-treated MS patients,

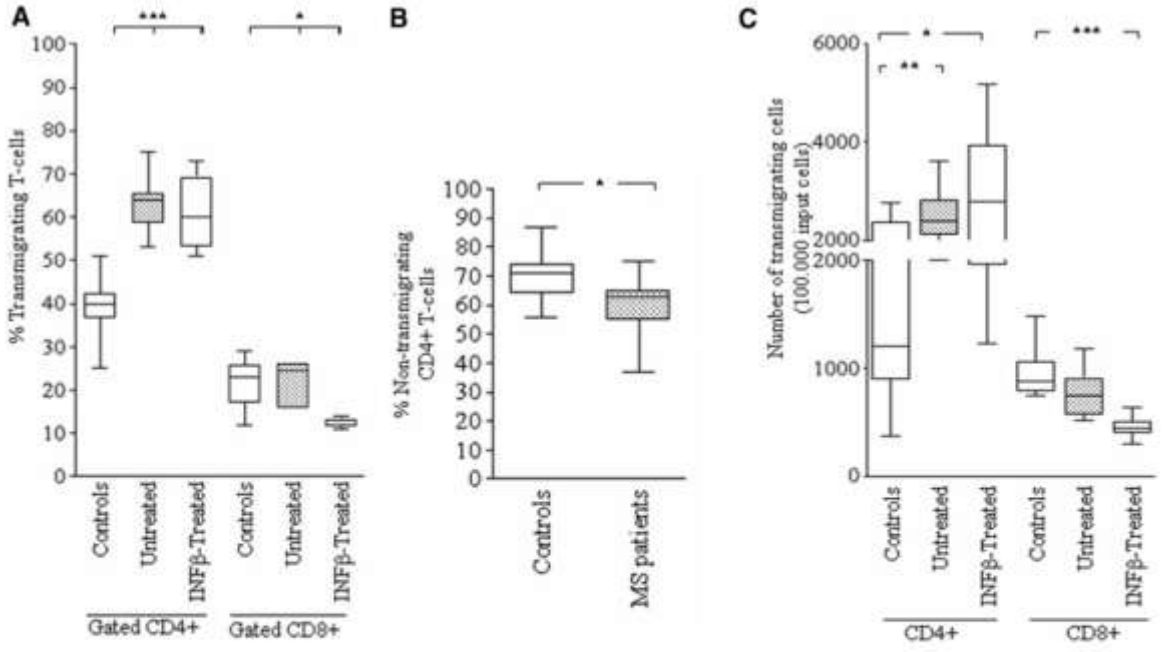
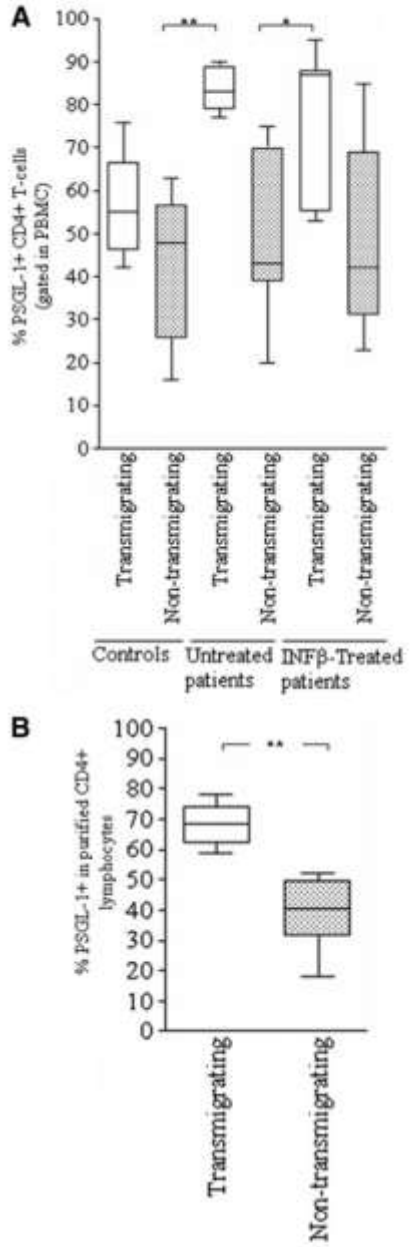


Figure 4. CD4+ T lymphocytes of MS patients transmigrated across resting hCMEC/D3 layers at a higher rate than those of HI. PBMC were added to the top of hCMEC/D3 monolayers and left to transmigrate to the bottom chamber for 8 h. The entire transmigrating and nontransmigrating cells were then collected, counted, and stained for the markers shown. (A) Percentages of CD4+ and CD8+ T lymphocytes in the transmigrating fractions of HI ($n=14$), untreated MS patients ($n=10$), and IFN-β-treated patients ($n=6$) pooled from independent experiments. (B) Percentages of CD4+ T lymphocytes in nontransmigrating fractions of HI and grouped MS patients. (C) Equivalent numbers (10^5 input cells) of purified CD4+ and CD8+ cells from HI ($n=13$), untreated MS patients ($n=11$), and IFN-β-treated MS patients ($n=6$) were left to transmigrate across hCMEC/D3 layers under conditions similar to those used for bulk PBMC. The entire transmigrating cells were counted by light microscopy and flow cytometry. Values were from three independent experiments. *, $P < 0.01$; **, $P < 0.001$; and ***, $P < 0.0001$.



e 5. Transmigrating CD4+ T lymphocytes of MS patients ex-ed PSGL-1 to a higher level than nontransmigrating cells. In all panels, the transmigrating and nontransmigrating cell popula- from each PBMC sample were stained for PSGL-1 or LFA-1 plus or CD8 markers. The mononuclear gate was defined, and then ercent of each marker was assessed. (A) Percentage of PSGL-1+ n transmigrating or nontransmigrating CD4+ and CD8+ T lym- tes of HI (n=7), untreated MS patients (n=7), and IFN-β- d MS patients (n=6) across resting endothelial layers. (B) Simi- periments were performed with purified CD4+ cells from un- d MS patients (n=6) to confirm PSGL-1 enrichment in the nigrating fraction. *, $P < 0.01$; **, $P < 0.001$.

would alter their TR. In accordance with total PBMC findings, purified CD8+ cells from untreated MS patients (749±210) and HI (958±243) had similar TR, and by contrast, those from IFN-β-treated MS patients transmigrated significantly less (447±108; Fig. 4C, P<0.0001). Untreated MS patients and HI could not thus be discriminated by CD8+ T cell transmigration levels.

PSGL-1+CD4+ T lymphocytes accumulate in transmigrating cell fractions from MS patients

Next, we asked whether PSGL-1+CD4+ T cells were more likely to transmigrate hCMEC/D3 layers, thus leading to a greater transmigration of the CD4+ T subpopulation in MS patients. To evaluate this possibility, percent PSGL-1+CD4+ or CD8+ T cells were compared between transmigrating and nontransmigrating fractions in MS patients and HI. A higher median of transmigrating CD4+ T cells from untreated MS patients expressed PSGL-1 (83%±5) compared with nontransmigrating CD4+ cells (43%±20; **Fig. 5A**, P<0.001). This difference was not found in HI (55%±13 vs. 48%±18). The frequency of transmigrating PSGL-1+CD4+ (87%±17) was additionally significantly higher than in nontransmigrating fractions (42%±20) of IFN-β-treated MS patients. Purified CD4+ cells from untreated MS patients also displayed a significant enrichment of PSGL-1 in their transmigrating fractions (69%±10) compared with the nontransmigrating ones (41%±12; P<0.001, Fig. 5B). In contrast, percent PSGL-1+CD8+ T cells did not differ significantly between transmigrating and nontransmigrating fractions from MS patients and HI (**Fig. 6A**). Considering the reduced percent LFA-1^{high} CD8+ T lymphocytes observed initially in PBMC of IFN-β-treated MS patients (Fig. 3), percent LFA-1^{high} cells were then evaluated in transmigrating and nontransmigrating fractions. LFA-1^{high} was not enriched in the transmigrating CD8+ cells of IFN-β-treated MS patients (Fig. 6B, 48%±5 vs. 35%±7, respectively). However, HI transmigrating CD8+ lymphocytes were enriched in LFA-1^{high} (48%±7) compared with the nontransmigrating cells (30%±5; Fig. 6B, P< 0.001). As a result, the PSGL-1 and LFA-1 enrichments in the transmigrating fractions suggest their positive involvement in hCMEC/D3 layer transmigration.

Accumulation of memory PSGL-1+CD45RO+CD4+ T lymphocytes in transmigrating fractions in MS patients

To confirm the PSGL-1 role in MS CD4+ T cell transmigration and as PSGL-1 is functionally activated on primed T lymphocytes, percent PSGL-1 was calculated in CD45RO+CD4+ memory lymphocytes before and after hCMEC/D3 transmigration by staining transmigrating and nontransmigrating fractions for CD4, PSGL-1, and CD45RO markers. In the untreated MS patients, memory percent PSGL-1+CD4+CD45RO+ lymphocytes were significantly higher in transmigrating fractions compared with the nontransmigrating ones (**Fig. 7A**). Higher percent PSGL-1+CD45RO+CD4+ cells in transmigrating fractions were still noted in untreated MS patients tested (85%±11) as compared with the nontransmigrating counterparts (36%±8; Fig.

