UNIVERSITE DE NANTES
FACULTE DE MEDECINE

ETUDE DU REPERTOIRE T ET DE LA SPECIFICITE DES LYMPHOCYTES T CD8+ CHEZ DES PATIENTS ATTEINTS DE SCLEROSE EN PLAQUES

THESE DE DOCTORAT

Ecole doctorale : CHIMIE BIOLOGIE
Discipline : SCIENCES DE LA VIE ET DE LA SANTE
Spécialité : IMMUNOLOGIE

Présentée et soutenue publiquement par

Laureline Berthelot

Le 25 juin 2007, devant le jury ci-dessous :

Présidente : Jeannine Choppin, Directrice de recherche, Paris

Rapporteurs :
Marie-Françoise Belin, Directrice de recherche, Lyon
Dominique Baeten, Docteur, Professeur associé, Pays Bas

Directeur de thèse : Jean-Paul Soulillou, Professeur, Nantes

Sommaire

Liste des figures

Liste des tableaux

Abréviations

BDNF : Facteur Neurotrophique Dérivé du Cerveau

BHE : Barrière Hémato-Encéphalique

CMH : Complexe Majeur d'Histocompatibilité

CDR : Région Déterminant la Complémentarité

CPA : Cellule Présentatrice d'Antigène

CTLA4 : *Cytotoxic T Lymphocyte Associated protein 4*

EAE : Encéphalomyélite Auto-immune Expérimentale

GA : Acétate de Glatiramère

HLA : Antigènes Humains des Leucocytes

HPRT : Hypoxanthine PhosphoRibosyl Transférase

ICAM : Molécule d'Adhésion InterCellulaire

IFN : Interféron

IL : Interleukine

IRM : Imagerie par Résonance Magnétique

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

NO : Monoxyde d'azote

PBMC : Cellules Mononucléées du Sang Périphérique

PGSL1 : P-Sélectine Glycoprotéine Ligand-1

PHA : Antigène PhytoHemaglutinine

PLP : Protéine Protéo-Lipidique

SEP : Sclérose En Plaques

SNC : Système Nerveux Central

TAP : Transporteur Associé à l'apprêtage des antigènes

TCR : Récepteur des Cellules T

TGFβ : *Transforming Growth factor β*

TNFα : *Tumor Necrosis Factor α*

VCAM : Molécule d'Adhésion Cellulaire Vasculaire

VLA-4: *Very Late Antigen-4* (nommé également α4β1 ou α4-intégrine)

Introduction

1- La Sclérose en Plaques

1.1 Définition

La sclérose en plaques (SEP) fut décrite pour la première fois par Robert Carswell et Jean Curveilhier 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 touchant le système nerveux central (SNC), caractérisée par une démyélinisation. Cette pathologie présente des formes différentes, 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 présentent une forme généralement circonscrite, bien délimitée et sont concentrées le plus souvent dans les zones périventriculaires de la substance blanche, le nerf optique, le tronc cérébral mais aussi au niveau de la moelle épinière. Les symptômes, causés par la défaillance de conduction nerveuse, correspondent en général à des pertes de fonctions motrices ou visuelles mais aussi cognitives. Ils peuvent se concentrer pendant une courte période, on appelle ce phénomène une poussée. Une personne sur 1000 en France est touchée par cette pathologie, les symptômes débutant habituellement entre 20 et 40 ans, les femmes étant deux fois plus touchées que les hommes. Le diagnostic clinique peut être établi lorsque le patient présente des plaques (zones démyélinisées) détectées par imagerie par résonance (IRM) disséminées à plusieurs endroits du SNC et dont les apparitions sont dispersées dans le temps (McDonald, Compston et al. 2001; Lublin 2005; Polman, Reingold et al. 2005). Un profil oligoclonal des immunoglobulines au niveau du liquide céphalorachidien (LCR) peut aussi être détecté lors d'une ponction lombaire (Freedman, Thompson et al. 2005). Il est à noter qu'aucun test biologique, clinique ou radiologique ne permet de confirmer de façon certaine le diagnostic, celui-ci reposant sur un faisceau d'arguments.

1.2 Variétés pathologiques

1.2.1 La sclérose en plaques de forme rémittente

Les personnes atteintes de sclérose en plaques de forme rémittente représentent 85% des cas en début de maladie. Les symptômes alternent entre phase de poussée et phase de rémission (fig 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.

1.2.2 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, Guttmann et al. 1994) (Filippi, Horsfield et al. 1995). La présence de poussée n'est pas systématique, mais habituellement suivie de l'augmentation du nombre de séquelles (fig 1).

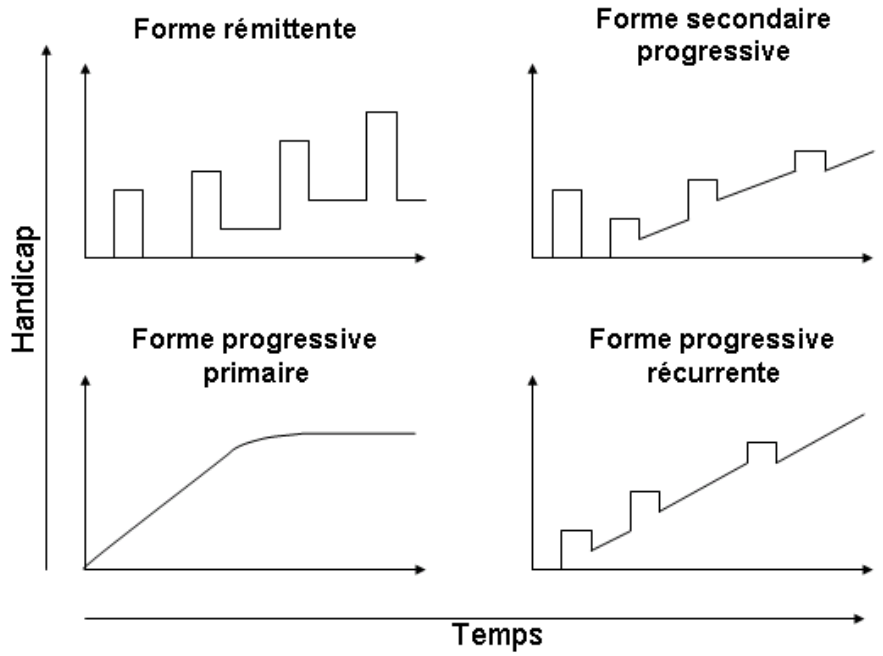


Figure 1: Les différentes évolutions au cours du temps du handicap pour les quatre formes de sclérose en plaques
La forme rémittente est caractérisée par l'alternance de poussées et de phases de rémission. Les formes secondaires progressives présentent une aggravation graduelle de l'état des patients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

1.2.3 La sclérose en plaques de forme progressive primaire

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

1.2.4 La sclérose en plaques de forme progressive récurrente

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

1.2.5 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 and Mancardi 2004). La maladie de type Marburg présente une évolution fulminante avec une démyélinisation importante du tronc cérébral ou des hémisphères cérébraux. Si les malades survivent à la phase aiguë, 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.

Dans le cas de la neuromyélite optique de Devic, les lésions démyélinisantes sont concentrées au niveau du nerf optique et de la moelle épinière. La présence d'anticorps anti-aquaporin-4, une protéine impliquée dans le maintien de l'équilibre hydrique du SNC permet de diagnostiquer cette forme de maladie (Weinshenker, Wingerchuk et al. 2006).

1.3 Etiologie

Les causes de la sclérose en plaques ne sont actuellement pas connues. Cependant l'étiologie de la sclérose en plaques semble indiquer l'influence de facteurs environnementaux et de la prédisposition génétique sur la survenue de la pathologie.

1.3.1 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 2004). L'hypothèse d'un facteur environnemental est étayée par des études sur les migrations de populations entre des zones de prévalence inégale: ceux qui migrent après l'âge de 15 ans ont le risque de la région d'origine, ceux qui migrent avant l'âge de 15 ans ont le risque de la région d'arrivée, comme si un évènement décisif se produisait à l'enfance (plusieurs années avant le début clinique de la maladie) (Alter, Leibowitz et al. 1966;

Hammond, English et al. 2000; Rosati 2001). Plusieurs facteurs de risque environnementaux ont été étudiés, incluant les infections, les vaccinations, le climat et les expositions à des toxines, les résultats sont parfois contradictoires. Aucun agent potentiel ne peut à lui seul être incriminé, le concours de plusieurs facteurs semble être une cause possible du déclenchement de la maladie (pour revue (Marrie 2004)).

1.3.2 Facteurs génétiques

De nombreuses études épidémiologiques sur la SEP ont montré que cette maladie n'est pas héréditaire, mais qu'il existe des facteurs génétiques influençant la survenue de la maladie. 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 de 25 à 30% (Willer, Dyment et al. 2003; Dyment, Ebers et al. 2004), les jumeaux dizygotes ont un risque de développer la maladie de 5% (Sadovnick, Armstrong et al. 1993). Par conséquent, la SEP semble se déclencher plus fréquemment chez des individus prédisposés génétiquement.

2. Immunologie de la sclérose en plaques

L'implication du système immunitaire dans la sclérose en plaques est certaine, mais elle présente de multiples facettes dont les mécanismes sous-jacents ne sont pas tous connus. Dans cette partie seront détaillées les modifications du système immunitaire dans la sclérose en plaques.

2.1 Génomique et transcriptome

La prédisposition génétique associée à la maladie a conduit à une recherche intense de gènes rendant les individus susceptibles à la survenue de la sclérose en plaques. Les seuls liens clairement établis entre SEP et génétique concernent des gènes codant des molécules du système immunitaire. De nombreuses études génétiques et épidémiologiques ont trouvé le locus du complexe majeur d'histocompatibilité (CMH) associé à la maladie SEP (Olerup and Hillert 1991; Sotgiu, Pugliatti et al. 2002) et plus particulièrement un consensus fait apparaître l'allèle HLA-DRB1*1501 comme gène de susceptibilité (Haines, Terwedow et al. 1998; Fogdell-Hahn, Ligers

et al. 2000; Oksenberg and Hauser 2005). Concernant les autres CMH de Classe II, les allèles DQA1*0102 et DQB1*0602 apparaissent également associés à la SEP (Fogdell, Hillert et al. 1995) ainsi que les allèles HLA-A3 et -B7 pour les CMH de Classe I (Jersild, Svejgaard et al. 1972; Naito, Namerow et al. 1972; Fogdell-Hahn, Ligers et al. 2000; Harbo, Lie et al. 2004). Pour le gène HLA-C, l'allèle HLAC*05 semble être associé à une protection (Yeo, De Jager et al. 2007). 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, Ebers et al. 2004)).

Concernant le transcriptome, d'autres gènes liés à des fonctions immunes ont été trouvés surexprimés dans les plaques au niveau du SNC des patients atteints de SEP comme les molécules de CMH de Classe II, le complément, des gènes exprimés dans les lymphocytes T et B, des cytokines (IL17) (Lock, Hermans et al. 2002). Dans les cellules mononucléées du sang (PBMC) de patients atteints de SEP, les taux d'ARN messagers des molécules de CMH de Classe II, des cytokines (IFNγ et TNFα), des molécules d'adhésion, des transcrits marqueurs des cellules T, B et NK (natural killer), des protéases impliquées dans l'apprêtage des antigènes voient leurs taux augmentés (Achiron, Gurevich et al. 2004).

2.2 Modèles animaux de la Sclérose en Plaques

Plusieurs modèles de démyélinisation chez l'animal sont provoqués par des induction immunologiques actives : soit par injection de virus, soit par injection d'auto-antigènes de la myéline ou de lymphocytes réactifs contre ces auto-antigènes.

2.2.1 Modèles animaux induits par injection de virus

2.2.1.1 Virus murin de l'hépatite

Le virus murin de l'hépatite est un coronavirus qui induit une démyélinisation inflammatoire du SNC de souris. Il y a une colocalisation de l'infection et de la démyélinisation qui conduit à un seul épisode symptomatique suivi dans la majorité des cas d'une rémission (Lavi, Gilden et al. 1984). Le virus se réplique en effet au niveau des oligodendrocytes (Powell

and Lampert 1975) provoquant l'enclenchement d'une réaction immunitaire et des dommages contre les oligodendrocytes (van Berlo, Warringa et al. 1989).

2.2.1.2 Virus de Theiler

Le virus de Theiler est un pathogène naturel de la souris. Il appartient à la famille des picornavirus. Il induit une infection persistante et une démyélinisation chronique et progressive chez des souches murines sensibles (SLJ), absentes des souches de souris résistantes (B6). Etant donné l'influence de l'environnement et des infections dans le déclenchement de la SEP, ce modèle montre la possibilité qu'une infection puisse induire une démyélinisation chez des sujets prédisposés. De plus, les réponses anti-virales sont détectées cinq à sept jours après l'infection et les réponses anti-myéline apparaissent au bout d'un mois après l'apparition des symptômes (Miller, Olson et al. 2001).

2.2.1.3 Semliki Forest Virus

Le semliki forest virus infecte les neurones et les oligodendrocytes chez la souris (Balluz, Glasgow et al. 1993). Au bout de 6 jours d'infection chez la souris adulte, le SNC semble débarrassé du virus au bout de 14 jours, un pic de démyélinisation et de symptômes a lieu (Amor, Scallan et al. 1996). Ce modèle viral est cependant uniquement monophasique, limitant les parallèles à la maladie humaine.

Ces modèles viraux chez l'animal prouvent qu'une infection virale ciblée au niveau du SNC peut induire des démyélinisations et enclencher des réponses auto-immunes. Chez l'Homme, de nombreuses études ont exploré l'influence des infections sur la survenue des symptômes et de la maladie. Plusieurs pathogènes ont été mis en cause, en particulier les infections touchant les enfants comme les virus de la rubéole, la varicelle, la rougeole et les oreillons. De nombreux autres virus ont également été étudiés dans la survenue de la maladie comme le virus d'Epstein-Barr (EBV) ou le virus de l'herpès de type 6 et dernièrement la bactérie *chlamydia pneumoniae*, la présence d'anticorps dirigés contre ces pathogènes ne prouvent cependant pas le lien de causalité à la survenue de la pathologie SEP (pour revue (Marrie 2004)).

2.2.2 Modèles d'encéphalomyélite auto-immune expérimentale

Le premier modèle d'encéphalomyélite auto-immune expérimentale (EAE) a été créé chez le singe par Thomas Rivers (Rivers 1933). Les modèles EAE sont induits chez les rongeurs et les singes par injection d'auto-antigènes de la myéline avec de l'adjuvant de Freund ou de la toxine pertussis (EAE dite active), ou par injection passive de cellules T, qui sont extraites d'animaux immunisés et qui sont spécifiques des auto-antigènes de la myéline (fig 2). La démyélinisation et les épisodes de paralysie sont associés à l'infiltrat de lymphocytes T CD4+ de type Th1 au niveau du SNC.