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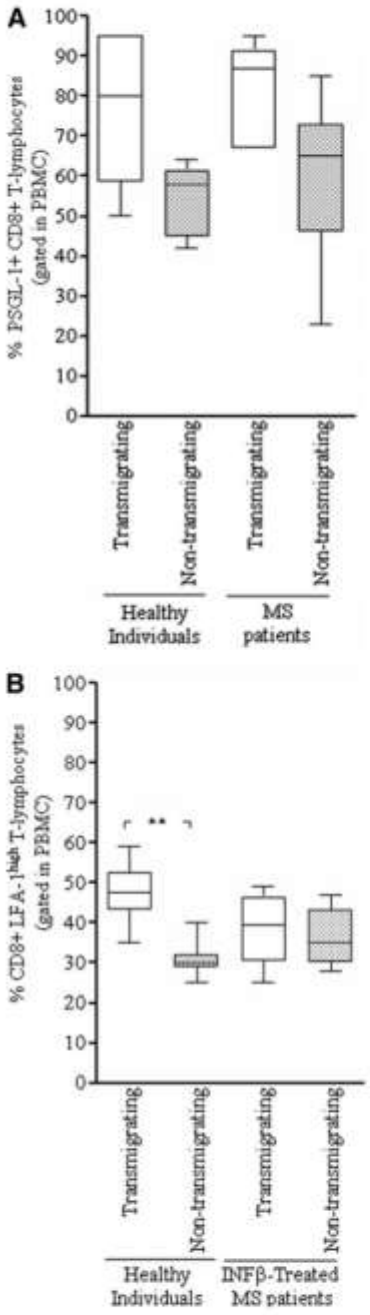


Figure 6. Transmigrating CD8+ T lymphocytes of IFN-β-treated MS patients did not become enriched for LFA-1^{high} after transmigration in contrast to those from HI. (A) Percentage of PSGL-1+ cells in transmigrating or nontransmigrating CD8+ T lymphocytes from HI (n=7) and IFN-β-treated MS patients (n=6). (B) Percentage of LFA-1^{high} cells in CD8+ T lymphocytes of transmigrating and nontransmigrating fractions of HI (n=8) and IFN-β-treated patients (n=6). $^{**}P < 0.01$.

7B, $P<0.0001$). In contrast, no significant difference could be found with CD4-CD45RO+ memory cells.

PSGL-1 and LFA-1 are functionally required for PBMC transmigration across resting hCMEC/D3 endothelial layers

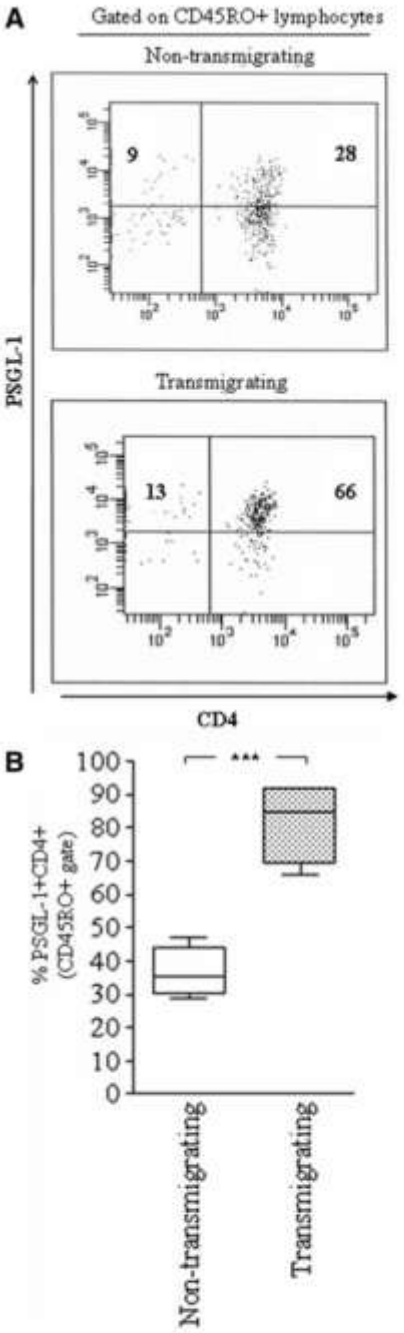
Next, PSGL-1 and LFA-1 functional roles in binding to and/or transmigration across hCMEC/D3 monolayers were analyzed. The PSGL-1 ligand P-selectin was expressed in 5–9% of hCMEC/D3 cells (**Fig. 8A**, ii) by comparison with a matching isotype control (Fig. 8A, i). PBMC and hCMEC/D3 coculture under transmigration assay conditions resulted in P-selectin up-regulation in gated hCMEC/D3 cells (18%, Fig. 8A, iii). This coculture also induced ICAM-1 up-regulation but not VCAM-1 in hCMEC/D3 cells (not shown). As expected, TNF-α induced a strong P-selectin up-regulation in hCMEC/D3 cells (Fig. 8A, iv). Blocking anti-PSGL-1 antibodies (100 ng/ml) reduced cell adhesion to hCMEC/D3 cells by three- to fourfold in CD4+ (40–12%) and CD8+ T lymphocytes (13–4%; Fig. 8B). PSGL-1 blocking also resulted in two- to threefold reduction in percent CD4+ and percent CD8+ T cells present in the transmigrating subpopulations (Fig. 8C). Furthermore, LFA-1 blocking resulted in a strong inhibition of hCMEC/D3 monolayer transmigration (Fig. 8D). These findings indicated that PSGL-1 and LFA-1 were instrumental for hCMEC/D3 transmigration. Although MS and HI PBMC differed by percent CD4+PSGL-1+ and percent CD8+ LFA-1^{high} T cells, the inhibition of their transmigration by PSGL-1 and LFA-1 blocking occurred at similar levels.

Small increase in PSGL-1 expression after complete transmigration of resting hCMEC/D3 monolayers

PSGL-1 enrichment in the transmigrating fractions may also suggest its induction by hCMEC/D3 transmigration. To test this hypothesis, indirectly isolated, PSGL-1-negative CD4+ cells (initial fraction; **Fig. 9A**) were left to transmigrate resting hCMEC/D3 layers, and then transmigrating fractions were stained for CD4/PSGL-1/CD45RO markers and compared with the initial fraction. Transmigrating fractions had more PSGL-1-expressing cells than the initial fraction (Fig. 9B), suggesting PSGL-1 up-regulation during transmigration. However, this PSGL-1 increase was low (<10%) and similar in HI and MS patients. Meanwhile, there was a threefold enrichment in CD45RO+ memory cells in the transmigrating cells (≈60%) compared with the initial fraction (≈20%) in HI and MS patients (Fig. 9C). As a result, PSGL-1 induction was too low to mediate the transmigration of PSGL-1-negative CD45RO+ memory cells across the hCMEC/D3 layers.

VLA-4 (CD49d) inhibition suppresses CD4+ and CD8+ T cell transmigration across resting but not TNF-α-activated hCMEC/D3 layers in untreated MS patients

Equivalent numbers of enriched CD3+ T cells from HI and untreated MS patients were used for VLA-4-blocking assays. CD4+ and CD8+ T cells were counted in nontransmigrating



7. Relative distribution of memory CD45RO+ lymphocytes essing CD4+ and PSGL-1 after transmigration across resting Z/D3 monolayers. (A) The transmigrating and nontransmi- cell subpopulations from PBMC samples were stained for O plus CD4 or CD8 markers. (A) A representative experi- howing the relative predominance of memory CD45RO+ PSGL-1+ T lymphocytes in transmigrating and nontransmi- fractions. (B) Result for the untreated MS patients ($n=7$). < 0.0001.

and transmigrating fractions to calculate TR. VCAM-1-binding inhibition by blocking antibodies did not inhibit HI and MS CD4+ and CD8+ T lymphocyte-resting transmigration. The CD4+ and CD8+ T cell TRs were indeed similar in the isotype- and anti-VCAM-1-treated cultures (**Fig. 10, A** and **B**). Despite unchanged transmigration with anti-VCAM-1, anti-VLA-4 did inhibit resting transmigration of CD4+ and CD8+ cells from MS patients specifically. The inhibitory effects of anti-VLA-4 were depicted in MS CD4+ (0.09%±0.019 with anti-VLA-4 vs. 3±1.4% isotype or 3%±2.3 with anti-VCAM-1; $P<0.01$) and MS CD8+ T cells (0.008%±0.03 with anti-VLA-4 compared with 1%±0.8 with the isotype or 1.4%±1 with anti-VCAM-1; $P<0.001$). These effects were lacking in HI CD4+ (0.1%±0.35 with anti-VLA-4 vs. 0.14%±1 with the isotype or 0.15%±0.35 with VCAM-1) and CD8+ T cells (0.8%±0.8 with anti-VLA-4 vs. 0.1±0.45% isotype or 0.2%±0.7 with anti-VCAM-1), probably as a result of the low levels of transmigration in HI.

The transmigrations assays with TNF-α-activated, BBB-derived hCMEC/D3 layers resulted in increased TR in MS patients and HI compared with resting conditions. MS CD4+ and CD8+ transmigrated activated endothelial layers two- to threefold more than related cells from HI (**Table 1**), thus indicating the greater MS T lymphocyte capacity to transmigrate resting or cytokine-activated, BBB-derived endothelial layers. Anti-PSGL-1-blocking antibodies displayed a nonsignificant trend to inhibit TNF-α-activated hCMEC/D3 transmigration by CD4+ and CD8+ T cells in MS patients (Table 1), and VLA-4 inhibition did not impact on the transmigration of HI and MS CD4+ or CD8+ T cells (Table 1).

Finally, purified CD4+ lymphocytes from MS patients and HI were enriched in PSGL-1 upon transmigration of TNF-α-activated hCMEC/D3 layers. As shown by representative experiments in Figure 10C, 83% of the transmigrating cells from the untreated MS patients were PSGL-1+ versus 70% of the nontransmigrating cells, and the equivalent values were 80% versus 48% in HI. This contrasted with transmigration across resting, nonstimulated hCMEC/D3 layers, where only transmigrating MS CD4+ T cells were enriched in PSGL-1, probably as a result of selectin overexpression induced by TNF-α so that cells adhering to selectin by PSGL-1 were more likely to transmigrate.

DISCUSSION

Although it remains a matter of controversy as to whether MS patients and HI differ in their frequency of myelin autoreactive CD8+ and CD4+ T lymphocytes [24–29] or regulatory cell function [30, 31], circulating blood effectors have been shown to present a different level of activation in this disease [32, 33]. Furthermore, a major difference between MS patients and HI may in fact lie in the capacity of activated blood T cells to penetrate the brain [34]. Only a few studies have focused on the transmigration of blood mononuclear cells across endothelial cells in MS [15, 17, 20], and only one has addressed the mechanism involved, highlighting the role of PSGL-1 and VLA-4 in T cell adhesion to BBB endothelium in a heterologous system in which human T cells were tested on

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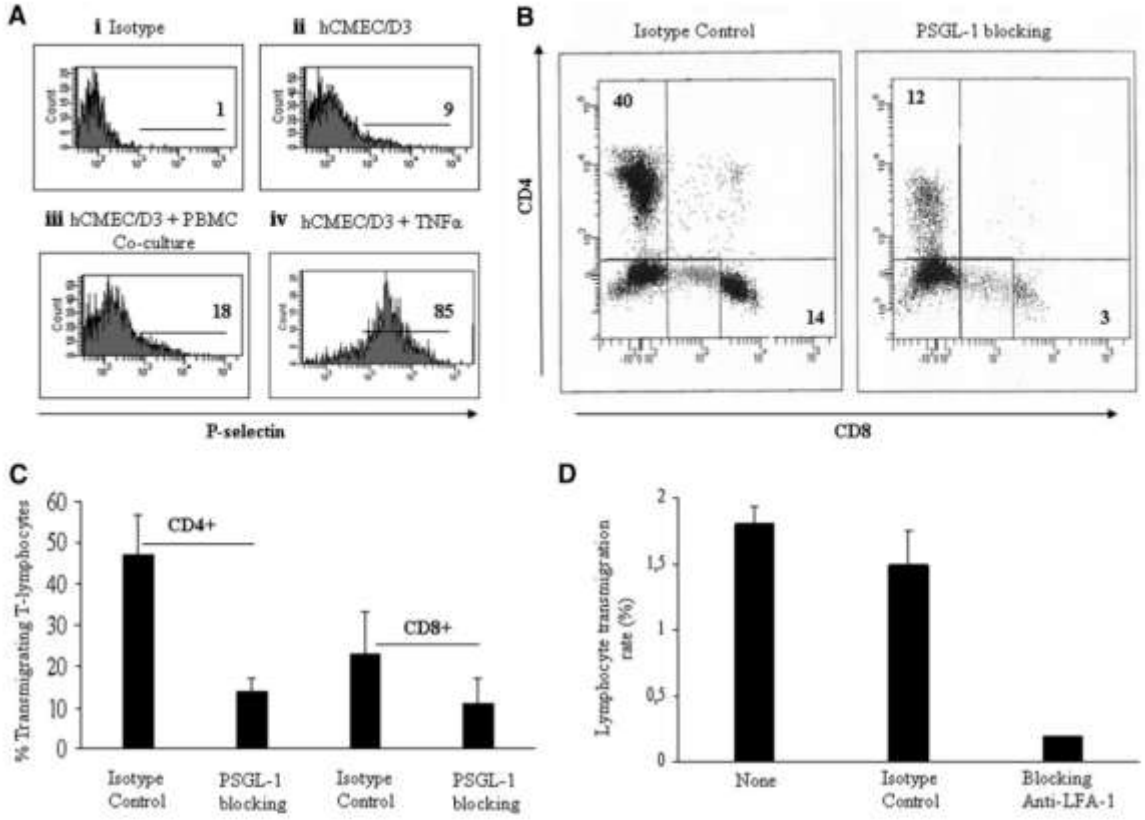

Figure 8. Detection of P-selectin in hCMEC/D3 cultured under different experimental conditions and the critical role of PSGL-1 in T lymphocyte adhesion to hCMEC/D3 layers. (A) Human BBB-derived hCMEC/D3 endothelial cell staining with anti-human P-selectin antibody. (i–iv) Isotype control-stained hCMEC/D3, P-selectin signal in hCMEC/D3 cultured alone, P-selectin in the hCMEC/D3 gate from hCMEC/D3 and PBMC cocultures as those used for the transmigration assay, and P-selectin in TNF-α-treated hCMEC/D3, respectively. (B) PBMC were pretreated for 2 h with anti-PSGL-1-blocking antibody or a matching isotype control and then left to adhere to hCMEC/D3 confluent monolayers for 8 h (blocking antibody replenished after 4 h). After washing unbound PBMC, the adherent PBMC and endothelial cells were detached by treatment with 0.1 M EDTA and stained for CD4 and CD8 markers. The T lymphocytes and hCMEC/D3 cells were discriminated on the basis of forward- and side-scatter properties. The percentage of positive mononuclear cells is stated in each panel. The experiments were repeated at least three times with similar results from the study groups (three- to fourfold inhibition of the adhesion of CD4+ and CD8+ T cells from the IFN-β-treated MS patients and HI). (C and D) PSGL-1 and LFA-1 mediate PBMC transmigration across the BBB-derived hCMEC/D3 endothelial cell layers. (C) PBMC pretreated for 2 h with a matching isotype control or the anti-PSGL-1-blocking antibody were allowed to transmigrate hCMEC/D3 for 8 h. The entire transmigrating cells were then stained for CD3, CD4, and CD8 markers, and then the percent of CD4+CD3+ or CD8+CD3+ was calculated in the mononuclear gate. Results are from three MS patients, and similar results were found with healthy donors or IFN-β-treated MS patients. (D) PBMC pretreated for 2 h with a matching isotype control or the blocking anti-LFA-1 antibody were allowed to transmigrate hCMEC/D3 for 8 h. As the numbers of cells in the transmigrating fractions from anti-LFA-1-treated cultures were too low to be analyzed for marker expression, the cells were counted by light microscopy and by flow cytometry. Results of LFA-1 inhibition are expressed by calculating the TR = (total number of transmigrating cells/total number of input cells) × 100 from three MS patients with similar results obtained with HI or IFN-β-treated MS patients (TR in all groups with anti-LFA-1-blocking antibodies were <0.2%).

mouse endothelial cells [16]. Two studies have examined CD4+ and CD8+ cell transmigration separately using in vitro models of human BBB and reported that CD4+ T cell transmigration is enhanced in MS [18] and that CD4+ and CD8+ T cells preferentially use VLA-4 and PSGL-1 to interact with the brain endothelium [16].