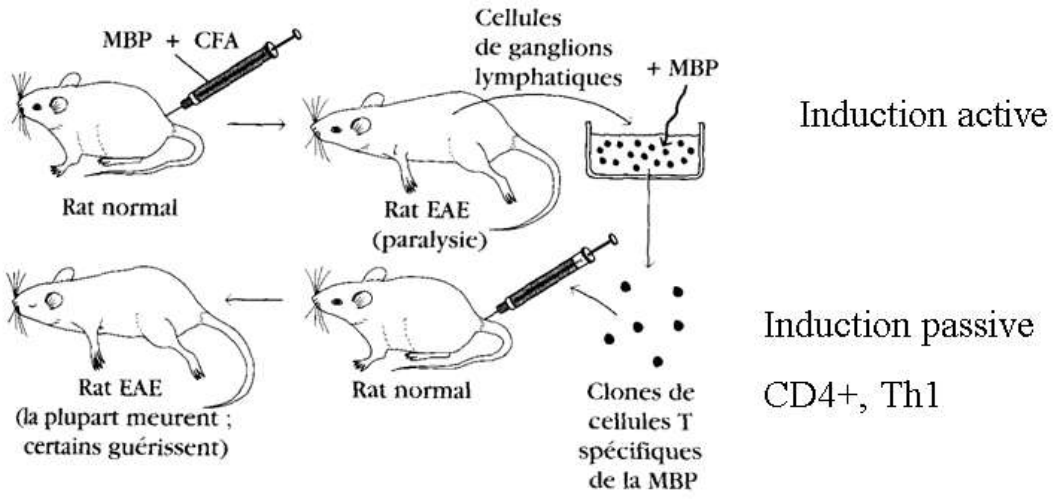


Figure 2: Modes d'induction de l'encéphalomyélite auto-immune expérimentale (EAE)
Figure extraite de (Goldsby RA 2001). L'induction active consiste à injecter l'auto-antigène avec de l'adjuvant de Freund (CFA) à l'animal, les symptômes débutent aux environs de 12 jours. Pour l'induction passive, des lymphocytes T spécifiques de l'auto-antigène sont extraits de différents compartiments lymphoïdes (rate ou ganglions) et injectés à un autre animal.

Aucun modèle EAE ne mime à lui seul toutes les caractéristiques de la maladie humaine, selon les souches animales et le mode d'induction de l'EAE, l'encéphalomyélite présente une démyélinisation, une atteinte axonale et une évolution (aiguë ou chronique) variables (Zamvil and Steinman 1990). Les rémissions, aussi bien des formes monophasiques que rémittentes, sont associées à la clairance des infiltrats au niveau du SNC. De façon intéressante, la susceptibilité aux différents sous-types d'EAE dépend de loci distincts (Butterfield, Blankenhorn et al. 1999). Les modèles EAE ont permis de décrypter certains mécanismes cellulaires lésionnels et

pathologiques de l'auto-immunité. Par exemple, dans un modèle chronique d'EAE, induit chez la souris SJL par immunisation avec le peptide PLP139-151 issu de la protéine protéolipidique, le phénomène d' « epitope spreading » a ainsi été révélé : au cours des évènements de poussée, les épitopes, dérivés d'auto-antigènes de la myéline, reconnus et induisant des réactivités changent (Vanderlugt and Miller 2002). Ainsi lors de la deuxième poussée de l'animal, l'épitope reconnu est PLP178-191 et lors de la troisième poussée l'épitope provient d'une autre protéine de la myéline :MBP84-104.

D'autre part, l'utilisation d'animaux transgéniques ou KO (knock-out) a permis de décrypter certains mécanismes moléculaires impliqués dans la formation des lésions ou de la réparation. Ainsi la surexpression de l'IL10 a pour conséquence la résistance à l'induction de l'EAE (Cua, Groux et al. 1999) et la surexpression du TNFα aggrave la démyélinisation (Akassoglou, Bauer et al. 1998). Des souris KO pour l'IFNγ sont plus sensibles au développement d'une EAE. Des modèles transgéniques dans lesquels une majorité des lymphocytes T expriment un récepteur à l'antigène (TCR) spécifique de la protéine basique de la myéline (MBP) ont montré la survenue d'une maladie spontanée, plus ou moins fréquente selon le sexe et la souche de l'animal (Goverman 1999). De plus, des souris transgéniques portant ce TCR dirigé contre la MBP développent spontanément une démyélinisation dans un environnement non stérile alors que ce n'est pas le cas dans un environnement stérile (Goverman, Woods et al. 1993), montrant l'influence de facteurs environnementaux sur la survenue de la maladie auto-immune. Un modèle transgénique portant un TCR dirigé cette fois contre la glycoprotéine de la myéline et des oligodendrocytes (MOG) présente quant à lui une démyélinisation au niveau du nerf optique. Croisées avec des souris portant des immunoglobulines dirigées contre la MOG, la descendance présente une encéphalomyélite spontanée sévère avec des zones du SNC atteintes comparables à celles de patients atteints de neuromyélite optique de Devic (Bettelli, Baeten et al. 2006). Ces modèles transgéniques montrent que les processus auto-immuns sont régulés de façon complexe.

Les modèles EAE ont par conséquent montré que l'injection de lymphocytes T auto-réactifs contre des antigènes de la myéline en périphérie peut induire des encéphalomyélites, étayant le concept d'auto-immunité évoqué dans la SEP. Les modèles EAE ont aussi permis l'émergence de plusieurs traitements utilisés chez les patients atteints de SEP comme l'acétate de glatiramère, la

mitoxantrone et l'anti-VLA-4 (Steinman and Zamvil 2006). Cependant, plusieurs molécules efficaces dans les modèles EAE ont eu des effets très délétères chez l'Homme comme l'injection de la cytokine IFNγ (Panitch, Hirsch et al. 1987) ou l'anti-TNFα (Group 1999). L'utilisation des modèles EAE doit être ainsi relativisée.

2.3 Physiopathologie humaine et immunologie

2.3.1 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, Racke et al. 2006). Les études de tissus humains sur des cas d'autopsies et de biopsies ont permis de décrire quatre types de lésions, impliquant des processus pathologiques différents (Lucchinetti, Bruck et al. 1996; Lucchinetti, Bruck et al. 1999; Lucchinetti, Bruck et al. 2000) (fig 3). Le type I est caractérisé par une démyélinisation et la présence de facteurs produits par les macrophages : le TNFα, des protéinases et des espèces oxygénées réactives intermédiaires. Dans le type II, sont présents des immunoglobulines et des molécules du complément. Le type III montre une perte précoce de la glycoprotéine associée à la myéline (MAG) et l'absence de remyélinisation, qui semble impliquer un dysfonctionnement des oligodendrocytes. L'apoptose des oligodendrocytes présentant une fragmentation de l'ADN est la spécificité du type IV.

Cependant les lésions d'un même patient peuvent présenter les caractéristiques d'un ou plusieurs types (Barnett and Prineas 2004). Au niveau de lésions dites actives, des transsections axonales ainsi que des dommages substantiels sont retrouvés (Trapp, Peterson et al. 1998) (Peterson, Bo et al. 2001). Certaines lésions présentent cependant une dégénérescence des oligodendrocytes en l'absence d'infiltrat de cellules inflammatoires (Barnett and Prineas 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 2007).

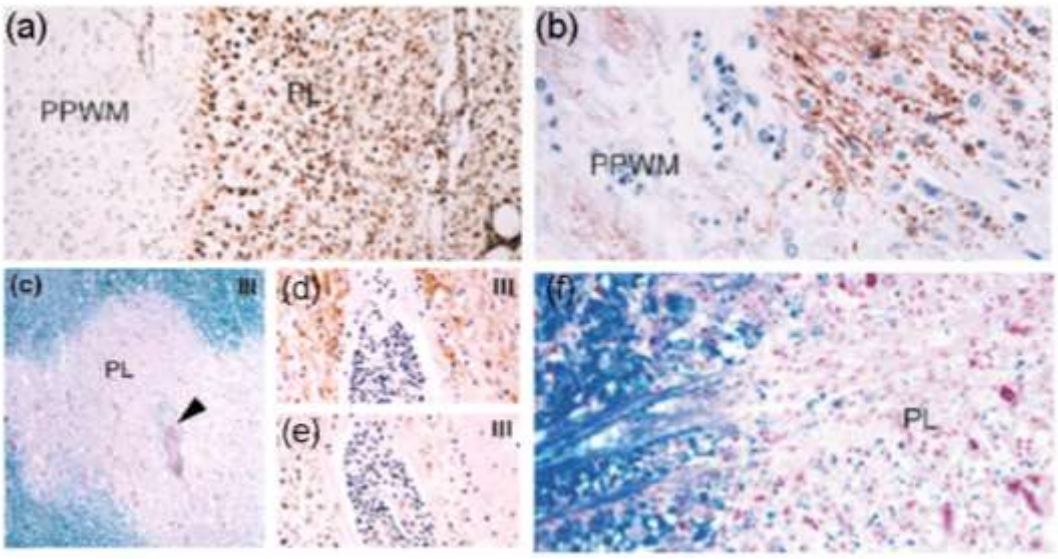


Figure 3: Histologies des quatre types de lésions chez des patients atteints de SEP
Photographies extraites de (Lassmann, Bruck et al. 2001). a) Plaque active (PL) de type I et II contenant des macrophages et des microglies, cellules marquées avec un anticorps anti-CD68. La substance blanche en périplaque (PPWM) est bien démarquée. Grossissement x200. b) Plaque active de type II présentant un dépôt de complément C9 (marquage marron). Grossissement x500. c) Plaque active de type III, la myéline est marquée au bleu de Luxol. Grossissement x30. La flèche indique un vaisseau sanguin grossit 300 fois sur (d) et (e). d) Marquage marron anti-MOG. e) Marquage anti-MAG. f) Plaque active de type IV. La plaque contient des macrophages contenant des débris de myéline (colorés par le bleu de Luxol). Grossissement x300.

2.3.2 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, De Groot et al. 2004), une perte axonale (Evangelou, Esiri et al. 2000; Kornek, Storch et al. 2000), un infiltrat de cellules T et les microglies présentes sont activées. 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, Arridge et al. 2002) et du cortex (Kidd, Barkhof et al. 1999) (Peterson, Bo et al. 2001). Ces atteintes neuronales peuvent être dues en partie aux transsections axonales visibles dans la substance blanche (dégénérescence wallérienne) qui cependant n'explique pas tout. Ces lésions sont moins inflammatoires avec peu de destruction de la matrice extracellulaire et uniquement des microglies comme cellules infiltrantes. La démyélinisation corticale touche 90% des patients, pourtant le tissu apparaît généralement normal à l'IRM standard (Kutzelnigg, Lucchinetti et al. 2005; Albert, Antel et al. 2007). L'utilisation d'appareils plus performants (en particulier à hauts champs) permet de visualiser ces lésions.

2.3.3 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, Braun 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 microglies, 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 2001; Streit 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'adhésion. Celles-ci leur permettent d'entrer en contact avec les cellules endothéliales (fig 4). 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 (fig 4).

L'augmentation de la prise de Gadolinium révélée par l'IRM reflète chez les patients atteints de SEP la rupture de la BHE (Katz, Taubenberger et al. 1993). De plus, chez les malades, la molécule d'adhésion intercellulaire (ICAM1) et la molécule d'adhésion cellulaire vasculaire (VCAM1) sont présentes à des taux élevés sur les cellules endothéliales des lésions (Sobel, Mitchell et al. 1990; Washington, Burton et al. 1994; Cannella and Raine 1995; Ransohoff 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, Peterson et al. 1996). Les cellules circulant dans le sang des patients présentent également des taux élevés de LFA1 et VLA-4 (Correale and Bassani Molinas Mde 2003). Les lymphocytes T des patients atteints de SEP ont des capacités d'adhésion plus grandes par rapport aux individus sains (Lou, Chofflon et al. 1997) et les lymphocytes T CD8+ des patients atteints de SEP en particulier expriment la molécule P-Sélectine Glycoprotéine Ligand-1 (PGSL1) (Battistini, Piccio et al. 2003).

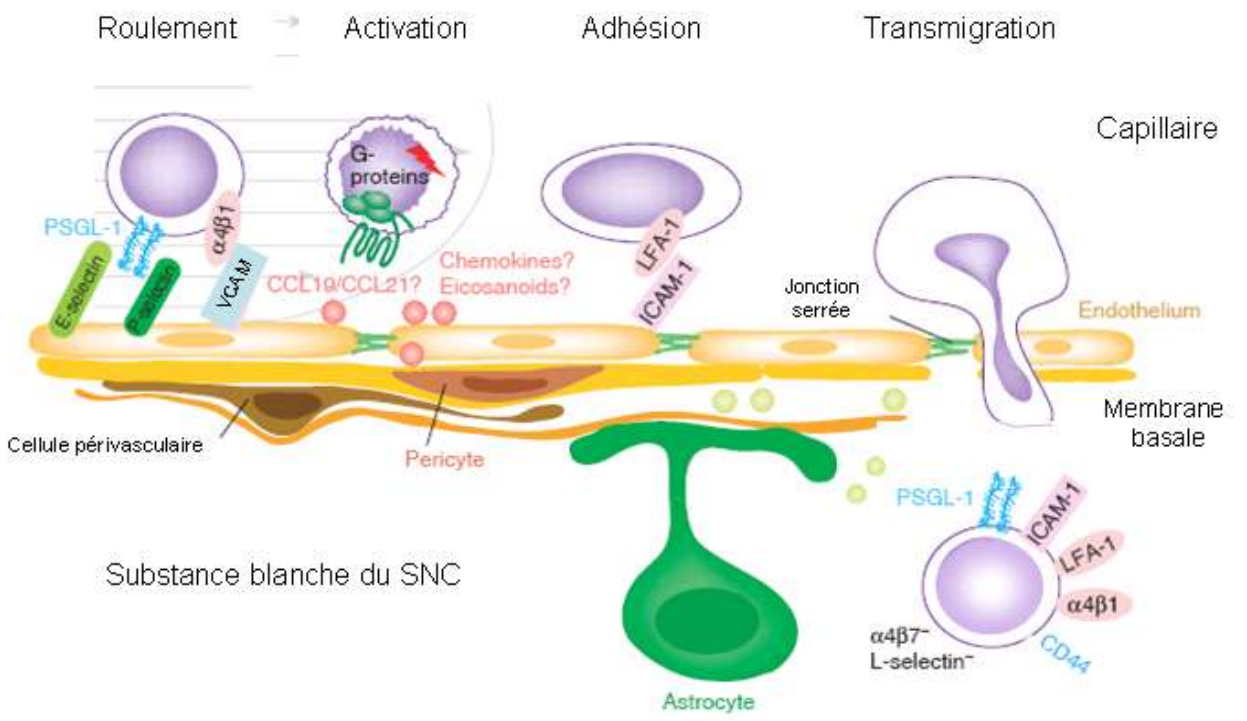


Figure 4: Passage de la BHE

Figure modifiée extraite de (Engelhardt and Ransohoff 2005). Lors de l'inflammation du SNC, les cellules immunitaires effectuent un roulement. Les molécules E- et P-sélectine, leur ligand PSGL-1 et l'α4β1 (α4-intégrine ou VLA-4) sont impliqués dans ce roulement des lymphocytes au niveau des vaisseaux superficiels. Les cellules sont activées via des protéines G et leur adhésion dépend de la liaison de la molécule LFA-1 à ICAM-1. Les cellules migrent à travers l'endothélium au niveau des jonctions serrées : c'est la diapédèse.

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, Ferguson et al. 1997; Cossins, Clements et al. 1997; Lindberg, De Groot et al. 2001; Vos, van Haastert 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, Palace et al. 1999; Lichtinghagen, Seifert et al. 1999; Waubant, Goodkin et al. 1999). Les leucocytes des patients présentent une augmentation des taux d'ARN messagers de MMP-1, -3, -7, -14 et -19 (Galboiz, Shapiro et al. 2001; Kouwenhoven, Ozenci et al. 2001; van Horssen, Vos 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, Shapiro et al. 2005).

2.4 Les effecteurs cellulaires chez l'Homme

2.4.1 Les microglies

Les microglies possèdent des propriétés de cellules présentatrices d'antigène et de phagocytes. Activées, elles sécrètent des cytokines pro-inflammatoires telles que l'IL1, l'IL6, l'IL12, l'IL23 et le TNFα et du monoxyde d'azote (NO) (John, Lee et al. 2003). Le TNFα et le NO peuvent causer des dommages aux oligodendrocytes. Les microglies se trouvent fréquemment dans un état activé au niveau des lésions actives de patients atteints de SEP (Lassmann, Bruck et al. 2001). Ces cellules chez les patients SEP sécrètent de grandes quantités d'IL23, une cytokine pro-inflammatoire, au niveau des lésions du SNC (Li, Chu et al. 2007).