T cells have been detected in CSF of HI [35] and shown to transmigrate resting BBB endothelium in vivo [36]. BBB inflammation possibly results from transmigration of autoimmune T lymphocytes across resting BBB endothelium. Here, we report on a significant increase, not only in the number of PSGL-1+CD4+ T lymphocytes circulating in the peripheral

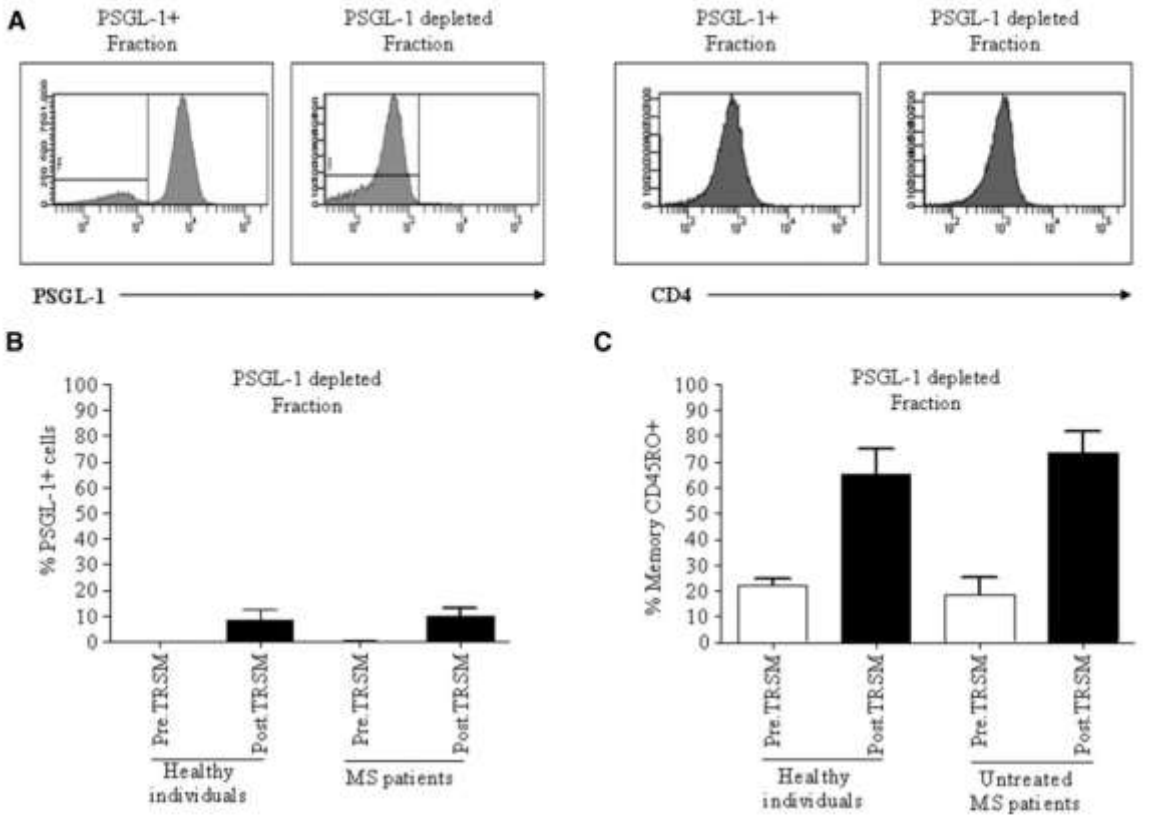

Figure 9. Low induction of PSGL-1 expression in PSGL-1-depleted CD4+ lymphocytes transmigrating across resting hCMEC/D3 layers. (A) Left panels show PSGL-1 expression in the initial fraction (used for PSGL-1 depletion) and CD4+ PSGL-1-depleted fractions. Right panels show equivalent CD4 staining in the two fractions. (B) PSGL-1-depleted CD4+ cells (10^6) were allowed to transmigrate resting, confluent hCMEC/D3 layers for 8 h, and then transmigrating cells were collected and stained for PSGL-1, CD4, and CD45RO or matching isotypes. The percent of PSGL-1 before transmigration (Pre.TRSM) and after transmigration (Post.TRSM) in PSGL-1-depleted fractions is shown. (C) The figure displays the percent of CD45RO expression in PSGL-1-depleted CD4+ fractions before and after transmigration. Data shown are from HI ($n=3$) and untreated MS patients ($n=3$).

blood of MS patients but also in their rate of transmigration across BBB endothelial cells. These properties of CD4+ T cells in MS patients were detected in purified CD4+ cells or in PBMC. The rates of T cell transmigration found (1–2%) were lower than those obtained in other studies with longer incubation times 18–24 h (TR 2–5% and 6–8%, respectively) [15, 17, 19]. However, preliminary data indicate that optimal conditions with our own BBB-derived cell line were 8 h with a lower TR. Furthermore, using blocking antibodies, we show that adhesion to and transmigration across resting hCMEC/D3 monolayers are functionally mediated by PSGL-1 and LFA-1 at similar levels in all of the groups studied. Despite variable frequencies of LFA-1 and PSGL-1, these molecules were key elements in T cell adhesion or transmigration, and transmigration inhibition by anti-PSGL-1-blocking antibodies was associated with the antibody's capacity to reduce the adhesion of CD4+ and CD8+ T lymphocytes to hCMEC/D3 layers.

Altogether, these findings suggest that PSGL-1, which is involved more in CD4+ T cell transmigration in MS patients, may be responsible for their higher transmigration capacity. This is in keeping with a report showing predominance of central memory CD4+ T cells expressing PSGL-1 in the CSF of HI [35]. Although our data imply that PSGL-1 functions as a cell adhesion molecule (not only restricted to leukocyte rolling [16]), previous reports have shown that E-, L-, and P-selectin are also capable of mediating leukocyte adhesion to endothelial layers under static and flow dynamic conditions using endothelial cells of cerebral or extracerebral sources [10, 37–40]. Anti-VLA-4-blocking antibodies also inhibited resting transmigration of MS CD4+ and CD8+ T cells despite the inability of anti-VCAM-1 antibodies to block transmigration. Altogether, these data suggest that BBB transmigration is a complex process involving different sets of adhesion molecules.

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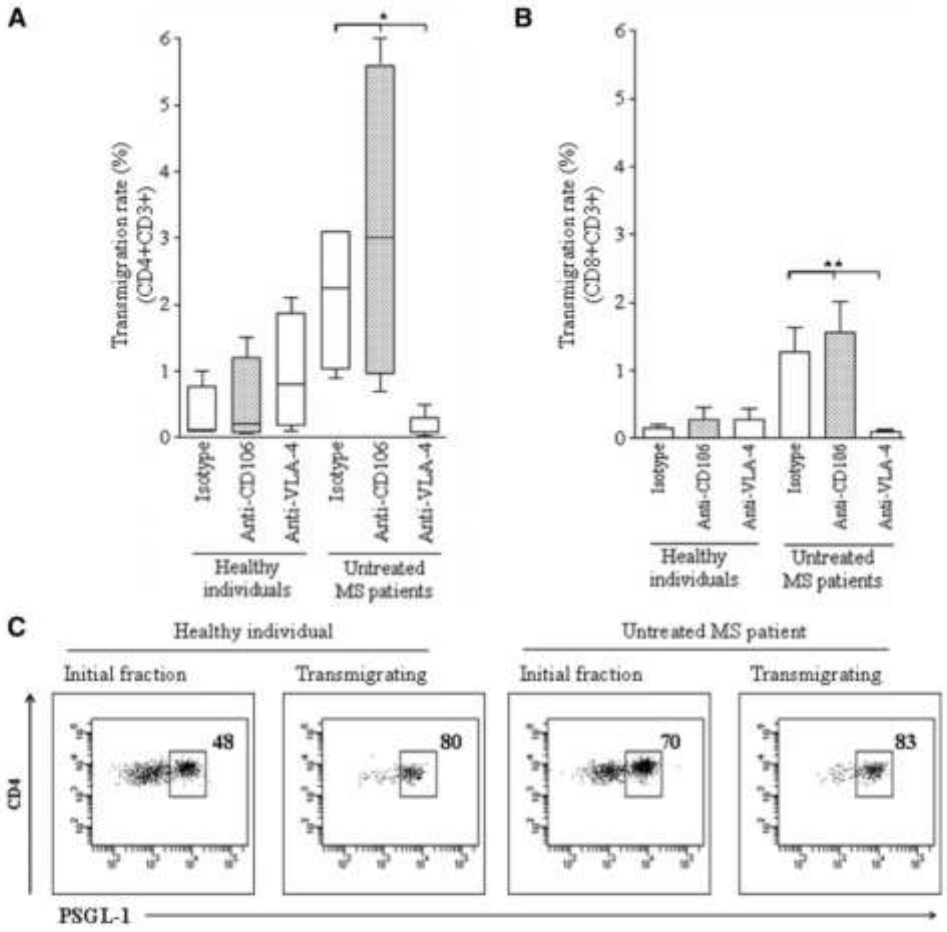

Figure 10. Effects of anti-VCAM-1 and anti-VLA-4-blocking antibodies on CD3+CD4+ or CD3+CD8+ transmigration across resting hCMEC/D3 layers. Enriched CD3+ T lymphocytes from PBMC samples of HI (n=5) or untreated MS patients (n=5) were left untreated or treated with anti-VLA-4-blocking antibodies or isotypes. Meanwhile, hCMEC/D3 layers were left untreated or treated with anti-VCAM-1-blocking antibodies (Anti-CD106). Treated or untreated cells were used for transmigration assays, the input and transmigrating cells were stained for CD3/CD4/CD8, and then TR were calculated. (A) Blocking effects on the CD3+CD4+ T cell subset. (B) Blocking effects on the CD3+CD8+ T cell subset. Median TR from each group is shown. (C) Representative examples from one HI and one untreated MS patient showing equivalent PSGL-1 enrichment in CD4+ lymphocytes transmigrating across TNF-α-treated hCMEC/D3 layers. *, $P < 0.01$; **, $P < 0.001$.