2.4.2 Les monocytes et macrophages

Les monocytes sont des cellules mononucléées circulantes qui migrent ensuite dans les tissus, devenant des macrophages. 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 un niveau élevé les molécules des voies de costimulation CD80 et CD86 (Kouwenhoven, Teleshova et al. 2001). Ils expriment également des taux importants de LFA1 et VLA-4 (Correale and Bassani Molinas Mde 2003), facilitant leur passage dans le SNC. On trouve effectivement des monocytes dans les infiltrats (Prineas and Wright 1978). Les macrophages, contenant des débris de myéline, sont retrouvés au centre des lésions (Lucchinetti, Bruck et al. 1996) et dans les ganglions cervicaux (Fabriek, Zwemmer 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 (Bajramovic, Plomp et al. 2000).

2.4.3 Les cellules dendritiques

Les cellules dendritiques représentent une population rare de cellules présentatrices d'antigènes (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. Dans les modèles EAE, la présence de cellules dendritiques au niveau du parenchyme semble cruciale pour l'invasion du SNC par les cellules du système immunitaire activées en périphérie (Greter, Heppner et al. 2005). Ces cellules circulantes sont naturellement présentes dans les méninges, le plexus choroïde et le LCR. Suite à une

inflammation du SNC, les cellules dendritiques activées sont capables de migrer du parenchyme aux ganglions lymphoïdes cervicaux et pourraient activer le système immunitaire périphérique dans la SEP. Chez l'homme, on peut distinguer deux types de cellules dendritiques: les cellules dendritiques myéloïdes (mDC) et les cellules dendritiques plasmacytoïdes (pDC). Ces dernières présentent un phénotype $CD4^+$, $CD11c^-$, $HLA\text{-}DR^+$ et les mDC sont quant à elles $CD4^+$, $CD11c^+$, et $HLA\text{-}DR^+$ (Bendriss-Vermare, Barthelemy et al. 2001). Chez les patients atteints de SEP rémittente, il y a un taux plus élevé de pDC dans le LCR que chez les individus sains (Pashenkov, Huang et al. 2001). Une diminution du nombre de cellules mDC circulantes chez les patients de forme secondaire progressive et de forme primaire progressive a été également observée (Lopez, Comabella et al. 2006). Les pDC extraites de patients SEP présentent des capacités de maturation faibles et des fonctions régulatrices altérées (Stasiolek, Bayas et al. 2006). Les mDC chez des patients atteints de forme secondaire progressive sont activées et présentent des anomalies de polarisation des cytokines vers Th1 (Karni, Abraham et al. 2006). Les cellules dendritiques préparées à partir de monocytes sécrètent plus de cytokines proinflammatoires comme du TNFα, de l'IFNγ, de l'IL6 (Huang, Xiao et al. 1999), de l'IL23 (Vaknin-Dembinsky, Balashov et al. 2006) et moins d'IL10 (Hussien, Sanna et al. 2001). Enfin, au niveau du SNC, des cellules dendritiques sont présentes dans les zones périvasculaires chez les patients atteints de SEP, aussi bien dans les lésions actives que chroniques et elles contiennent dans leur cytoplasme des composants de la myéline (Serafini, Rosicarelli et al. 2006).

2.4.4 Les lymphocytes B

Les lymphocytes B interviennent dans la réponse immunitaire de type humorale. Une fois activés, ils se transforment en plasmocytes et sécrètent des anticorps. Le système humoral est fortement impliqué dans la pathologie SEP. En effet, l'augmentation de la production des immunoglobulines au niveau du LCR est observée chez 90% des patients atteints de SEP et est caractérisée par un profil oligoclonal lorsque les protéines sont séparées par électrophorèse (Correale and de los Milagros Bassani Molinas 2002). Des anticorps auto-réactifs ont été décrits, réagissant contre diverses protéines de la myéline comme la protéine basique de la myéline (MBP) (Gerritse, Deen et al. 1994), la glycoprotéine des oligodendrocytes et de la myéline (MOG) (Genain, Cannella et al. 1999), la protéine protéolipidique (PLP) (Laman, Visser et al. 2001), la glycoprotéine associée à la myéline (MAG) (Baig, Jiang et al. 1991), la protéine spécifique des oligodendrocytes (OSP) (Bronstein, Lallone et al. 1999), la transaldolase TAL (Banki, Colombo et al. 1994), l'αβ-crystalline (Agius, Kirvan et al. 1999) mais aussi contre des

protéines neuronales (Ehling, Lutterotti et al. 2004) et des protéines ubiquitaires comme celles impliquées dans le protéasome (Mayo, Arribas et al. 2002), ou des protéines chaperones (protéines de choc thermique) (Cid, Alvarez-Cermeno et al. 2004). Les anticorps les plus décrits sont ceux anti-MOG (pour revue (Reindl, Khalil et al. 2006)). Il a été efffectivement suggéré que les anticorps anti-MOG étaient un critère de diagnostic (Berger, Rubner et al. 2003) mais récemment une étude plus importante n'a pu retrouver ces résultats (Kuhle, Pohl et al. 2007). Des anticorps humains dirigés contre la MOG native et présents dans le LCR de patients atteints de SEP ont été injectés à des rats ayant déjà développé une EAE, la démyélinisation et la perte axonale ont ainsi été aggravées (Zhou, Srivastava et al. 2006).

Les lymphocytes B, et en particulier des plasmocytes ont été détectés au niveau des lésions et des infiltrats lymphocytaires chez des patients atteints de SEP (Prineas and Wright 1978). De plus, des expansions clonales de lymphocytes B présentant des mutations somatiques au niveau des régions variables des immunoglobulines ont également été observées au niveau des lésions du SNC (Owens, Kraus et al. 1998; Baranzini, Jeong et al. 1999) et au niveau du LCR (Qin, Duquette et al. 1998; Colombo, Dono et al. 2000). L'augmentation du nombre de lymphocytes B dans le LCR est associée à une progression plus rapide de la maladie (Cepok, Jacobsen et al. 2001). Dans le LCR, on trouve tous les types de différenciation de cellules B, du lymphocyte B naïf au plasmocyte (Corcione, Casazza et al. 2004). En outre, il y a plus de lymphocytes B mémoires dans le LCR que dans le sang des patients atteints de SEP (Cepok, Rosche et al. 2005).

2.4.5 Les mastocytes

Les mastocytes, cellules présentes au niveau des muqueuses, sont surtout impliqués dans des phénomènes d'allergie mais aussi dans l'inflammation. Ils expriment à leur surface le récepteur de haute affinité pour les immunoglobulines de type IgE (FcεRI). Au niveau du SNC des patients atteints de SEP, les mastocytes sont trouvés plus fréquemment dans des lésions chroniques et moins fréquemment au niveau des lésions actives (Ibrahim, Reder et al. 1996). Au niveau du LCR des patients, ont été trouvés des taux élevés de facteurs sécrétés par les mastocytes comme l'histamine (Tuomisto, Kilpelainen et al. 1983), la trypase et la protéase spécifique des mastocytes (Rozniecki, Hauser et al. 1995). Les mastocytes sont capables de dégranuler en réponse à la MBP et d'induire une démyélinisation *in vitro* (Johnson, Seeldrayers et al. 1988; Theoharides, Dimitriadou et al. 1993). De plus, les mastocytes peuvent réguler la rupture de la BHE (montré expérimentalement dans (Zhuang, Silverman et al. 1996)).

2.4.6 Les cellules NK

Les cellules tueuses naturelles (NK) sont une population hétérogène qui contribue à la défense de l'organisme contre les infections et les cancers. 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 1998). Des défauts dans la fonction et au niveau du nombre de cellules NK chez les patients atteints de SEP ont été décrits (Merrill, Jondal et al. 1982; Vranes, Poljakovic et al. 1989; Munschauer, Hartrich et al. 1995). Les phases de rémission sont associées à une production accrue d'IL5 par ces cellules (Takahashi, Miyake et al. 2001). Les cellules NK sont également capables *in vitro* de lyser des oligodendrocytes (Morse, Seguin et al. 2001). D'autre part, des cellules régulatrices NK $CD56^{bright}$ ont été décrites chez des patients atteints de SEP et traités avec un anti-récepteur de l'IL2 (Daclizumab). Ces cellules agiraient en inhibant la survie des lymphocytes T de manière contact dépendant (Bielekova, Catalfamo et al. 2006).

2.4.7 Les cellules NKT

Les cellules NKT constituent une population présentant des caractéristiques des cellules NK et des lymphocytes T (van der Vliet, Pinedo et al. 2002) (Cava, Kaer et al. 2006). Elles expriment un TCR invariant constitué des segments Vα24 et Vβ11 et des marqueurs NK tels que CD161 ou NKR-P1. Elles reconnaissent les molécules CMH de classe I non classiques : CD1d qui présentent des glycolipides (Godfrey, Hammond et al. 2000). Chez les patients atteints de SEP, une forte diminution du taux de cellules NKT a été observée (Illes, Kondo et al. 2000). 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 (Araki, Kondo et al. 2003). Chez un patient, une diminution de la diversité de Vα24 a été observée (Demoulins, Gachelin 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, Shimamura et al. 2004).

2.5 Implication des lymphocytes T

2.5.1 Le récepteur des cellules T (TCR)

Les lymphocytes T expriment à leur surface un récepteur spécifique, le TCR, qui reconnaît un antigène. Deux types de TCR ont été identifiés : le TCRαβ et le TCRγδ. Il s'agit d'hétérodimères formés respectivement de deux chaînes : soit α et β, soit γ et δ, associées au complexe CD3 impliqué dans l'expression du TCR à la surface des lymphocytes et indispensable à la transduction du signal. Le récepteur TCRαβ est impliqué dans la reconnaissance du complexe CMH/peptide. La molécule de CMH est exprimée à la surface des cellules présentatrices d'antigènes (CPA). La chaîne α et la chaîne β du TCR sont composées de plusieurs domaines : V (Variable), D (Diversité), J (Jonction) et C (Constant) (fig 5).

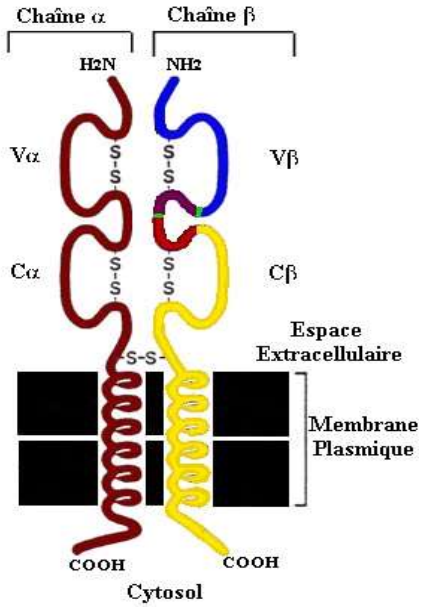


Figure 5: Schéma du récepteur des cellules T (TCR).
Deux chaînes α et β composent le TCR. Elles possèdent un domaine intracellulaire, un domaine transmembranaire et un domaine extracellulaire. Ce dernier possède une partie constante (C) et une partie variable (V).

Au niveau des domaines variables des chaînes du TCR, il existe des boucles hypervariables CDR1, CDR2, CDR3 (Région déterminant la complémentarité) impliquées dans l'interaction avec le CMH et le peptide. La chaîne β est plus fortement impliquée dans la reconnaissance du peptide grâce à la grande variabilité de sa région CDR3 (Davis, Boniface et al. 1998). Dans le thymus, les thymocytes (progéniteurs des lymphocytes T circulants), subissent un réarrangement du gène codant pour la chaîne β qui aboutit à la juxtaposition aléatoire des quatre segments nucléotidiques : V, D, J et C, parmi un grand nombre de combinaisons possibles (Spits

2002). Les jonctions VDJ constituent la région CDR3. La synthèse du TCR commence par le réarrangement au niveau somatique des segments Dβ et Jβ (fig 6), suivi de l'assemblage de ce segment réarrangé DβJβ avec un segment Vβ et enfin l'ajout d'un segment constant C. Cette chaîne β est ensuite exprimée à la surface des thymocytes et s'associe avec la chaîne α, issue elle aussi du réarrangement des gènes Vα et Jα. Lors du réarrangement, la jonction des segments VDJ se produit avec une imprécision pouvant entraîner un décalage du cadre de lecture et est accompagnée de l'addition d'un nombre aléatoire de nucléotides au niveau des jonctions par une enzyme: la TdT (Bogue and Roth 1996). Ces deux mécanismes sont source de diversités qualifiées respectivement de diversité combinatoire et jonctionnelle. A ces deux mécanismes s'ajoute une diversité liée à l'association d'une chaîne β à une chaîne α. On estime ainsi que 2,5 10^6 TCRαβ différents peuvent être générés chez l'homme (Arstila, Casrouge et al. 1999). De plus, du fait de la diversité jonctionnelle, cette région CDR3 est de différentes longueurs pouvant aller de 10 à 33 nucléotides.

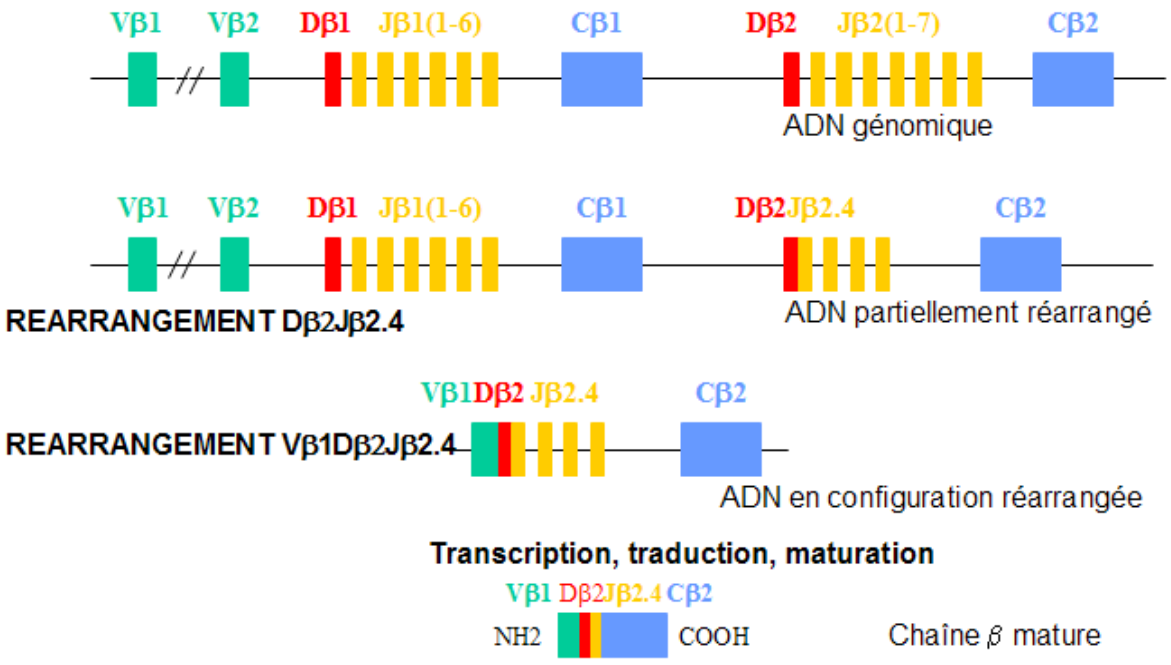


Figure 6: Exemple de réarrangements de la chaîne β du TCR
Le locus codant la chaîne β du TCR dans les lymphocytes T subit un premier réarrangement aboutissant au segment DβJβ, un second réarrangement résulte en la production du segment VDJ. Après transcription, traduction et maturation, la chaîne β est exprimée à la surface des lymphocytes T.

2.5.2 Le répertoire T dans la sclérose en plaques

Le terme de « répertoire » désigne l'ensemble des TCR exprimés par les lymphocytes T. Les techniques d'analyse du répertoire T permettent de visualiser la diversité des TCR et donc indirectement la diversité d'une population de cellules T et d'en suivre l'évolution. Etudier la répartition des différentes longueurs de CDR3 de la chaîne β du TCR permet d'appréhender la diversité du répertoire T. Ce type d'études chez les patients atteints de SEP a montré des expansions clonales de lymphocytes T dans le LCR (Hafler, Duby et al. 1988). Des biais d'usage du répertoire Vβ ont été également détectés dans le sang des patients atteints de SEP (Utz, Biddison et al. 1993; Gran, Gestri et al. 1998; Laplaud, Ruiz et al. 2004). Les patients SEP présentent un changement au niveau du compartiment T naïf détectable en début de maladie (Haegert, Cowan et al. 1999). Ces expansions clonales chez les patients corrèlent avec les réponses contre la MBP (Muraro, Bonanni et al. 2002; Matsumoto, Yoon et al. 2003). De plus, une étude du répertoire T dans le LCR d'un patient a montré que les altérations du répertoire étaient augmentées pendant une poussée (Muraro, Cassiani-Ingoni et al. 2006). Certaines études ont décrits des expansions clonales chez les patients au niveau de familles Vβ particulières comme Vβ 5.2 chez les patients porteurs de l'allèle HLA-DR2 (Matsumoto, Yoon et al. 2003) ou Vβ13 (Demoulins, Mouthon et al. 2003) et Vβ5.3 (Musette, Bequet et al. 1996). Une autre étude chez des patients HLA-DR2, s'est également attachée à analyser les familles Vβ 5 et Vβ 17 au niveau du LCR et du sang et n'a pas détecté ces expansions (Lozeron, Chabas et al. 1998). De plus, une étude clinique utilisant les anticorps anti-Vβ5.2 et Vβ5.3 a montré peu d'effets sur l'amélioration de la maladie (Killestein, Olsson et al. 2002). Il semble difficile d'incriminer une famille Vβ particulière dans la pathologie et notre équipe a d'ailleurs montré que l'ensemble du répertoire T des patients SEP présente des altérations différentes d'un patient à l'autre (voir annexe I (Laplaud, Ruiz et al. 2004)).

En ce qui concerne le répertoire Vα, il semble que le phénomène d'altérations dans la diversité des chaînes α se produit également. Des réarrangements limités de la chaîne α ont en effet été détectés au niveau du SNC de patients atteints de SEP (Oksenberg, Stuart et al. 1990).

Certains épitopes de la myéline ont été décrits dans le cadre de la pathologie SEP et les cellules T réactives à ces épitopes ont été cultivées *in vitro* afin d'analyser les TCR exprimés par ces cellules. Ainsi, des expansions clonales de lymphocytes T réactifs contre le peptide MBP84-102 ont été décrites (Giegerich, Pette et al. 1992; Wucherpfennig, Zhang et al. 1994; Vandevyver, Mertens et al. 1995). Pour l'épitope MBP83-99, les lignées cellulaires T spécifiques présentent

une restriction du répertoire Vα (Zang, Kozovska et al. 1998) et expriment principalement Vβ13.1 avec un motif CDR3 particulier (Hong, Zang et al. 1999). Les cellules T spécifiques de la protéine MBP, voient quant à elles leur TCR caractérisé par la présence de Vα8 et Vβ5 (Offner and Vandenbark 1999). Dans les cerveaux de patients SEP, ont été détectés des lymphocytes T spécifiques de la MBP grâce à des anticorps anti-TCR : ces anticorps reconnaissent une partie du TCR spécifique de la MBP (Oksenberg, Panzara et al. 1993). Outre la présence de ces lymphocytes auto-réactifs au niveau des lésions, une étude longitudinale des réponses des cellules T à différents antigènes de la myéline a clairement montré un phénomène d'« epitope spreading » et l'étude du répertoire T sur les lignées a confirmé un changement de clones impliqués dans ces réponses auto-réactives (Goebels, Hofstetter et al. 2000).

2.5.3 Les lymphocytes T γδ

Les TCRγδ 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 (Wucherpfennig, Newcombe et al. 1992) et sont plutôt du type δ2 alors qu'au niveau du sang des patients on retrouve le type δ1 (Battistini, Borsellino et al. 1997). Les lymphocytes T γδ sont capables de lyser des oligodendrocytes *in vitro* (Zeine, Pon et al. 1998). Des expansions clonales des lymphocytes T γδ ont été trouvées chez les patients SEP en tout début de maladie (Shimonkevitz, Colburn et al. 1993) et au niveau des lésions du SNC (Hvas, Oksenberg et al. 1993).

2.5.4 Les lymphocytes T αβ auto-réactifs

2.5.4.1 Sélection thymique

Au cours de leur différenciation intrathymique, les lymphocytes T subissent une sélection positive suivie d'une sélection négative. Ces étapes vont permettre l'expansion sélective des cellules T capables de reconnaître le CMH du soi exprimé par les cellules endothéliales corticales du thymus et la mort des cellules présentant un TCR de très faible affinité (sélection positive) (fig 7). Enfin, les thymocytes interagissant avec une trop forte affinité avec les complexes CMH/peptide du soi et donc potentiellement auto-réactifs subissent soit une délétion clonale (sélection négative), soit une nouvelle édition de leur TCR utilisant le deuxième allèle, soit une anergie (c'est le cas des cellules T régulatrices, détaillé par la suite). Si l'interaction des

thymocytes via leur TCR avec les complexes CMH/peptide du soi présentés dans le thymus se situe dans une gamme d'avidité "acceptable", il y a sélection et différentiation de ces thymocytes en cellules matures exportables en périphérie (Hogquist, Baldwin et al. 2005). On considère que plus de 95% des thymocytes sont éliminés à ce stade (fig 7).

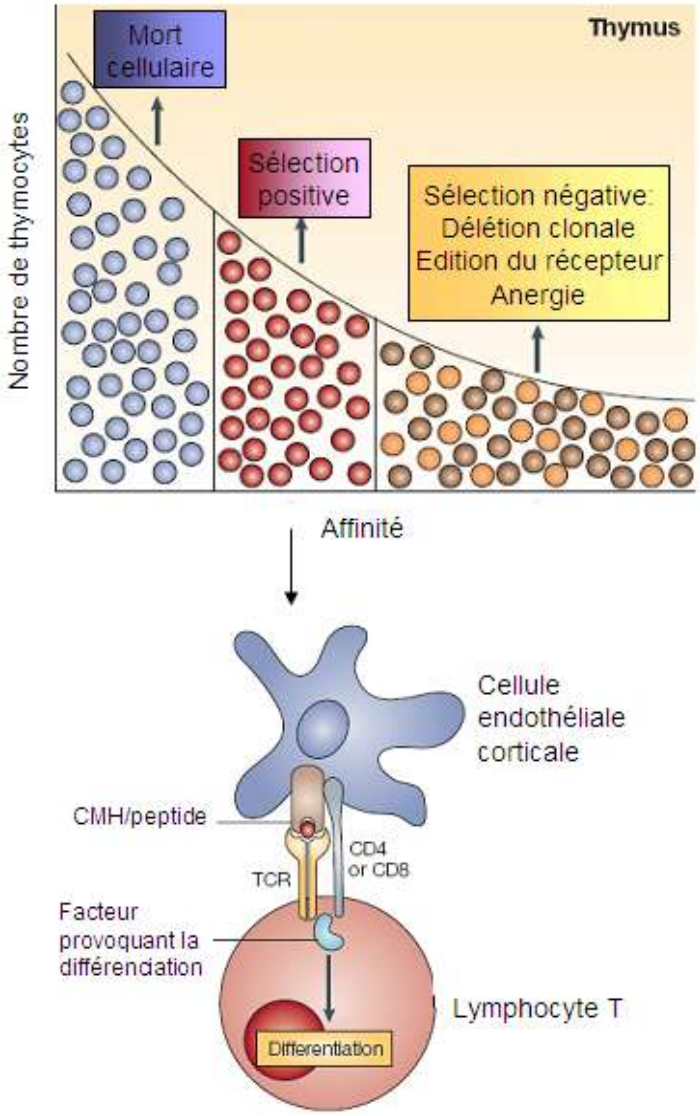


Figure 7: Sélection thymique des lymphocytes
Les thymocytes de très faible affinité pour le CMH/peptide du soi présenté par les cellules endothéliales corticales du thymus meurent. Ceux de forte affinité subissent la sélection négative. Enfin, seuls les lymphocytes possédant un TCR de faible affinité pour le CMH subissent la sélection positive.

2.5.4.2 Les lymphocytes T auto-réactifs dans la SEP

Une fréquence élevée de lymphocytes T réactifs contre la myéline est notée dans le LCR des patients atteints de SEP (Lovett-Racke, Trotter et al. 1998; Scholz, Patton 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 nombreux laboratoires en établissant des lignées cellulaires spécifiques, en particulier contre :

- la protéine basique de la myéline (MBP) (Burns, Rosenzweig et al. 1983; Martin, Jaraquemada et al. 1990; Ota, Matsui et al. 1990; Pette, Fujita et al. 1990; Jingwu, Medaer et al. 1992),
- la glycoprotéine des oligodendrocytes et de la myéline MOG (Kerlero de Rosbo, Milo et al. 1993; Lindert, Haase et al. 1999),
- la protéine protéolipidique (PLP) (Sun, Olsson et al. 1991; Markovic-Plese, Fukaura et al. 1995)
- la protéine basique associée aux oligodendrocytes et à la myéline (MOBP) (Arbour, Holz et al. 2003)
- l'αβ-cristalline (Chou, Burrows et al. 2004)

Cependant, les lymphocytes T auto-réactifs contre la myéline font également partie du répertoire immun d'individus sains adultes (Tsuchida, Parker et al. 1994; Burns, Bartholomew et al. 2002), malgré la sélection thymique. La présence de ce type de lymphocytes n'est pas à l'origine du déclenchement pathologique de la SEP 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, Nicholson et al. 2005) (Goebels, Hofstetter et al. 2000) (Muraro, Wandinger et al. 2003).

2.5.5 Les lymphocytes T CD4+

2.5.5.1 Caractéristiques des lymphocytes T CD4+

Les lymphocytes T CD4+ jouent un rôle de cellules auxiliaires et reconnaissent des antigènes présentés par les CPA exprimant des CMH de Classe II. Trois types d'effecteurs CD4+ se distinguent : Th1, Th2 et Th17 (Hunter and Reiner 2000; Steinman 2007). Les cellules de type Th1 sécrètent des cytokines plutôt pro-inflammatoires comme l'IL2, IL6, IFNγ et TNFα et médient une réaction immunitaire cellulaire en activant les lymphocytes cytotoxiques et les macrophages (fig 8). Les cellules de type Th2 sécrètent quant à elles les cytokines IL4, IL5, IL10 et TGFβ et induisent une réponse immunitaire de type humoral en activant les lymphocytes B.

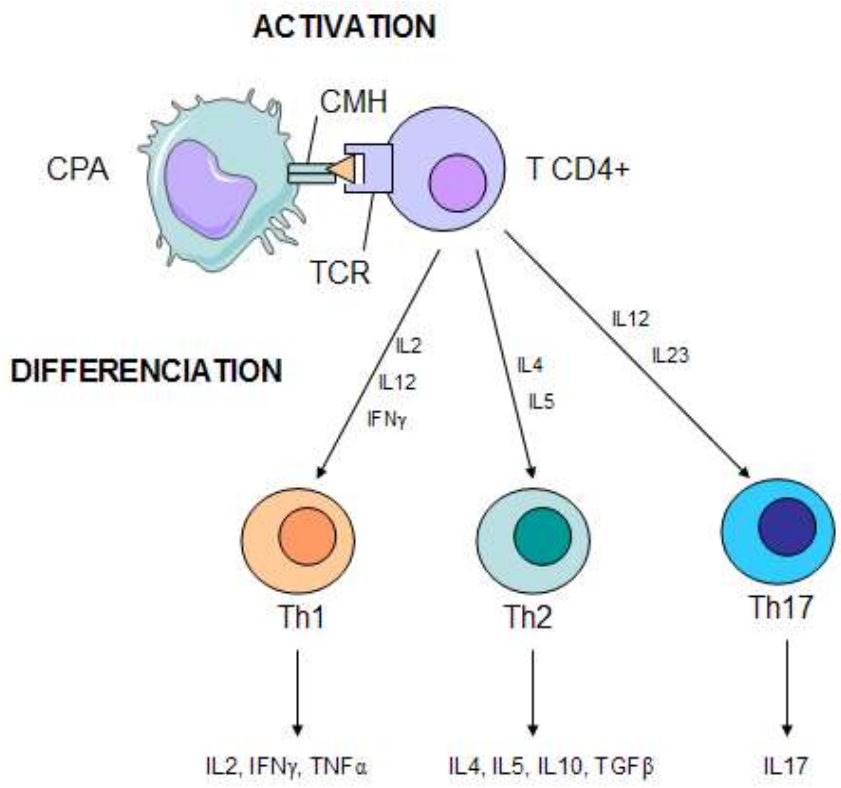


Figure 8: Les trois types d'effecteurs CD4+
Après activation via leur TCR, les lymphocytes T CD4+ se différencient selon l'environnement cytokinique. Les cellules Th1 sécrètent des cytokines activant les lymphocytes cytotoxiques et macrophages. Les cellules Th2 activent plutôt les lymphocytes B. Les cellules de type Th17 sont induites par la production d'IL12 et d'IL23 dans leur environnement.

2.5.5.2 Les lymphocytes T CD4+ de type Th1

Le gène de susceptibilité reconnu de la maladie humaine codant une molécule de CMH de Classe II et les modèles animaux d'EAE étant induits uniquement par l'injection ce type de lymphocytes T, un postulat général place les lymphocytes T CD4+ 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, Paroli et al. 1991) que dans le cerveau (Hofman, Hinton et al. 1989) que dans le sang des patients atteints de SEP et une baisse des cytokines anti-inflammatoires de type Th2 (IL4, IL5, IL10). De plus, la production de TNFα, d'IFNγ et d'IL2 par les cellules circulantes corrèle avec les lésions actives à l'IRM (Chofflon, Roth et al. 1997; Calabresi, Fields et al. 1998; Killestein, Kalkers et al. 2001; Killestein, Rep et al. 2001). 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, Sorensen et al. 2006). Des infiltrats de cellules de type Th1 ont été détectés au niveau de cerveau de patients atteinte de SEP (Kivisakk, Mahad et al. 2003). Les lymphocytes T CD4+ 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, Becher et al. 2000). Enfin, un essai thérapeutique utilisant un IFNγ recombinant a aggravé la maladie chez les patients testés (Panitch, Hirsch et al. 1987).

2.5.5.3 Les lymphocytes T Th17

Récemment, un nouveau type de lymphocytes T a été décrit : les lymphocytes de type Th17 ayant un rôle particulièrement important dans les modèles EAE (pour revue (Steinman 2007)). Les souris KO pour l'IL23 ou déficiente pour l'IL17 sont en effet résistantes à l'induction d'une EAE (Cua, Sherlock et al. 2003; Komiyama, Nakae et al. 2006). Chez les patients atteints de SEP, une augmentation des taux d'IL17 dans les cellules mononucléées du sang et du LCR a été notée (Matusevicius, Kivisakk et al. 1999). La voie IL23/IL17 semble une piste prometteuse pour établir de nouvelles thérapeutiques dans la SEP.