Nevertheless, despite the absence of exogenous proinflammatory cytokines under resting conditions, hCMEC/D3 cells cocultured with PBMC (in the adhesion or the transmigration assays) were not in a complete resting state and did overexpress markers such P-selectin and ICAM-1. These observations suggest that a "physiological" production of local cytokines by PBMC or cell–cell contacts with PBMC provided some activation signals in the assay. The PBMC of MS patients were found to display alterations in their trafficking properties associated with an elevated CD4+ PSGL-1+ frequency and the higher TR of these cells, which was independent of artificial in vitro cytokine stimulation of BBB endothelial cells similar to previous reports [15, 17]. Even after TNF-α activation of hCMEC/D3 layers, CD4+ and CD8+ T cells from MS patients still transmigrated at higher levels than controls. On the contrary to resting conditions, PSGL-1/VLA-4-blocking antibodies were unable to block transmigration across cytokine-stimulated hCMEC/D3. Previous reports have indeed shown that VLA-4 interaction with VCAM-1 is essential for adhesion but not for the diapedesis step [41–45]. Similar to our findings, increased transmigration of CD4+ T cells from MS patients in comparison with healthy control individuals has been reported recently by Dressel et al. [18]. However, the authors did not investigate the adhe-

TABLE 1. Neither Anti-PSGL-1 Nor Anti-VLA-4 Significantly Blocked CD3+ T Lymphocyte Transmigration Across TNF-α-Pretreated hCMEC/D3 Layers

CD4+ T cell	Isotype	Anti-PSGL-1	Anti-VLA-4
HI	3.2 ± 0.3	4 ± 0.46	3.4 ± 0.66
Untreated MS patients	7.6 ± 4.9	3.75 ± 0.07	11.4 ± 1.18
CD8+ T cell	**Isotype**	**Anti-PSGL-1**	**Anti-VLA-4**
HI	4.5 ± 5.9	1.25 ± 1.4	5 ± 0
Untreated MS patients	13.3 ± 2.25	3.7 ± 0.51	12.3 ± 3.5

sion molecules involved in this process. It can be argued that the enhanced PBMC transmigration reported previously in MS disease was largely a result of the increased trafficking properties of CD4+ T cells. In addition, BBB endothelium is well reported to be more activated and permeable in the course of MS disease [46, 47], which would increase PBMC influx further to the brain.

In our study, PSGL-1+ cells were significantly more numerous in transmigrating CD4+ T lymphocytes of MS patients compared with their corresponding nontransmigrating cells under resting conditions, and this difference was not observed with the CD4+ lymphocytes of HI. PSGL-1+ cells were therefore recruited to a greater extent in the transmigration of the CD4-positive lymphocytes in MS patients, a phenomenon likely enhanced by the expansion of the PSGL+CD4+ T cell pool in the peripheral blood of patients with MS. In addition and in accordance with the fact that post-translationally activated PSGL-1 is preferentially expressed on memory cells [48–50], we found that transmigrating CD4+CD45RO+ memory cells expressed PSGL-1 at a level that significantly exceeded that of the nontransmigrating memory CD45RO+ cells. Conversely, TNF-α stimulation of hCMEC/D3 layers resulted in PSGL-1 enrichment in the transmigrating fractions of HI and MS patients, suggesting an important role of PSGL-1 in stimulated transmigration. Although our data fit with the previous report of Battistini et al. [16], we did not confirm that PSGL-1 has a similar importance in hCMEC/D3 transmigration by CD8+ T lymphocytes, which did not exhibit enriched PSGL-1+ cell frequency after transmigration. Remarkably, a reduced transmigration of CD8+ T cells in IFN-β-treated MS patients was associated with reduced percentages of LFA-1^{high} CD8+ T lymphocytes in their peripheral blood. LFA-1 down-regulation has actually been hypothesized to be one potential mechanism by which IFN-β modulates PBMC transmigration across the brain endothelium [51, 52]. In contrast to LFA-1, the higher expression of PSGL-1 in bulk PBMC or purified CD4+ T cells from MS patients was unchanged by the patient's exposure to IFN-β. Although LFA-1 was a key element on its own for PBMC transmigration across hCMEC/D3 layers, it may synergize with PSGL-1 at the cell surface. Atarashi et al. [53] have demonstrated that PSGL-1 enhances LFA-1 binding to ICAM-1, which may also explain why CD4+ cells transmigrated in higher numbers than CD8+ T lymphocytes in MS patients.

It should also be noted that other adhesion molecules not analyzed may be involved in PBMC transmigration, such as the recently reported activated-leukocyte-cell-adhesion-molecule [54], which might also have a distinctive role in CD4+ and CD8+ cell transmigration. Of note, a minority of CD4+ and CD8+ T lymphocytes still adhered/transmigrated hCMEC/D3 layers, despite the addition of anti-PSGL-1-blocking antibodies. In addition, PSGL-1-negative memory CD45RO+CD4+ cells were able to transmigrate hCMEC/D3 layers. Although transmigration induced PSGL-1 re-expression in some transmigrating cells, as reported previously for CCR5 on BBB-transmigrating monocytes [55], it was too low to account for the overall transmigration of memory PSGL-1-negative CD4+ cells. These data therefore suggest that some PSGL-1-independent mechanisms were also crucial for the ex vivo CD4+ T cell transmigration across resting BBB. Blocking selectin and integrin functions improve the inflammation process in animal models [56]. Although humanized antibodies against the α4-integrin subunit were proven efficient in autoimmune diseases such MS, there is a controversy about the PSGL-1 or P-selectin role in brain inflammation. On one hand, PSGL-1 has been reported to be unnecessary for the development of EAE in animal studies [57, 58]; on the other hand, Kerfoot and Kubes [59] have shown that P-selectin expression in brain vasculature is increased early during EAE and mediates leukocyte rolling on brain venules and that α4-integrin-mediated leukocyte adhesion was entirely dependent on P-selectin.

Finally, the present study was not performed under dynamic shear stress conditions. However, in the dynamic system, the cells with low-affinity receptors roll without adhesion/transmigration [40, 60, 61]. In the dynamics study by Battistini et al. [16], a large dose of LPS (25 μg/ ml) had to be administered to experimental animals to promote activation of the BBB endothelium and to increase the detection sensitivity of the rolling/adherent cells.

In conclusion, our data suggest that an expanded pool of PSGL-1-expressing CD4+ T lymphocytes would increase the rate of leukocyte rolling and transmigration across the brain endothelium, thus potentially leading up to a pathological immune infiltration in the brain.

ACKNOWLEDGMENT

This work was supported in part by the Association pour la Recherche sur la Sclérose En Plaques (ARSEP; Paris, France).

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KEY WORDS:
LFA-1 · VLA-4 · p-selectin · VCAM-1 · ICAM-1 · inflammation · endothelium

ANNEXE III : Implication de TRIB1 dans la physiopathologie de la Sclérose en Plaques

TRIB1, aussi connu sous la désignation C8FW, GIG2 ou SKIP1, est un homologue intracellulaire de Drosophila tribbles, une famille comprenant 3 gènes. Chez les mammifères, il a été suggéré que TRIB1 pourrait être un régulateur de la signalisation cellulaire et, notament, agirait sur la voie des MAPkinases. En effet, il inhiberait spécifiquement la voie menant à l'activation d'AP-1, un facteur de transcritpion impliqué dans l'apoptose, la prolifération et la différentiation (Kiss-Toth et al. 2004). Néanmois, l'expression et le rôle endogène de TRIB1 n'a pas encore été bien établi. Le profil d'expression du transcrit de TRIB1 a été étudié dans la greffe de rein et il a été montré que TRIB1 était un biomarqueur à la fois dans le sang périphérique et le tissu du rejet chronique humoral (Ashton-Chess et al. 2008). Même si la physiopathologie du rejet de greffe et de la SEP est différente, il existe certaines caractéristiques communes comme l'allo et l'auto-immunité.

Nous avons donc cherché à caractériser l'expression de TRIB1 dans les différentes formes et phase de la SEP. Nos résultats préliminaires suggèrent que ce gène est surexprimé dans le sang de patient ayant cette maladie (Figure 1).