2.5.5.4 Les lymphocytes T CD4+ régulateurs

Une sous-population particulière de lymphocytes T CD4+ présente des propriétés régulatrices. Ces cellules sont générées naturellement dans le thymus et sont sélectionnées positivement pour leur reconnaissance avec le CMH du soi présent sur les cellules épithéliales corticales mais leurs TCR présentent une affinité moyenne envers le complexe CMH/antigène du soi (Cabarrocas, Cassan et al. 2006). Elles expriment fortement le récepteur à l'IL2 (CD25) (Baecher-Allan, Brown et al. 2001) et surtout le facteur de transcription FOXP3 (Khattri, Cox et al. 2003). Les cellules régulatrices T CD4+CD25+ semblent agir sur tous les types cellulaires immunitaires : elles inhibent en particulier la prolifération et la fonction des lymphocytes T CD4+CD25-, des T CD8+ (Camara, Sebille et al. 2003; Suvas, Kumaraguru et al. 2003). En ce qui concerne le nombre de cellules régulatrices, les taux circulants de ces cellules chez les patients SEP sont normaux (Putheti, Pettersson et al. 2004) et elles ne sont pas plus sensibles à l'apoptose déclenchée par CD95L (Fas ligand) que les cellules régulatrices des individus sains (Fritzsching, Korporal et al. 2006). A l'inverse du compartiment sanguin, la fréquence des cellules T régulatrices au niveau du LCR semble plus élevée chez les patients atteints de SEP (Feger, Luther et al. 2007). De plus, l'augmentation du taux de leptine dans le LCR des patients

semble corréler inversement avec le taux de cellules T régulatrices (Matarese, Carrieri et al. 2005). Appuyant ce fait, les lignées cellulaires T spécifiques de la MBP sécrètent de la leptine, et les souris KO pour la leptine ont un taux plus élevé de cellules T régulatrices (Matarese, Carrieri et al. 2005). Concernant la fonction cellulaire, les lymphocytes T régulateurs (CD4+CD25+) des patients atteints de SEP de forme rémittente présentent des capacités diminuées de régulation de la prolifération des cellules CD4+CD25- stimulées de façon polyclonale par l'anti-CD3 (Viglietta, Baecher-Allan et al. 2004) mais aussi de la prolifération induite par la MBP ou la MOG chez les patients SEP (Kumar, Putzki et al. 2006) (Haas, Hug et al. 2005). Selon le stade de la maladie, la fonction des cellules régulatrices des patients atteints de SEP varie. En effet, les cellules régulatrices des patients atteints de SEP de forme secondairement progressive de SEP présentent des fonctions suppressives de prolifération comparables à celles des témoins, contrairement aux cellules des patients atteints de SEP de forme rémittente (Venken, Hellings et al. 2006). Cette altération de fonction des cellules régulatrices pourrait être expliquée par la diminution du taux d'expression de FOXP3 dans les cellules T CD4+CD25+ des patients atteints de SEP (Huan, Culbertson et al. 2005).

Il existe également d'autres cellules régulatrices T CD4+ non pas naturelles mais induites : les lymphocytes T CD4+ régulateurs de type 1 (Tr1). Les cellules Tr1, après activation via le TCR, exercent leur capacité suppressive par l'intermédiaire des cytokines qu'elles produisent telles que l'IL10 et le TGFβ (Groux, O'Garra et al. 1997). Le groupe de David Hafler a montré un défaut d'induction des cellules Tr1 par stimulation avec un anticorps anti-CD3 et un anti-CD46 chez les patients SEP comparés aux témoins (Astier, Meiffren et al. 2006).

2.5.6 Implication des lymphocytes T CD8+

2.5.6.1 Caractéristiques des lymphocytes T CD8+ cytotoxiques

Les lymphocytes T CD8+ sont des cellules cytotoxiques, capables de détruire des cibles cellulaires (exprimant des CMH de Classe I). Leur activation s'effectue grâce à l'interaction entre leur TCR et le complexe CMH de Classe I/antigène porté par la CPA. Ils peuvent alors agir de plusieurs façons sur leurs cellules cibles (fig 9) :

- voie FasLigand/Fas : l'interaction entre FasLigand présent à la membrane du lymphocyte T CD8+ et Fas à la membrane de la cellule cible entraîne 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 des granzymes entraînant également l'activation des caspases et l'apoptose (Trapani and Smyth 2002)

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

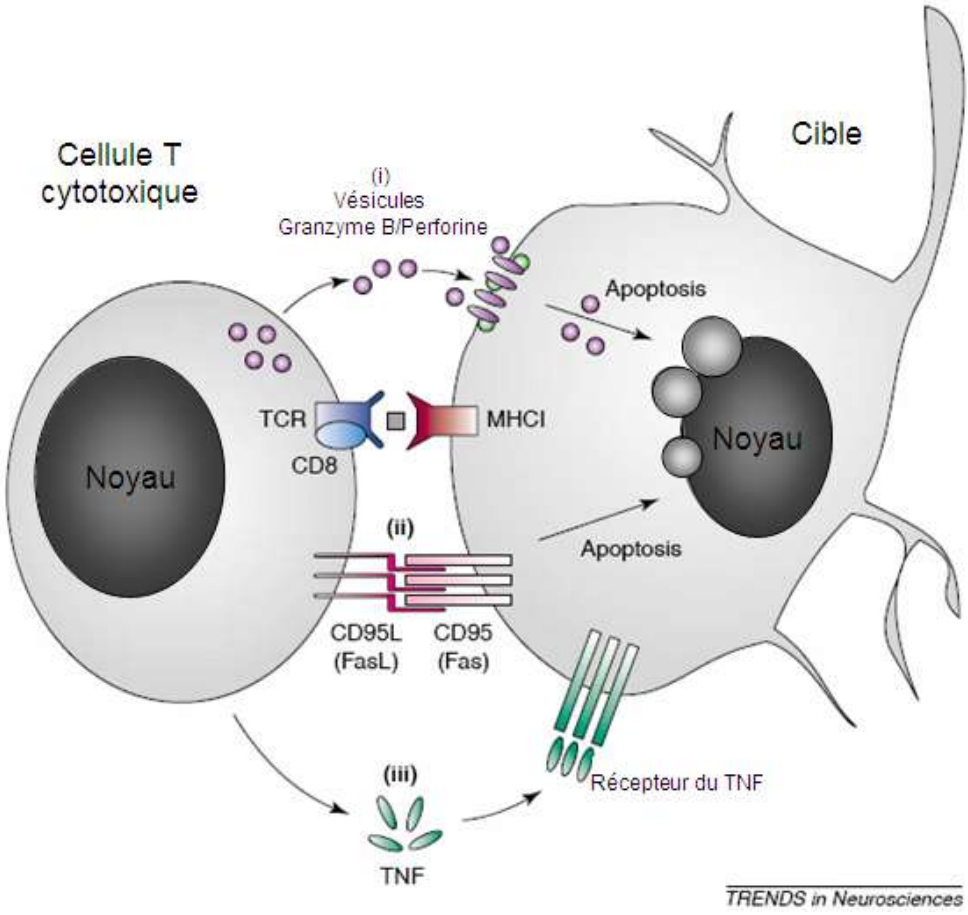


Figure 9: Voies cytotoxiques des lymphocytes T CD8+

Figure extraite de (Neumann, Medana et al. 2002). Trois voies cytotoxiques, non mutuellement exclusives, utilisées par les lymphocytes T CD8+ cytotoxiques pour détruire une cellule cible, exprimant à sa surface un complexe MCH/peptide reconnu par le lymphocyte. (i) sécrétion de granules cytotoxiques libérant perforine/granzyme. (ii) activation de Fas par Fas/ligand et (iii) activation du récepteur du TNF, entraînant une activation en cascade des caspases et aboutissant à l'apoptose de la cellule.

2.5.6.2 Les lymphocytes T CD8+ effecteurs dans la sclérose en plaques

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. Cependant en 2001, deux nouveaux modèles d'EAE ont été induits par l'injection de lymphocytes T CD8+ réactifs contre la MBP (Huseby, Liggitt et al. 2001) et la MOG (Sun, Whitaker et al. 2001). Chez les patients atteints de SEP, les infiltrats cellulaires au niveau des lésions sont majoritairement constitués de lymphocytes T CD8+ (Booss, Esiri et al. 1983; Gay, Drye et al. 1997; Babbe, Roers et al. 2000). Les clones CD8+ infiltrant peuvent persister dans le liquide céphalo-rachidien et le sang de ces patients (Jacobsen, Cepok et al. 2002; Skulina, Schmidt et al. 2004). Dans le compartiment sanguin, la production de cytokines par les lymphocyte T CD8+ corrèle avec la formation de lésions détectées par IRM (Killestein, Eikelenboom et al. 2003) et avec les scores de handicap (Moldovan, Rudick et al. 2003; Sepulcre, Sanchez-Ibarrola et al. 2005). Notre équipe a montré que les altérations du répertoire T présentes dans le sang des patients atteints de SEP concernaient principalement le compartiment T CD8+ (voir annexe I (Laplaud, Ruiz et al. 2004)). Il a également été montré la présence de lymphocytes T CD8+ auto-réactifs contre des peptides dérivés de la myéline chez les patients atteints de SEP et également chez des individus sains (Tsuchida, Parker et al. 1994; Honma, Parker et al. 1997; Crawford, Yan et al. 2004; Zang, Li 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, Biddison et al. 1998). Neumann et al. ont également montré que des lymphocytes T 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 (fig10) (Neumann, Medana et al. 2002).

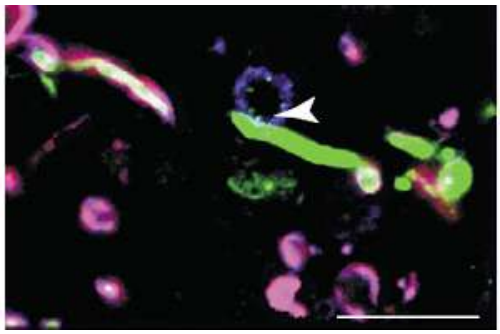


Figure 10: Photographie d'un lymphocyte T cytotoxique accolé à un axone démyélinisé au niveau d'une lésion active d'un patient atteint de SEP.
Photographie extraite de (Neumann, Medana et al. 2002). Cette section a fait l'objet de quatre marquages : CD3 (bleu), PLP (rose), neurofilament (vert), granzyme B (points verts). Les granules contenant du granzyme B sont polarisées vers la surface de l'axone démyélinisé. La barre d'échelle correspond à 50µm.

Les lymphocytes T CD8+ cytotoxiques, présents en nombre au niveau du SNC chez les patients SEP, sont donc capables de causer des dommages aux oligodendrocytes mais aussi potentiellement aux axones. 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 des axones (fig 11). 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, Schuchardt et al. 2000; Kuhlmann, Lingfeld et al. 2002).

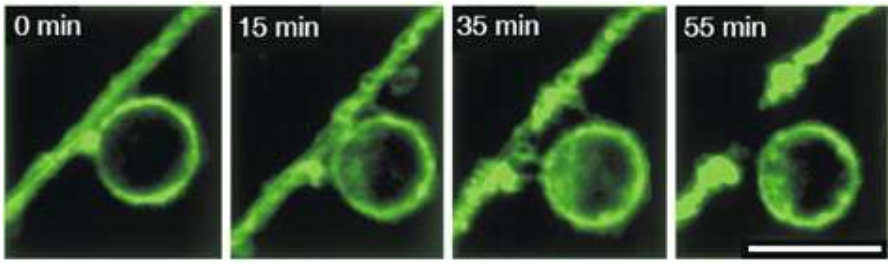


Figure 11: Transsection d'un axone par un lymphocytek T cytotoxique
Photographie extraite de (Neumann, Medana et al. 2002). Barre d'échelle à 10µm. La destruction de cet axone est dépendante du contact entre le TCR du lymphocyte T et le complexe CMH/ peptide.

2.5.6.3 Les lymphocytes T CD8+ suppresseurs

Les cellules T CD8+ suppressives sont capables de diminuer les réponses immunes en détruisant les cellules réactives impliquées dans la réponse. Chez des patients atteints de SEP, les lymphocytes T CD8+ activés ont des propriétés suppressives altérées (Balashov, Khoury 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, Giacomini et al. 1999).

3. Traitements de la sclérose en plaques

A l'heure actuelle, aucun traitement curatif de la SEP n'a été découvert. Seuls les traitements s'attaquant à la composante immunologique de la maladie ont montré des effets bénéfiques chez l'homme, ils se partagent en deux grandes classes de traitements : les immunosuppresseurs et les immunomodulateurs. Les immunosuppresseurs sont des agents ciblant la prolifération des cellules immunitaires. Ces traitements sont prescrits en général lors des poussées et certaines fois en traitement de fond. Les immunosuppresseurs contrôlent l'inflammation et la survenue des poussées mais ne préviennent pas la neurodégénérescence et la progression irréversible du handicap. Leur durée de prescription est limitée étant donné le risque de cancers et les effets secondaires importants. Les immunomodulateurs sont utilisés quant à eux comme traitement de fond principalement pour les formes rémittentes de la maladie. Les effets secondaires des immunomodulateurs sont principalement des douleurs et traces au niveau des sites d'injection, mais aussi des syndromes pseudogrippaux dans le cas des interférons β et des cas de réactions allergiques pour l'acétate de glatiramère.

3.1 Immunosuppresseurs

3.1.1 Azathioprine

C'est l'immunosuppresseur historiquement utilisé le premier comme thérapie de la SEP et le plus largement prescrit en France (12% des patients). Utilisé à l'origine chez des individus transplantés, il a été testé empiriquement dans la SEP. Ce traitement diminue la progression du handicap et la survenue de poussées après 2 à 3 ans de thérapie (Yudkin, Ellison et al. 1991; Palace and Rothwell 1997). Ce traitement est également prescrit en combinaison avec les IFNβ dans les cas de formes non agressives.

3.1.2 Le mycophénolate mofétil (Cellcept)

Cet immunosuppresseur récent est utilisé dans la prévention du rejet de greffe. Sans essai clinique dans le cas de la pathologie SEP, il représente une alternative de traitement.

3.1.3 Mitoxantrone

La mitoxantrone est le seul immunosuppresseur autorisé depuis 2003 dans le traitement de la SEP pour des formes agressives, à évolution rapide. Il diminue les poussées, la progression de la maladie et le nombre de lésions prenant le Gadolinium (Edan, Miller et al. 1997; Goodin, Arnason 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 effet de toxicité cardiaque et hématologique.

3.1.4 Cyclophosphamide (Endoxan)

Donné dans les cas de forme secondairement progressive avec aggravation rapide du handicap, cet immunosuppresseur montre une efficacité moindre que la mitoxantrone. En effet, le cyclophosphamide a peu d'effets bénéfiques pendant les poussées (Drachman, Paterson et al. 1975), mais diminue le taux de poussées (Gonsette 1986).

3.1.5 Corticothérapies

La méthylprédnisolone est généralement injectée sur trois à quatre 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, Newcombe et al. 1987). Ce traitement permettrait d'accélérer la récupération des symptômes (Brochet 2001).

3.2 Immunomodulateurs

3.2.1 Les interférons β

Depuis les années 1990, trois protéines recombinantes interférons β (IFNβ) sont autorisées comme traitements de la SEP. Leurs effets semblent assez similaires malgré quelques différences dans leur structure (comme la glycosylation) et dans leur posologie. Les études cliniques de phase III ont montré une diminution d'environ 30% du taux annuel de poussée comparé à un groupe placebo et 30% des patients après 2 ans de traitement étaient indemnes de poussées (Group 1993). Plusieurs effets des IFNβ ont été décrits, comme une suppression de la prolifération des

cellules T (Rep, Hintzen et al. 1996; Pette, Pette et al. 1997) et une diminution de l'expression des CMH de Classe II induites par IFNγ (Joseph, D'Imperio et al. 1988; Lu, Riley et al. 1995). La production de cytokines aux effets plutôt anti-inflammatoires est augmentée après traitement aux IFNβ (Rudick, Ransohoff et al. 1996; Rep, Schrijver et al. 1999). Les IFNβ semblent inhiber l'activation des monocytes (Van Weyenbergh, Lipinski et al. 1998) et agir également sur les cellules dendritiques via l'inhibition de la production d'IL12 (Bartholome, Willems et al. 1999). Ces traitements sont capables également de bloquer le trafic T en bloquant les protéines MMP (Stuve, Chabot et al. 1997) et en diminuant l'expression de VLA-4 au niveau des lymphocytes T des patients (Calabresi, Pelfrey et al. 1997).

3.2.2 Natalizumab (Tysabri)

Le natalizumab est un anticorps monoclonal anti-VLA-4, molécule impliquée dans le passage des lymphocytes T dans le SNC. Testée dans un premier temps dans des modèles animaux, (modèle EAE et modèle du virus de Theiler), les essais cliniques chez l'homme ont montré une diminution de 83% du nombre de nouvelles lésions visibles à l'IRM en T2 et Gadolinium positives, de 68% du nombre de poussées et une diminution de 42% de la progression de la maladie dans les formes rémittentes (Polman, O'Connor et al. 2006; Rudick, Stuart et al. 2006). Malheureusement, deux patients atteints de SEP traités par l'anti-VLA-4 ont développé une leucoencéphalopathie due à une infection par le virus JC, la survenue de cette leucoencéphalopathie est estimée à 1 sur 1000 (Yousry, Major et al. 2006).

3.2.3 L'acétate de glatiramère

3.2.3.1 De la découverte aux essais cliniques

L'acétate de glatiramère (GA) est un copolymère randomisé de quatre acides aminés (Acide Glutamique, Lysine, Alanine et Tyrosine avec les ratios respectifs suivants: 4,2 ; 3,4 ; 1,4 ; et 1,0) dont la longueur moyenne varie de 45 à 100 acides aminés et le poids de 4.7 à 11kDa (Teitelbaum, Meshorer et al. 1971). Découverte par Michael Sela et al., cette molécule sensée mimer la séquence peptidique de la protéine basique de la myéline (MBP) n'a non seulement pas induit d'EAE mais au contraire a prévenu la survenue de l'EAE induite par l'injection de MBP chez le rongeur (Teitelbaum, Meshorer et al. 1971). Depuis, le GA a montré sa capacité à inhiber (de 50 à 100% selon le modèle) l'induction d'EAE chronique ou aiguë, chez le rongeur, le lapin et le singe (Teitelbaum, Arnon et al. 1997). Plusieurs essais cliniques chez l'Homme ont suivi

cette découverte dans des cas de SEP de forme rémittente (Bornstein, Miller et al. 1987; Johnson, Brooks et al. 1995; Johnson, Brooks et al. 1998). Le traitement au GA a pour principaux effets bénéfiques chez les patients atteints de SEP de forme rémittente une diminution de 30% de la survenue des poussées (Johnson, Brooks et al. 1995; Comi, Filippi et al. 2001) et un ralentissement de la progression de la maladie (Johnson, Brooks et al. 2000). Par imagerie par résonance magnétique, on a pu détecter une diminution du nombre de lésions et des volumes lésionnels T1 et T2 ainsi que de l'atrophie générale du cerveau (Comi, Filippi et al. 2001; Filippi, Rovaris et al. 2001; Rovaris, Codella et al. 2002; Sormani, Bruzzi et al. 2005). Le score de handicap EDSS chez les patients diminue également après un traitement d'une durée de huit ans au GA (Johnson, Ford et al. 2005).

3.2.3.2 Les mécanismes d'action possibles de l'acétate de glatiramère

3.2.3.2.1 Signal altéré

Dans cette hypothèse, la molécule GA est capable d'entrer en compétition avec des auto-antigènes dérivés de la myéline et induirait un signal altéré au niveau des cellules auto-réactives. Il existe en effet une réaction croisée entre la MBP et le GA au niveau des anticorps (Teitelbaum, Aharoni et al. 1991) et au niveau cellulaire (Webb, Teitelbaum et al. 1973; Webb, Teitelbaum et al. 1976). De plus, le copolymère GA induit des réponses prolifératives T de CMH de Classe II chez des patients SEP ou des témoins et agit comme compétiteur avec le peptide MBP 85-99 pour la liaison à la molécule HLA-DR (Fridkis-Hareli, Teitelbaum et al. 1994; Fridkis-Hareli, Stern et al. 2001). Le copolymère GA inhibe également les réponses de lignées cellulaires T réactives contre la MBP (Fridkis-Hareli, Stern et al. 2001). Chez l'animal, la molécule GA est un antagoniste de l'épitope MBP82-100 (Aharoni, Teitelbaum et al. 1999) et prévient l'EAE induite par deux peptides dérivés de la protéine PLP : PLP139-151 et PLP178-191 par compétition avec ces peptides (Teitelbaum, Fridkis-Hareli et al. 1996). Le même phénomène se produit avec le peptide MOG35-55 injecté en même temps que la molécule GA (Ben-Nun, Mendel et al. 1996). Ces modèles animaux montrent que le GA n'est pas seulement un antagoniste de la MBP mais serait un super antagoniste des antigènes de la myéline.

3.2.3.2.2 Induction d'un changement de Th1 à Th2

La maladie étant associée à une production de cytokines de type Th1, plusieurs études se sont attachées à observer les changements induits à ce niveau par le traitement au GA et ont montré un changement de profil de cytokines sécrétées vers le type Th2 (Duda, Schmied et al.

2000; Qin, Zhang et al. 2000). Chez les patients traités par le copolymère GA, la sécrétion d'IL5 et IL13 par les lymphocytes T CD4+ est augmentée (Wiesemann, Klatt et al. 2001) et cette augmentation corrèle avec la réponse au traitement des patients (Wiesemann, Klatt et al. 2003). Dans le sérum, on note une augmentation du taux d'IL10 ainsi que des taux d'ARNm du TGFβ et IL4 dans les leucocytes du sang (Miller, Shapiro et al. 1998). Il y a également une diminution de la production de cytokines de type Th1 comme le TNFα (Fellay, Chofflon et al. 2001). *In vitro*, les lignées des patients SEP réactives contre la molécule GA produisent des cytokines de type Th2 et Th0 (Duda, Krieger et al. 2000) (Neuhaus, Farina et al. 2000) et sont capables d'inhiber la prolifération des lymphocytes T réactifs contre la MBP (Dabbert, Rosner et al. 2000; Gran, Tranquill et al. 2000).

3.2.3.2.3 Induction d'anergie, d'hyporéponse et de mort cellulaire

Après une durée prolongée de traitement au GA, les cellules des patients montrent une capacité à proliférer en présence de GA diminuée et une hyporéponse au copolymère (Ragheb, Abramczyk et al. 2001; Schmied, Duda et al. 2003). De plus, le traitement au GA induit de l'apoptose (Rieks, Hoffmann et al. 2003). Il y a en effet une augmentation du taux des molécules pro-apoptotiques au niveau des lymphocytes circulants (Ruggieri, Avolio et al. 2006).

3.2.3.2.4 Effets sur les capacités migratoires

Les capacités de migration des lymphocytes T de patients traités au GA au travers d'une barrière de cellules endothéliales sont réduites comparées aux patients non traités (Prat, Al-Asmi et al. 1999). Les lignées cellulaires spécifiques du GA possèdent des capacités de transmigration *in vitro* (Kim, Biernacki et al. 2004). De plus, une fois les cellules endothéliales humaines dérivées de la barrière hémato-encéphalique traitées avec le copolymère, les lignées cellulaires humaines de type Th1 migrent moins que les lignées Th2 au travers de cette barrière de cellules endothéliales traitée (Prat, Biernacki et al. 2005).

3.2.3.2.5 Effet sur les lymphocytes T CD8+

Le traitement au GA a montré des effets variés sur les différents sous-types de lymphocytes T CD8+. Une première étude a montré que le traitement au GA restaure les fonctions immunes défectueuses des lymphocytes T CD8+ IFNγ+ (Karandikar, Crawford et al. 2002). Il y a une

augmentation du taux de lymphocytes T CD8+CD28-CD57+ perforine+ circulants chez les patients traités (Ratts, Lovett-Racke et al. 2006). Le traitement au GA *in vitro* induit des lymphocytes T CD8+ aux capacités suppressives : elles inhibent la prolifération de lymphocytes T CD4+ et ces cellules sont cytotoxiques (Tennakoon, Mehta et al. 2006). Chez quatre patients atteints de SEP, l'étude du répertoire T sur des lignées générées *in vitro* par le GA montre que, dans le compartiment T CD8+, le copolymère GA aboutit à l'apparition de familles oligoclonales (Biegler, Yan et al. 2006). Egalement sur des lignées T CD8+ induites par le GA, une autre équipe a montré qu'il y avait une augmentation de la production d'IL4 (Dressel, Vogelgesang et al. 2006).

3.2.3.2.6 Induction de cellules T régulatrices CD4+CD25+

Analysées en cytométrie de flux, les sous-populations de lymphocytes T ont montré une augmentation du taux de cellules régulatrices CD4+CD25+ produisant de l'IL10 chez les patients traités par le GA (Putheti, Soderstrom et al. 2003). De plus, *in vitro*, chez des individus normaux la stimulation par la molécule GA a déclenché l'induction des cellules régulatrices (Hong, Li et al. 2005).

3.2.3.2.7 Effets sur la composante humorale

Le traitement par le copolymère GA induit des anticorps réactifs contre le GA chez les patients atteints de SEP (Brenner, Arnon et al. 2001; Salama, Hong et al. 2003). Il y a en particulier une augmentation du taux d'immunoglobulines IgG4 chez des patients atteints de SEP de forme rémittente (Farina, Vargas et al. 2002). Après un suivi de trois ans, chez des patients atteints de SEP de forme primaire progressive traités au GA, une diminution des immunoglobulines du type IgG1 et une augmentation des immunoglobulines de type IgG4 ont été observées (Basile, Gibbs et al. 2006).

3.2.3.2.8 Effets sur les monocytes et microglies

Le traitement au GA induit une augmentation de la production d'IL10 et IL12 au niveau des monocytes et, par l'intermédiaires des lymphocytes T, une différentiation des monocytes et des microglies de type 2 (Kim, Ifergan et al. 2004). *In vitro*, la molécule GA change l'interaction et la production de cytokines entre les lymphocytes T et les microglies (Chabot, Yong et al. 2002).

3.2.3.2.9 Effets neuroprotecteurs

Chez l'animal, il a été montré que les lymphocytes T spécifiques du GA sont capables de sécréter in situ au niveau du SNC le facteur neurotrophique dérivé du cerveau (BNDF) (Aharoni, Kayhan et al. 2003). Chez l'homme, les lignées spécifiques du GA sécrètent également du BNDF *in vitro* (Chen, Valenzuela et al. 2003; Ziemssen, Kumpfel et al. 2005). Le traitement au GA a pour effet d'augmenter le taux de BDNF dans le sérum et le LCR, taux qui est anormalement diminué chez les patients SEP non traités (Azoulay, Vachapova et al. 2005). Outre la sécrétion de facteur neurotrophique, un effet plus global de neuroprotection est la récupération du métabolisme axonal chez les patients traités au GA comparés aux patients non traités (Khan, Shen et al. 2005).

3.2.3.3 Différences entre répondeurs et non répondeurs au traitement

Les effets bénéfiques ne sont malheureusement pas retrouvés chez tous les patients traités par le GA et on peut distinguer deux groupes de patients : un groupe dit de répondeurs et un groupe de non répondeurs au traitement. En séparant en deux groupes distincts les patients traités au GA, une étude a montré des différences entre les deux groupes : chez les patients répondeurs au traitement au GA suivis longitudinalement, le taux de lymphocytes T aussi bien CD4+ que CD8+ produisant la cytokine pro-inflammatoire IFNγ diminue tandis que le taux de lymphocytes T CD4+ CD45RA+ (naïfs) et la production de BDNF augmente (Blanco, Moral et al. 2006).

3.3 Essais thérapeutiques

D'autres thérapies sont actuellement en cours d'essais cliniques, ciblant d'autres mécanismes impliqués dans la SEP et pouvant cibler entre autre la composante neurologique. Le tableau 1 résume les thérapies existantes et nouvelles de la SEP, qui sont détaillées ensuite.

Composante de la maladie	Mécanismes	Agents	Cibles
Immunologie	Blocage de la prolifération des leucocytes	Immunosuppresseurs	
	Altération des fonctions T	Acetate de glatiramère	
		Statines	HMG-CoA réductase
		Teriflunomide	Dihydro-orotate deshydrogénase
		Abatacept	B7
		IFNβ	IFNAR1 et IFNAR2
	Séquestration des lymphocytes	FTY720-P	Récepteurs des sphingosine1-phosphatases
	Déplétion des leucocytes	Campath-I	CD52
		Cladribine	Adénosine déaminase
		Rituximab	CD20
	Blocage du passage des vaisseaux	IFNβ	IFNAR1 et IFNAR2
		Natalizumab	VLA-4
Neurodégénérescence	Renversement de la diminution de l'Acétylcholine	Inhibiteur d'AchE	AchE
	Bloquage de la voie de neurotransmision Glu/Asp	Riluzole	
	Augmentation de l'expression de facteurs neurotrophiques	Acetate de glatiramère	

Tableau 1 : Thérapies de la sclérose en plaques
Tableau modifié extrait de (De Jager and Hafler 2007) Abbrévations : AchE = Acétylcholinestérase, IFNAR= récepteur de l'interféron β

3.3.1 Les statines

Les statines sont des inhibiteurs de l'HMG-CoA réductase, molécule impliquée dans le cycle du cholestérol. Lors des essais cliniques, ce traitement semble diminuer le nombre de lésions prenant le Gadolinium (Vollmer, Key et al. 2004). Les mécanismes d'action de ce traitement sont surtout une inhibition la prolifération des monocytes et des lymphocytes T.

3.3.2 Teriflunomide

Inhibant la dihydro-orotate deshydrogénase, le teriflunomide possède des propriétés immunomodulatrices, il diminue la prolifération des lymphocytes. En phase II, ce traitement a eu pour effet la diminution du nombre de lésions à l'IRM. Une phase III est en cours.

3.3.3 Abatacept

L'Abatacept (CTLA4-Ig) bloque la voie de costimulation CD28-C80/86 et est utilisé comme thérapie dans la polyarthrite rhumatoïde. Il semble prometteur dans la pathologie SEP.

3.3.4 FTY720-P

Le FTY720 est un agoniste de quatre des cinq récepteurs sphingosine 1-phophastases. Ce traitement inhibe la marginalisation des lymphocytes T : son action de rétention au niveau des organes lymphatiques, aboutit à l'absence des lymphocytes au niveau de la circulation, les éloignant de leur cible. Les essais cliniques sont en cours, la phase II ayant montré l'efficacité du traitement.