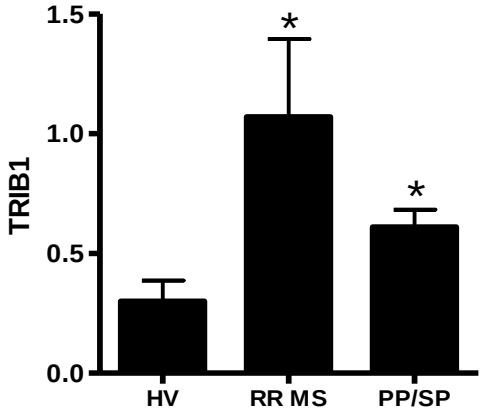

Figure 1: Expression de l'ARNm de TRIB1 au sein des PBMC.
L'expression de l'ARNm de TRIB1 est étudiée pour les patienst atteints de SEP de forme rémittente (RR MS), et de forme progressive (primaire et secondaire, PP/SP) et chez les individus sains appariés selon l'âge (HV). Cette expression est mesurée par RT-PCR. Les résultats sont exprimés en unités arbitraires. * indique $p < 0.05$. Entre 11 et 16 patients sont inclus par groupes.

Dans la suite de l'étude, la caractérisation de l'expression de TRIB1 dans la SEP a été limitée aux patients atteints de SEP de forme rémittente, non-traités (RRMS n=5). Un nouveau groupe de témoins a été ajouté. Celui-ci est composé de patients atteints de la maladie de Devic (NMO n=5). L'inclusion de ce groupe a été rendue possible grâce à la collaboration de l'équipe de Lille du Professeur P. Vermersch. Nous avons aussi observé l'expression de TRIB1 au sein des PBMC de sujets sains (HV n=5).

De plus, cette étude a été approfondie en mesurant l'expression de l'ARNm des deux autres membres de la famille TRIB : TRIB2 et TRIB3, pour les trois cohortes d'individus.

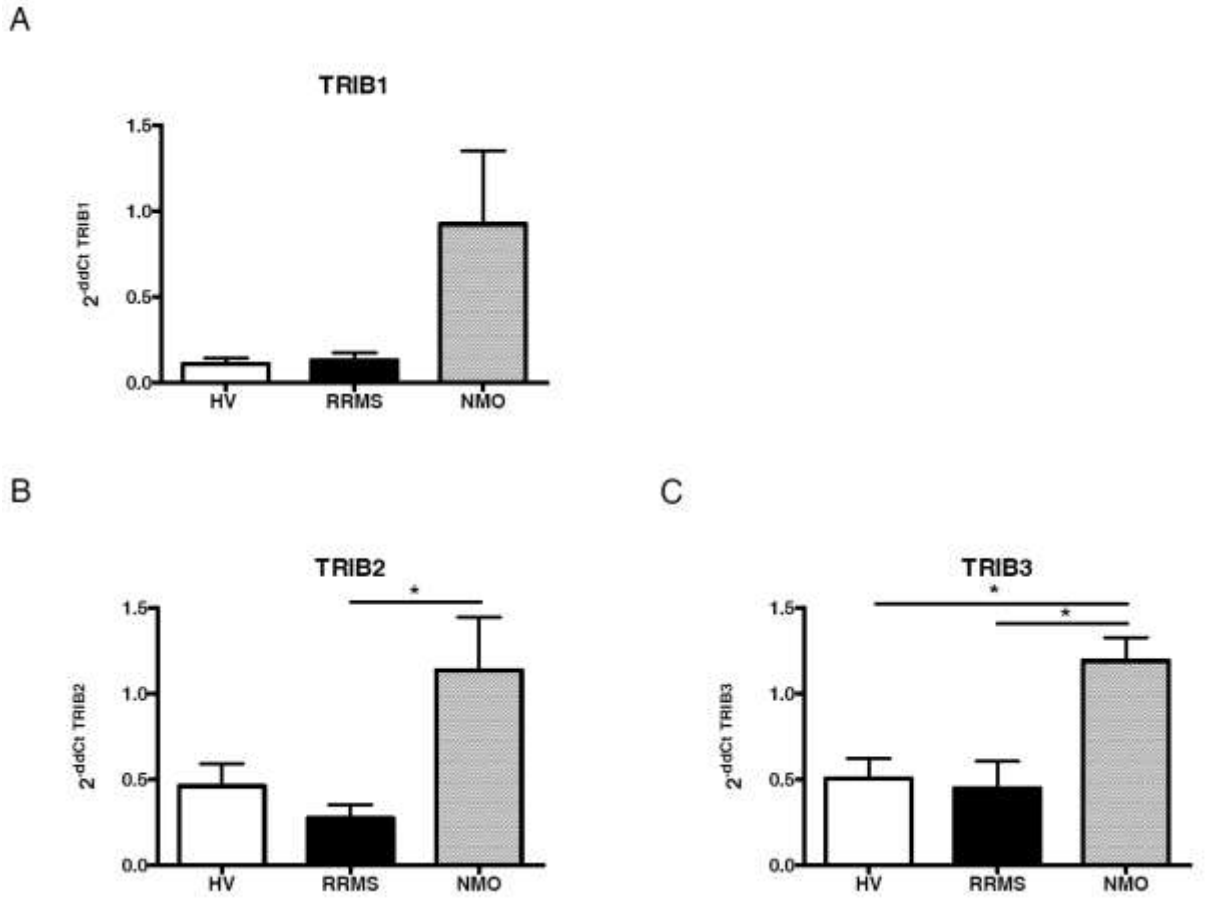

Figure 2 : Expression de l'ARNm de TRIB1, TRIB2 et TRIB3 au sein des PBMC.
L'expression des transcrits de chacun des trois membres de la famille TRIB est mesurée par RT-PCR et rapporté à l'expression du gène rapporteur HPRT. Ces expressions de transcrits sont mesurées chez cinq individus sains (HV), cinq patients atteints de SEP de forme rémittente et non-traités (RRMS) et chez cinq patients atteints de la maladie de Devic (NMO). **A.** L'expression de l'ARNm de TRIB1 est comparable pour les trois groupes étudiés. **B.** L'expression de l'ARNm de TRIB2 est mesurée chez les témoins sains, les patients atteints de SEP et les patients atteints de la maladie de Devic. **C.** L'expression de l'ARNm de TRIB3 est mesurée dans les trois groupes d'intérêt. * indique $p < 0.05$.

Comme nous pouvons l'observer sur la Figure 2A, aucune différence significative n'est obtenue concernant le niveau d'expression de l'ARNm de TRIB1 entre les trois cohortes d'individus. Le niveau d'expression de TRIB2 est, quant à lui, significativement augmenté dans les PBMC des patients atteints de maladie de Devic comparé à celui des patients atteints de SEP. Aucune différence d'expression n'est observée avec le groupe des individus sains (Figure 2B). Le niveau d'expression de TRIB3 est significativement augmenté chez les patients atteints de la maladie de Devic comparé aux patients atteints de SEP mais aussi comparé aux témoins sains (Figure 2C).

Ces résultats seront à confirmer en augmentant le nombre d'individus au sein des trois populations étudiées.

Cette étude sera complétée en isolant différentes populations cellulaires du système immunitaire à partir de prélèvements sanguins de patients. Nous chercherons ainsi à identifier le(s) type(s) cellulaire(s) responsable de l'augmentation de l'expression des membres de la famille TRIB. Enfin, si l'augmentation de TRIB1 est confirmée dans la SEP, nous chercherons s'il existe une corrélation entre cette surexpression de TRIB1 et la présence d'auto-anticorps anti-myéline, comme cela est suggéré par l'association de ce gène avec des anticorps dans le rejet de greffe de rein.

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ANNEXE IV : Caractérisation phénotypique des Lymphocytes B dans le sang de patients atteints de Sclérose en Plaques

Les lymphocytes B (LB) interviennent dans la réponse immunitaire de type humorale. Ils jouent un rôle central dans le développement et le contrôle de l'immunité. Ils peuvent agir dans l'ontogénèse des cellules dendritiques folliculaires mais également comme cellule présentatrice d'antigènes dans l'activation des lymphocytes T CD4+ et CD8+. Il est même suggéré l'existence d'une catégorie de cellules B régulatrices productrices d'IL10 (Serra et al. 2006). Ces cellules pourraient à l'instar des cellules T CD4+CD25high, réguler les différents phénomènes immuns et auto-immuns. Le système humoral est fortement impliqué dans la SEP. En effet, les lymphocytes B, en particulier les plasmoytes, ont été détectés au niveau des lésions et des infiltrats lymphocytaires chez les patients atteints de SEP (Prineas et al.1978). Tous les types de différentiation des cellules B, c'est-à –dire du lymphocyte B naïfs au plasmocyte, sont présents dans le LCR des malades (Corcione et al. 2004).