3.3.5 Campath-I (Alemtuzumab)

Le Campath-I est un anticorps monoclonal anti-CD52 ciblant les monocytes et les lymphocytes. La déplétion drastique montre des effets bénéfiques sur le nombre de lésions à l'IRM et diminue le taux de poussées chez des patients atteints de SEP de forme rémittente et de forme secondaire progressive (Coles, Cox et al. 2006) mais semble avoir peu d'effets sur la progression de la maladie. Les lymphocytes B sont rapidement régénérés. De plus, étant donné la forte suppression du système immunitaire, les effets secondaires sont importants.

3.3.6 Cladribine

La cladribine (2-chlorodeoxyadenosine) est un analogue des purines possédant une activité cytoxique sur les lymphocytes et monocytes. La thérapie résulte en une amélioration des symptômes et une diminution du nombre de lésions actives dans les formes rémittentes et secondaires progressives (Brousil, Roberts et al. 2006). Les lymphocytes B sont également touchés faiblement et de manière transitoire par ce traitement. La cladribine est actuellement testée en phase III.

3.3.7 Rituximab

Le Rituximab est une anticorps monoclonal anti-CD20, ciblant par conséquent les lymphocytes B. Actuellement testé en phase III, cette thérapie a déjà montré des effets bénéfiques chez les patients atteints de neuromyélite optique (Cree, Lamb et al. 2005), 80% des cas de neuromyélite étant associés à la présence d'anticorps anti-aquaporine 4.

3.3.8 Inhibiteurs de l'acétylcholinestérase

Les inhibiteurs de l'acétylcholinestérase surtout utilisés dans des cas de maladie d'Alzheimer, ont montré des effets bénéfiques sur les troubles de cognition chez les patients atteints de SEP (Krupp, Christodoulou et al. 2004; Porcel and Montalban 2006).

3.3.9 Riluzole

Le Riluzole est un inhibiteur du relargage du glutamate, molécule accumulée au niveau du SNC des patients atteints de SEP. Le glutamate a en effet des propriétés cytotoxiques causant des dommages aux neurones et cellules gliales. Ce traitement a montré des effets bénéfiques dans les cas de formes progressives primaires (Kalkers, Barkhof et al. 2002).

Méthodologie et objectifs

1. Etudes du répertoire T

1.1 Le Tclandscape® : outil d'exploration du répertoire T

Notre laboratoire a développé une représentation graphique combinée des données qualitatives (distribution des différentes longueurs de CDR3) et quantitatives (accumulation des ARN messagers), le Tclanscape®, cartographie du répertoire Vβ (Guillet, Sebille et al. 2001).

L'analyse qualitative consiste en une étude de la distribution des différentes longueurs de CDR3 dans chaque famille Vβ. Chez l'Homme, le locus du gène β comporte 66 exons V dont 45 effectivement traduits. Les 26 amorces Vβ utilisées pour amplifier les différentes régions CDR3 reconnaissent en tout 44 Vβ. La population lymphocytaire T αβ est ainsi divisée artificiellement en 26 familles Vβ. Le logiciel Immunoscope® permet d'analyser la distribution de tailles CDR3 au sein d'une famille Vβ et représente les résultats sous forme de profil (Pannetier, Even et al. 1995) (fig 12). Chaque profil d'une famille Vβ est représenté par 7 à 13 pics correspondant à 7 à 13 tailles différentes de CDR3, séparées chacune de trois nucléotides et par conséquent d'un acide aminé dans la séquence protéique. Cette analyse du répertoire des cellules T permet de détecter des familles Vβ présentant certaines tailles de CDR3 particulièrement impliquées dans la réponse étudiée et qui sont représentatives de clones T sélectionnés et amplifiés lors de réponses immunes. Cette analyse permet également de discerner deux types de répertoires, altérés ou non. Un profil est non altéré ou de type gaussien lorsque que la totalité des familles Vβ présente une distribution gaussienne des différentes tailles de CDR3 (profil (a) sur la figure 12). Le terme altéré désigne un répertoire dans lequel certaines familles Vβ présentent des expansions clonales (profil (b) sur la figure 12) ou des délétions, les profils apparaissent alors perturbés.

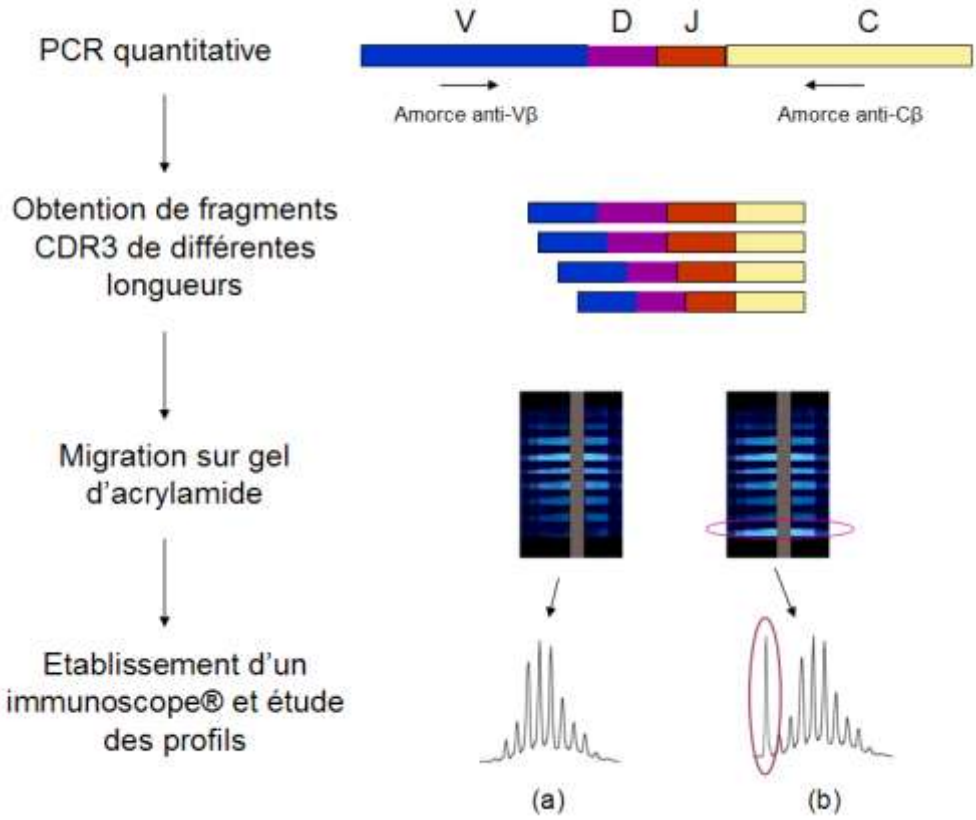


Figure 12: Méthode d'analyse du répertoire

La méthode choisie pour l'analyse quantitative est une PCR quantitative en temps réel faisant appel à la technologie TaqMan®. Cette PCR permet de détecter et de mesurer la fluorescence émise par un marqueur non spécifique (Syber Green), qui se loge dans le petit sillon de l'ADN au fur et à mesure de l'amplification. Les valeurs obtenues pour chaque échantillon sont rapportées à la valeur mesurée pour le gène de l'Hypoxanthine PhosphoRibosyl Transférase (HPRT, gène de ménage) afin de s'affranchir des variations de quantité en ADN complémentaire de chaque échantillon.

Le TcLandscape® permet d'obtenir une vision globale du répertoire: l'axe des x représente les familles Vβ, l'axe des y les différentes longueurs de CDR3 et l'axe des z le rapport des transcrits Vβ/HPRT. Elle permet de suivre non seulement les familles Vβ présentant une altération clonale mais également les familles Vβ présentant une distribution gaussienne des différentes tailles de CDR3 et fortement exprimées (fig 13).

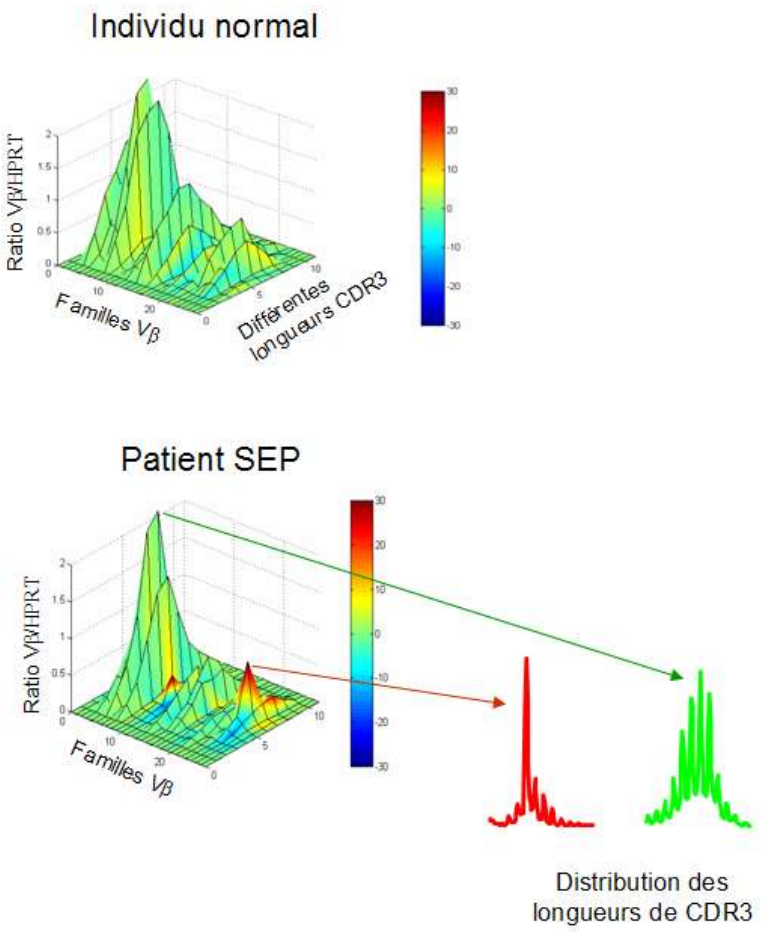


Figure 13: Tclanscapes® d'un individu normal et d'un patient atteint de SEP
Représentations graphiques des répertoires Vβ. L'axe des x correspond aux 26 familles Vβ étudiées, l'axe des y à la répartition des différentes longueurs de CDR3 et l'axe des z au rapport des transcrits Vβ/HPRT. Le code couleur indique les altérations dans la répartition des longueurs de CDR3, du bleu au rouge. En rouge apparaissent les pics altérés, en vert une répartition gaussienne.

1.2 Objectifs

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 (Annexe I, (Laplaud, Ruiz et al. 2004)), un exemple représentatif est donné dans la figure 13. Ces altérations touchent un grand nombre de familles Vβ, qui diffèrent selon les individus. Analysant plus particulièrement les familles Vβ altérées des patients, notre équipe a observé plus fréquemment ce phénomène 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.

A partir de ces observations, un premier objectif de ma thèse a été de déterminer si les altérations du répertoire T dans le sang des patients atteints de SEP pouvaient être liées à l'activité de la maladie. Ce travail a abouti à une publication (Article I dans la partie résultats).

Un autre objectif a été d'étudier le répertoire T de patients traités par l'acétate de glatiramère. Ce traitement a en effet montré des propriétés immunomodulatrices entraînant des modifications au niveau des lymphocytes T. La question de ses effets *in vivo* sur le répertoire T a motivé ce travail (Article III en préparation dans la partie résultats).

Concernant le répertoire T, nous avons également réalisé une étude des cellules T infiltrant le SNC en collaboration avec l'équipe de Silva Markovic-Plese (Chapell Hill, Etats-Unis). Notre partie a consisté à analyser le répertoire T de cellules isolées de quatre lésions différentes et de la substance blanche d'apparence normale, cellules amplifiées par l'antigène phytohemaglutinine (PHA) après leur extraction. Ces échantillons proviennent d'une jeune patiente décédée, atteinte de SEP.

Enfin, 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 chercher à déterminer les épitopes issus de la myéline et reconnus par les lymphocytes T CD8+ dans ce contexte.

2. Recherche d'épitopes issus de la myéline restreints pour les molécules CMH de Classe I

En collaboration avec l'équipe de Jeannine Choppin (Institut Cochin, Paris), notre étude a consisté à déterminer les épitopes issus de la myéline restreints pour les molécules de CMH de Classe I entraînant des réponses cellulaires chez les patients atteints de SEP. Ce travail est l'objet d'une publication soumise (Article II, partie résultats). La méthode utilisée dans cette étude est détaillée ci-dessous.

2.1 La présentation peptidique par les molécules de CMH de Classe I

Les molécules de CMH de Classe I présentent des peptides d'une longueur de 8 à 11 acides aminés reconnus par les lymphocytes T CD8+, ces peptides reflètant les composants intracellulaires des cellules qui les présentent. Cette présentation résulte de plusieurs processus biochimiques. Trois voies d'apprêtage ont été décrites : la voie endogène (fig14) et deux voies exogènes : la présentation croisée et le dressage croisé.

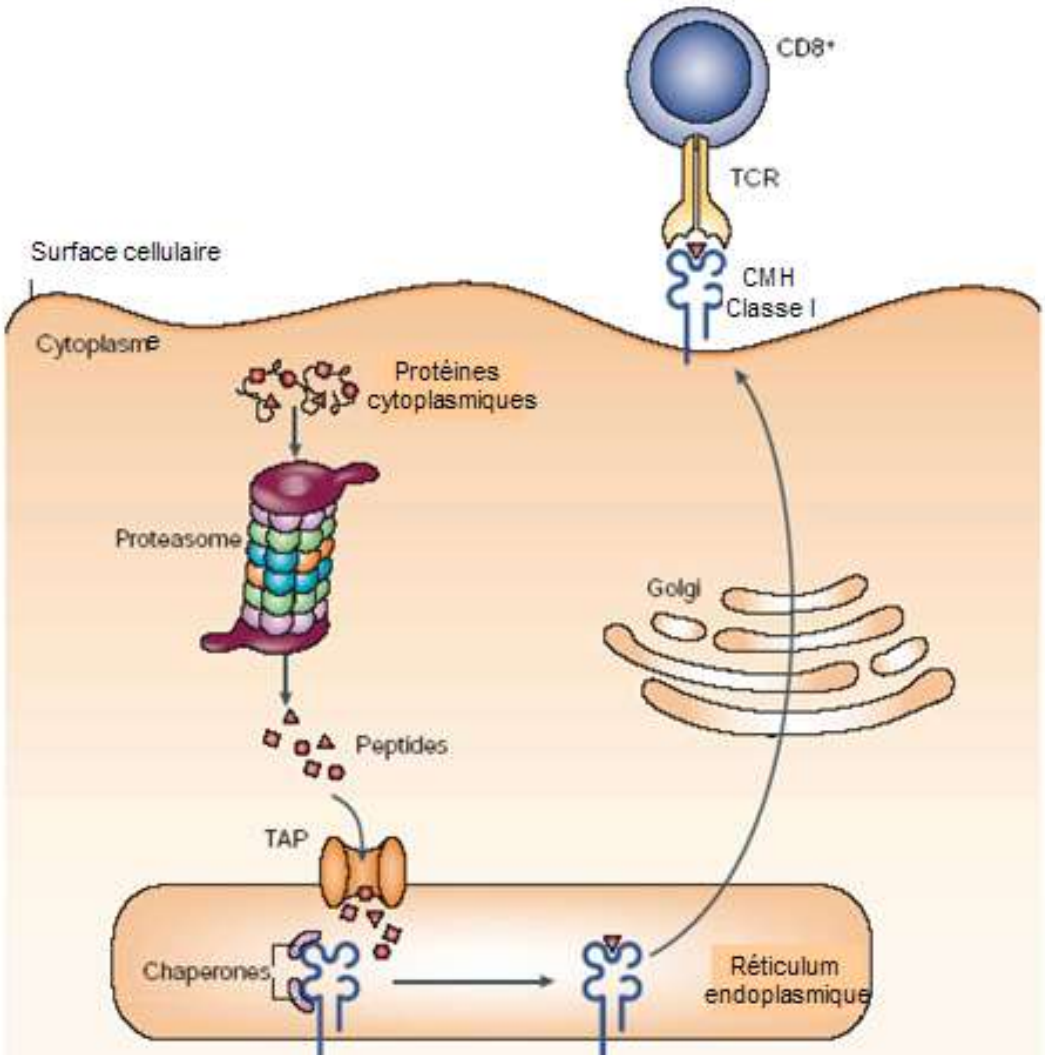


Figure 14: Présentation d'antigènes par les molécules de CMH de Classe I: voie endogène
Figure extraite de (Yewdell, Reits et al. 2003). Les protéines cytoplasmiques sont dégradées par le protéasome, les fragments peptidiques subissent une translocation vers le réticulum endoplasmique grâce au transporteur associé à l'apprêtage des antigènes (TAP). Les peptides sont chargés ensuite sur les molécules de CMH couplées à la β-2-microglobuline. Les complexes CMH/peptides sont ensuite exprimés à la surface des cellules où ils sont reconnus par des TCR de lymphocytes T CD8+.