Notre étude a pour objectif de caractériser phénotypiquement les LB chez les patients atteints de SEP de forme rémittente non-traités. L'analyse fine des différentes sous-populations n'a pour l'instant jamais été réalisée dans le contexte de la SEP

L'ontogenèse des LB se déroule dans la moelle osseuse. Au terme de leur maturation, les LB quittent la moelle pour migrer vers la périphérie en tant que LB transitionnels. Les LB matures sont présents dans les organes lymphoïdes secondaires (OLII). Ce sont eux qui participent à la réponse immunitaire et constituent les follicules lymphoïdes. Les LB matures vont suivre une voie de différenciation qui va donner naissance aux LB mémoires et aux plasmocytes sécréteurs d'anticorps. Ces sous-populations peuvent être identifiées grâce a un marquage IgD/CD38 qui caractérisent fonctionnellement les sept populations de B matures (Hillion, Saraux et al. 2005) : les LB naifs Bm1 $IgD^{+}/CD38^{-}$, les LB activés Bm2 $IgD^{+}/CD38^{+}$, les cellules fondatrices du centre germinatif Bm2' $IgD^{+}/CD38^{high}$, les cellules du centre germinatif (centroblastes et centrocytes Bm3 et Bm4) $IgD^{-}/CD38^{high}$, les cellules B mémoires précoces EBm5 ($IgD^{-}/CD38^{+}$) et les cellules B mémoires Bm5 $IgD^{-}/CD38^{-}$. Les plasmocytes sont $IgD^{-}/CD38^{high}$ $CD138^{+}$ (Figure 1). On peut retrouver les diversités des phénotypes des LB dans le sang périphérique, elle peut constituer une image fonctionnelle de l'activation LB d'un individu. En effet, elle peut être anormale dans certaines pathologies

auto immunes (Youinou, Hillion et al. 2006) ou dans des infections virales comme l'hépatite C (Fournillier, Freida et al. 2004).

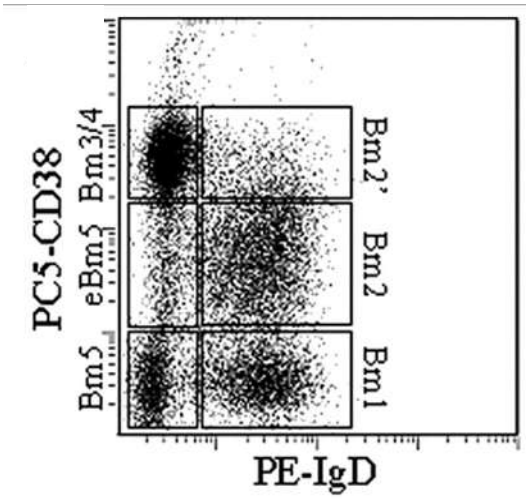

Figure 1 : Distribution des LB matures par cytométrie en flux selon le marquage IgD et CD38.

J'ai réalisé un monitorage spécifique des LB pour mettre en évidence et caractériser les différentes populations de LB matures à l'aide de marqueurs membranaires. Des marqueurs associés aux différentes sous-populations de B matures comme les marqueurs associés à la présentation d'antigène CD86, CD80 ont été analysés. Les marqueurs des LB mémoires ont été évalués comme CD27 ou CD62L. 27 patients atteints de SEP de forme rémittente et non-traités (MS) ont été inclus à l'étude. Deux groupes de témoins ont été constitués : l'un composé de 21 individus sains (HV) l'autre de 22 patients atteints de la maladie de Devic (NMO). Ce dernier groupe a pu être constitué grâce à la colaboration des équipes de Lille du Professeur P. Vermersch et de celle Strasbourg du Professeur J. De Sèze.

Dans un premier temps, je me suis interessée la fréquence des LB CD19+ au sein des trois cohortes d'individus. Cette population cellulaire présente une fréquence similaire que ce soit chez les patients atteinst de SEP, les patients atteinst de NMO ou bien les témoins sains. Nous faisons le même constat concernant la répartition des six sous-populations composant les LB (Figure 2).

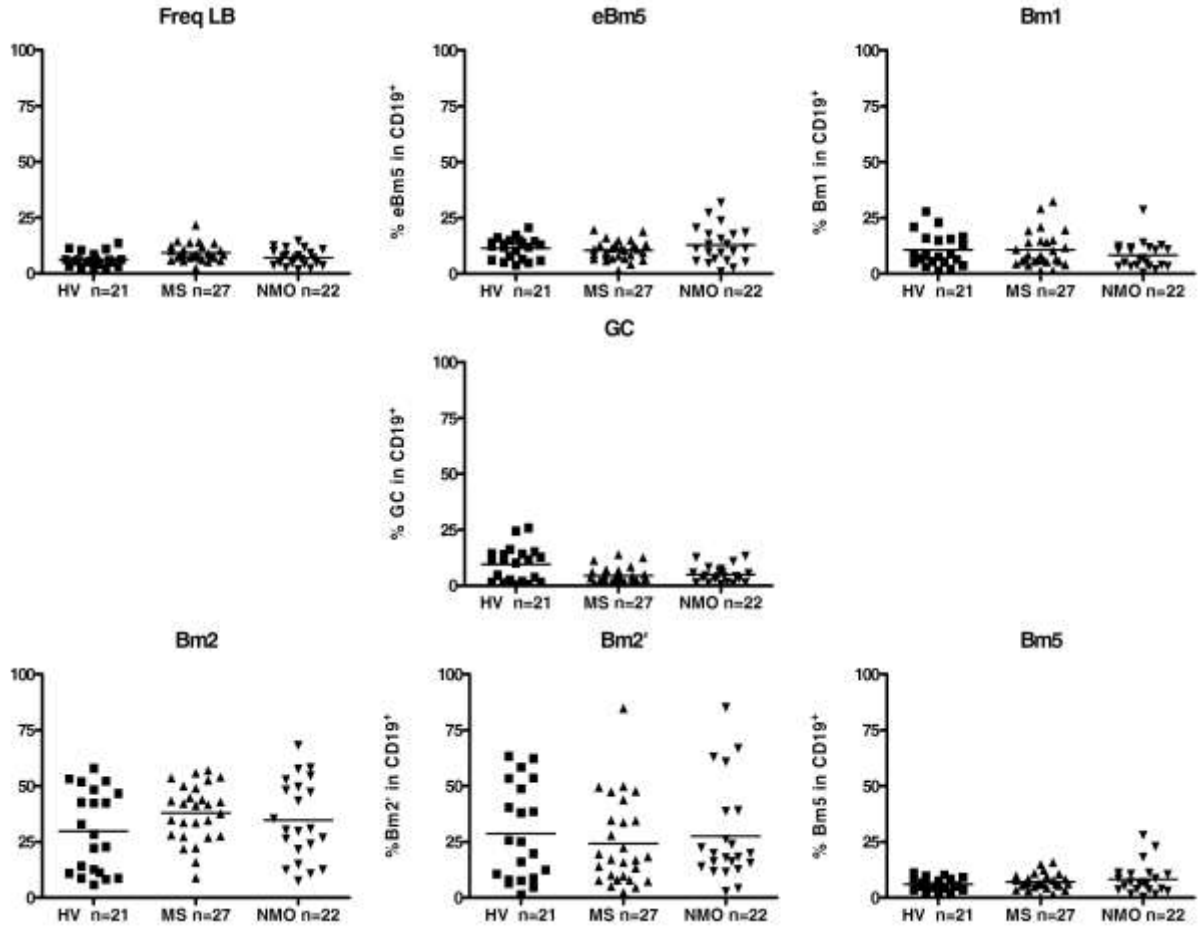

Figure 2 : Répartition des différentes sous-populations de lymphocytes B périphériques
Les PBMCsont marqué avec des anticorps anti-CD19-PE-Cy7, anti-IgD-FITC et antiCD38-PE-Cy5. Les cellules sont analysées par cytométrie en flux en positionnant une fenêtre de sélection sur les lymphocytes B CD19+. Aucune différence significative n'est observée pour l'ensemble des sous-populations étudiées entre les patienst atteints de SEP non-traités (MS=27), les patients atteints de la maladie de Devic (NMO=22) et les sujets sains (HV=21). **Freq LB** = Fréquence des LB, **eBm5** = Fréquence de la population eBm5 au sein des LB, **Bm5** = Fréquence de la population Bm5 au sein des LB, **GC**= Fréquence de la population GC (Bm3/4) au sein des LB, **Bm2** = Fréquence de la population Bm2 au sein des LB, **Bm2'**= Fréquence de la population Bm2' au sein des LB et **Bm5** = Fréquence de la population Bm5 au sein des LB.

L'étude phénotypique des lymphocytes B a été complétée par la caractérisation de l'expression des différents marqueurs : CD27, CD62L, CD80 et CD86 à la surface des LB CD19+ (Figure 3). Aucune différence significative n'a été observée pour l'expression de ces quatre marqueurs au sein des LB des patients atteints de SEP, des patients atteints de NMO et des sujets sains.

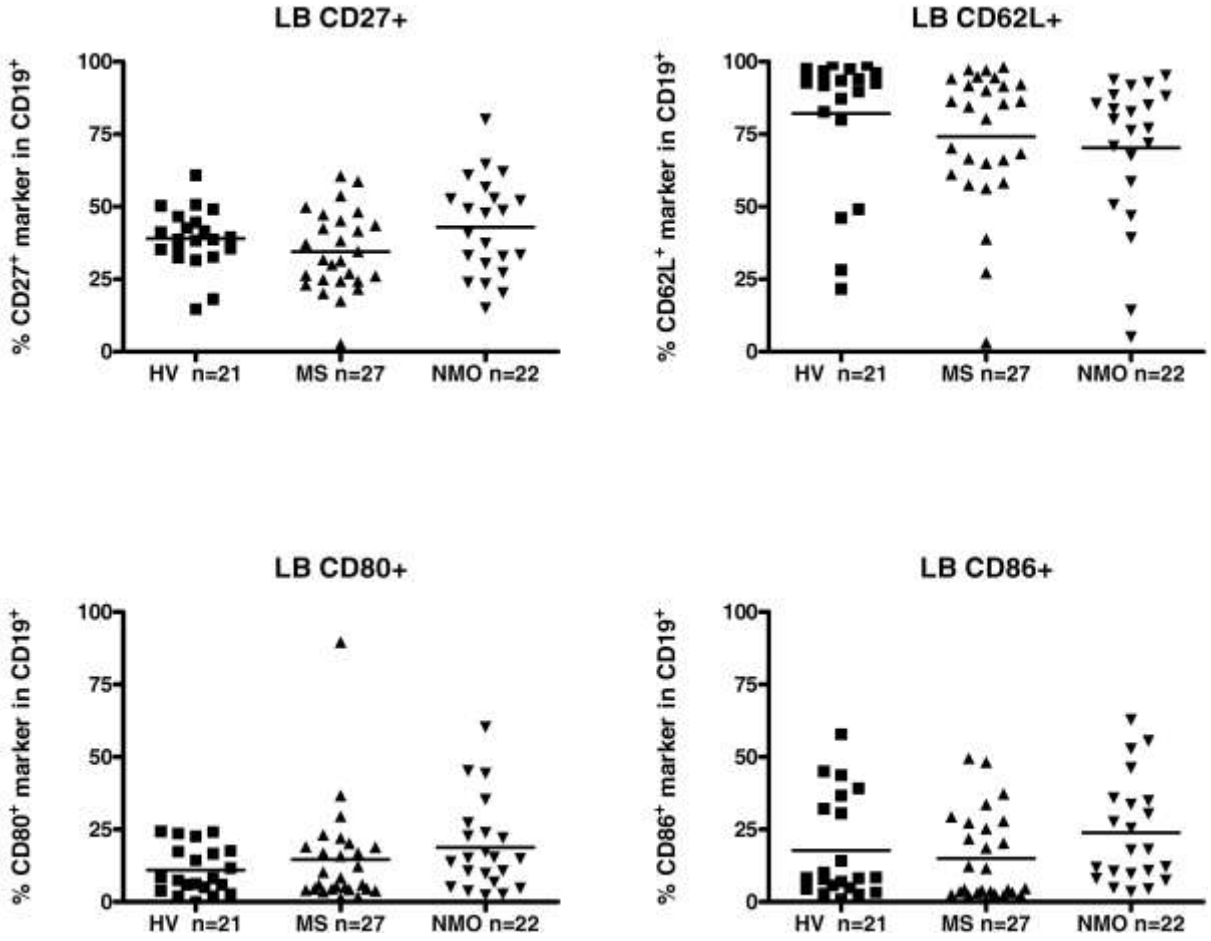

Figure 3 : Fréquence des cellules exprimant CD27, CD62L, CD80 et CD86 au sein des lymphocytes B périphériques. Les PBMC sont marqué avec un mix d'anticorps composé d'anti-CD19-PE-Cy7, anti-IgD-FITC et d'anti-CD38-PE-Cy5. Puis, un quatrième anticorps couplé PE est ajouté (ciblant soit CD27, soit CD62L, soit CD80 ou soit CD86). Les cellules sont analysées par cytométrie en flux en positionnant une fenêtre de sélection sur les lymphocytes B CD19+. Aucune différence significative n'est observée pour l'ensemble des sous-populations étudiées entre les patienst atteints de SEP non-traités (MS=27), les patients atteints de la maladie de Devic (NMO=22) et les sujets sains (HV=21).

Les conclusions de ces résultas préliminaires sont en concordance avec ceux obtenus par Fraussen et ses collaborateurs. En effet, dans leur travail, la distribution des LB périphériques est comaprable entre les patients atteints de SEP et les individus sains (Fraussen et al. 2009). Cependant, chez les sujets sains, les cellules B sont rarement présentes dans le LCR à l'inverse des patients où l'on observe une expansion clonale de cette population cellulaire.

Cette étude préliminaire sera complétée par l'analyse de la présence et la caractérisation des cellules B régulatrices dans nos trois cohortes d'intérêt. La population de LB régulatrice sera caractérisée sur sa capacité à secréter de l'IL-10 en association avec d'autres marqueurs tels CD1D. Les molécules de costimulation du récepteur d'antigène (BCR, B cell receptor)

seront également analysées : les stimulateurs positifs comme CD21 ou CD19 et les régulatrices négatives comme CD22 ou CD32. Les plasmocytes circulants seront identifiés grâce au marqueur CD138.

Le rôle de ces populations B sur d'autres populations lymphocytaires comme les lymphocytes T pourra ensuite être évalué par des cultures mixtes.

Les différentes sous-populations de LB matures seront séparées grâce au trieur cellulaire FACSAria®. Le profil transcriptionnel de chacune d'entre elles sera établi. Nous ciblerons l'analyse transcriptionnelle des LB sur l'expression différentielle de cytokines pro- ou anti-inflammatoires. Une analyse globale sur les PBMCs de l'expression des différents récepteurs pour le fragment Fc des immunoglobulinnes de type activateurs CD32a ou inhibiteur CD32b sera également effectuée.

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Étude du rôle effecteur et régulateur des lymphocytes T dans la Sclérose en Plaques

La sclérose en plaques (SEP) est une maladie chronique inflammatoire du système nerveux central associée à la présence de lymphocytes T auto-réactifs. En dépit de la prépondérance des lymphocytes CD8+ dans les altérations du répertoire T dans la SEP, nous avons observé une fréquence similaire des cellules CD8+ auto-réactives dans le sang des patients atteints de SEP et des individus sains. Ceci nous a conduit à étudier la fonction régulatrice des cellules T CD4+CD25high en incluant le marqueur CD127 (chaîne α du récepteur à l'IL7) et à proposer d'auters approches de calcul de fréquence. Notre étude a montré que les cellules CD4+CD25highCD127- des patients atteints de SEP possédaient la même capacité régulatrice que celles des individus sains. En outre, les cellules CD4+CD25highCD127+ des patients atteints de SEP semblent être hyperprolifératives et présenter un profil cytokinique pro-inflammatoire. Pour la première fois, notre équipe a mis au point la technique TRAP (T Recognition of APC by Protein Transfer) dans le contexte de la SEP, limitée à l'utilisation de la myéline totale comme antigène. Cette nouvelle méthode a permis de mettre en évidence une fréquence très élevée des cellules T auto-réactives dans la SEP. Les lymphocytes T auto-réactifs positifs au TRAP sont caractérisés par un niveau d'expression plus important de CD40L et de $CD62L^{low}$. À la différence de la technique de référence qu'est l'ELISPOT-IFNγ, le TRAP présente l'avantage de s'affranchir des différents CMH et peptides et d'étudier directement tous les lymphocytes auto-réactifs quelques soient les peptides reconnus. Nos résultats portant sur des patients non-traités en début de maladie plaident fortement pour une étiologie auto-immune de la SEP.

Mots-clés : sclérose en plaques, lymphocyte T, auto-immunité

Study of effectory and regulatory role of T lymphocytes in Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of central nervous system associated with auto-reactive T cells. Even though MS patients show increased alterations in T cell repertoire, predominantly in CD8+ T cell compartment, we observed that there was no difference between the auto-reactive CD8+ T cell frequency in blood of MS patients and healthy individuals. Furthermore, we used CD127 (IL7 receptor α chain) in order to describe the regulatory function of CD4+CD25high T cells. We have shown that CD4+CD25highCD127- T cells present similar regulatory capacity in MS patients compared to healthy individuals. Moreover, CD4+CD25highCD127+ T cells from MS patients seem to be hyperproliferative and present a pro-inflammatory cytokine profile. We are the first laboratory to use a new method, called TRAP (T Recognition of APC by Protein Transfer) in the context of MS. This new assay revealed a significantly increased frequency of auto-reactive T cells in MS patients with respect to healthy individuals. Auto-reactive T lymphocytes are characterized by higher CD40L and $CD62L^{low}$ expression. Compared to the classically used ELISPOT-IFNγ assay, the main interest of TRAP employing whole myelin as an antigen is to study the entire population of auto-reactive T cells allowing at the same time to distinguish between CD8+ and CD4+ T cells. In conclusion, activation and/or regulation of cell effectors seem to be important in MS pathology.

Key words: Multiple sclerosis, T lymphocyte, auto-immunity,