Lors de la présentation par voie endogène, les fragments peptidiques issus de la dégradation de protéines cytosoliques des CPA par le protéasome sont dirigés vers le réticulum

endoplasmique grâce au Transporteur Associé à l'apprêtage des antigènes (TAP). Plusieurs enzymes (aminopeptidases) clivent alors les peptides qui sont chargés sur les molécules CMH de Classe I / β-2-microglobuline. Ces complexes sont ensuite exprimés à la surface des cellules.

Lors de la présentation croisée, des protéines exogènes sont captées par des CPA professionnelles par endocytose et dirigées vers le cytosol où elles sont dégradées par le protéasome et subissent la translocation via TAP (Ackerman and Cresswell 2004). Enfin un troisième processus a été récemment décrit : le dressage croisé (Dolan, Gibbs et al. 2006). Des CPA dans ce processus portent des CMH de Classe I provenant de cellules mortes et activent ainsi des lymphocytes T CD8+ naïfs.

2.2 Analyse des séquences peptidiques des protéines de la myéline

Les trois protéines de la myéline que j'ai étudiées représentent dans la myéline 30% des protéines pour la MBP, 0,01 à 0,05% pour la MOG et 50% pour la PLP. Ces trois protéines sont présentes au niveau des gaines de myéline du SNC (fig 15). Ce sont les trois protéines les plus étudiées comme auto-antigènes de la myéline et des modèles EAE ont été créés par l'injection de peptides dérivant de ces trois protéines.

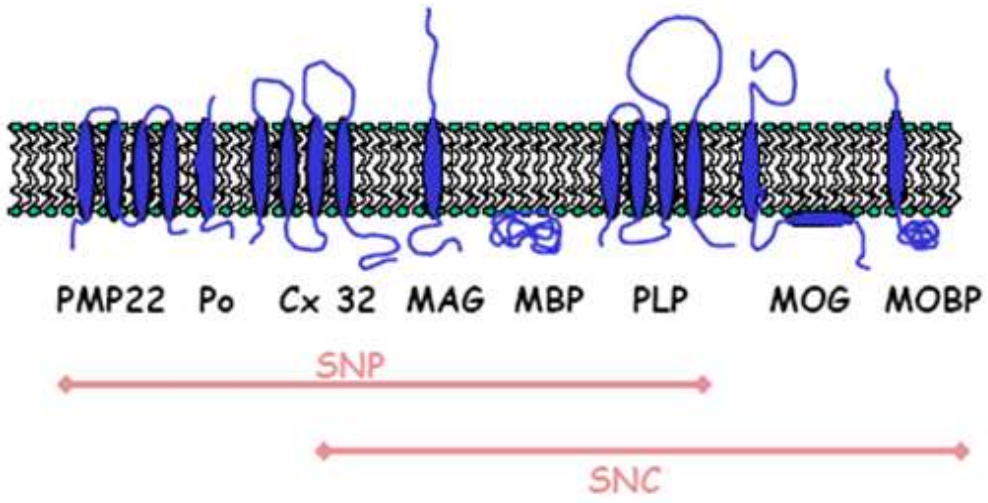


Figure 15: Protéines composant la myéline
SNP= Système Nerveux Périphérique, SNC= Système Nerveux Central. Les gaines de myéline produites par les cellules de Schwann au niveau du SNP sont principalment composées par les protéines : Protéine de la myéline périphérique 22 (PMP22), Protéine zéro de la myéline (P0), Connexine 32 (Cx32), Glycoprotéine Associée à la myéline (MAG), Protéine basique de la myéline (MBP) et Protéine ProtéoLipidique (PLP). Pour les gaines de myéline du SNC produites par les oligodendrocytes, la composition protéique est la suivante : MBP, PLP, Glycoprotéine de la Myéline et des Oligodendrocytes (MOG) et la Protéine Basique de la Myéline et des Oligodendrocytes (MOBP).

L'analyse des séquences des trois protéines de la myéline : MBP, PLP et MOG a été réalisée selon la méthode utilisant les motifs connus dans les séquences peptidiques pour leur capacité de liaison aux molécules de CMH. D'après Sette et al., les molécules de présentation CMH peuvent être regroupées dans des supertypes (Sette and Sidney 1999). Chaque supertype se lie au niveau des séquences des peptides à des motifs particuliers. Par exemple pour le supertype HLA-A2, les peptides se liant aux molécules appartenant à ce supertype (-A2, -A26, -A68…) présentent en général en position 2 un acide aminé hydrophobe ou aliphatique (Alanine, Leucine Isoleucine, Méthionine, Valine ou Thréonine) et en position C terminale les mêmes acides aminés. L'objectif de cette analyse est de sélectionner les peptides qui se lient potentiellement aux molécules de CMH étudiées.

2.3 Tests de liaison aux molécules de CMH

Les tests de liaison aux molécules CMH utilisés dans notre étude sont des tests biochimiques dans lesquels les peptides sont incubés avec la molécule CMH étudiée et la β-2-microglobuline dans des plaques préalablement recouvertes d'anticorps dirigés contre la molécule de CMH de Classe I. Ces anticorps sont spécifiques d'une conformation et fixent la molécule CMH uniquement si un complexe CMH/β-2-microglobuline/peptide est formé. Les complexes formés sont ensuite détectés par l'ajout d'un anticorps anti-β-2-microglobuline.

2.4 Tests de réponse cellulaire : Elispot

Les peptides sélectionnés sont ensuite testés pour leur induction d'une réponse cellulaire des lymphocytes T CD8+ dans des tests Elispot IFNγ. Cette cytokine pro-inflammatoire semble effectivement représentative des réponses contre l'antigène des lymphocytes T CD8+ et corréler avec la cytotoxicité (van Lier, ten Berge et al. 2003). Le test Elispot est effectué *ex vivo* sans pré-stimulation sur des PBMC frais.

Résultats

Partie I : Etude du répertoire T

Article I : Etude sériée des altérations du répertoire T dans le sang de patients atteints de sclérose en plaques, corrélations avec les paramètres cliniques et données issues de l'IRM.

Par : Berthelot L*, Laplaud DA*, Miqueu P*, Bourcier K, Moynard J, Oudinet Y, Guillet M, Ruiz C, Oden N, Brouard S, Guttmann CR, Weiner HL, Khoury SJ, Soulillou JP.

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

Publié dans : Journal of neuroimmunology. 2006. 177(1-2):151-60.

ELSEVIER

Journal of Neuroimmunology 177 (2006) 151–160

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Serial blood T cell repertoire alterations in multiple sclerosis patients; correlation with clinical and MRI parameters☆

David-Axel Laplaud [a,1], Laureline Berthelot [a,1], Patrick Miqueu [a,b,1], Kasia Bourcier [c], Julien Moynard [a], Yannick Oudinet [a], Marina Guillet [a,b], Catherine Ruiz [b], Neal Oden [d], Sophie Brouard [a], Charles R.G. Guttmann [c], Howard L. Weiner [c], Samia J. Khoury [c,2], Jean-Paul Soulillou [a,*,2]

[a] *Institut National de la Santé Et de la Recherche Médicale (Inserm) Unité 643: "Immunointervention dans les allo- et Xénotransplantations", Institut de Transplantation Et de Recherche en Transplantation (ITERT) CHU Hôtel Dieu, 30 Bd Jean Monnet, 44093 Nantes Cedex, France*

[b] *TcLand SA, 30 Bd Jean Monnet, 44093 Nantes Cedex, France*

[c] *Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*

[d] *The EMMES Corporation, 401 N. Washington St., Suite 700, Rockville, MD 20850, USA*

Received 3 January 2006; received in revised form 14 April 2006; accepted 2 May 2006

Abstract

A significant skewing of the peripheral T cell repertoire has been shown in relapsing–remitting multiple sclerosis (MS). Most of the studies already performed in this field are cross-sectional and therefore, little is known of the T cell repertoire evolution over time in MS and the correlation of T cell repertoire variation with clinical and MRI parameters. This study was performed on serially harvested frozen PBMC from nine untreated MS patients (27 samples) and 14 healthy individuals. The blood T cell repertoire of each patient was analysed at the complementarity determining region 3 (CDR3) level and compared with a monthly MRI scan performed over a six month period with assessment of T2 lesion load and gadolinium enhancing lesions. A highly significant blood T cell repertoire skewing was observed in MS patients as compared with healthy controls ($p<0.01$). In addition, the number of altered Vβ families correlated significantly with both the T2 lesion volume and the number of gadolinium enhancing lesions as assessed by MRI (Spearman correlation tests, $r=0.51$ and $r=0.44$, $p<0.01$ and $p<0.05$ respectively). Furthermore, the variation of the number of altered Vβ families over time also correlated with the appearance of new gadolinium enhancing lesions ($r=0.36$, $p=0.05$). These findings which need confirmation on larger serial cohorts, suggest an association between the magnitude of TCRBV CDR3 length distribution alterations in the peripheral blood of MS patients and the disease process.

Keywords: Multiple sclerosis; T cell repertoire; V beta genes; CDR3; Spectratyping; Immunoscope; MRI

Abbreviations: BBB, Blood Brain Barrier; CDR3, Complementarity Determining Region 3; CNS, Central Nervous System; EDSS, Expanded Disability Status Scale; HPRT, Hypoxanthin-Phosphoryl-Ribosyl-Transferase; HI, Healthy Individuals; LD, Length Distribution; MBP, Myelin Basic Protein; MS, Multiple Sclerosis; PBMC, Peripheral Blood Mononuclear Cells; RR, Relapsing–Remitting; SP, Secondary-progressive; TCR, T Cell Receptor.

☆ This research was performed as a project of the Immune Tolerance Network (#TOOA563254), a collaborative clinical research project headquarted at the University of California San Francisco and supported by the national institute of Allergy and Infectious Diseases, the National Institute of Diabetes, Digestive and Kidney Diseases, the Juvenile Diabetes Research Foundation and the French association for multiple sclerosis research (ARSEP).

* Corresponding author. INSERM U643, 30 Bd Jean-Monnet, 44093 Nantes Cedex 01, France. Tel.: +33 2 40 08 74 10; fax: +33 2 40 08 74 11.
E-mail address: jean-paul.soulillou@univ-nantes.fr (J.-P. Soulillou).

[1] These authors contributed equally.

[2] Both are senior authors.

0165-5728/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2006.05.006

1. Introduction

Multiple sclerosis (MS) is a central nervous system (CNS) disease associated with auto-immunity against myelin determinants (Noseworthy et al., 2000; Zamvil et al., 1985). A large body of evidence indicates that myelin reactive T cells are implicated at least in the early phase of the disease process, such cells being predominant in the active MS plaques (Booss et al., 1983). Recently, a humanized antibody directed against the T cell molecule VLA4 has shown its ability to reduce the formation of new gadolinium enhancing lesions in the CNS of MS patients, suggesting that activated autoreactive blood T cells cross the blood brain barrier (BBB) and contribute to MS lesions (Polman et al., 2006)(Miller et al., 2003). Investigations into the selective T cell repertoire of MS patients have shown preferential usage of certain selected Vβ families, with different individual-specific (private) TCR repertoire restrictions (Gran et al., 1998; Laplaud et al., 2004; Lozeron et al., 1998; Matsumoto et al., 2003; Muraro et al., 2002; Musette et al., 1996). Analysis of the entire T cell repertoire has also shown a significant global alteration at the very onset of the disease (Laplaud et al., 2004). However, the precise location where these T cells undergo selection, whether it be in the periphery or in the CNS itself, is still controversial (see (Prat and Antel, 2005) for review) as is the issue of cell migration fluxes from the blood to the CNS (and vice versa) (de Vos et al., 2002; Melchior et al., 2005). T cell clones with similar TCRBV chain hypervariable regions, as assessed by the complementarity determining region 3 (CDR3) length or sequence, have been observed in the blood as well as in the CNS of MS patients (Babbe et al., 2000). We have also shown that most of the T cells from the blood TCR Vβ families presenting an altered CDR3 length distribution pattern are in fact CD8 cells (Laplaud et al., 2004). CD8+ cells expressing adhesion molecules such as P-selectin ligand have been shown to efficiently cross the BBB (Battistini et al., 2003). In addition, we have shown that a high frequency of sorted T cells belonging to Vβ families with strongly biased TCR usage are MBP-reactive (6000/10^6) when tested in vitro (Laplaud et al., 2004).

In this study, we collected serial blood samples from MS patients and longitudinally studied TCR repertoire skewing at the Vβ CDR3 length distribution (LD) level on a patient by patient basis. We confirm and extend previous observations that MS patients' exhibit altered blood CDR3-LD compared to normal individuals. Moreover, we observed a significant positive correlation between T2 lesion volume, the number of gadolinium enhancing lesions as assessed by MRI, and the proportion of altered Vβ families in MS samples. Furthermore, a correlation between appearance of new gadolimium enhancing lesions with time and newly altered Vβ families was also observed. These data, which need confirmation by larger prospective studies, suggest that blood T cell repertoire analysis could be used as an additional non invasive method for disease follow-up in MS.

2. Patients and methods

2.1. Patients and study design

2.1.1. Patients

Nine MS patients were included in this longitudinal study between October 1994 and April 1995; 6 patients with Relapsing–Remitting (RR) MS and 3 patients with Secondary Progressive (SP) MS. All were followed at the MS Center of the Brigham and Women's Hospital in Boston, and were selected from the placebo arm of a randomized, double-blind, placebo-controlled, phase II trial of linomide in the treatment of MS. All gave their informed consent before participating in this study, which was approved by the local IRB. None of the patients were on treatment with interferon, steroids or other immunosuppressive drugs for at least 3 months prior to entering the study. All were clinically examined by a neurologist at baseline, 12, 24, 36 and 48 weeks (Table 1).

2.1.2. Healthy controls

Blood from 14 healthy individuals, under the age of 35 years at the time of sampling, for whom PBMC were stored in liquid nitrogen tank since 1996, corresponding to a same time period of freezing than MS patients, were used. For three other healthy individuals, blood was drawn every month for 3 months, to determine the variations in CDR3-LD alterations (landscape Topview, see below) with time in a healthy population (see Supplementary Figure 1). All HI gave their informed consent to this study according to the local IRB.

2.2. Blood sampling and processing

2.2.1. PBMC preparation and freezing

Blood from MS patients was drawn at baseline and then every 4 weeks until week 48. Peripheral Blood Mononuclear Cells (PBMC) were subsequently isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation and re-suspended at 2.10^6/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 25 mM Hepes buffer, 50 units/ml penicillin, and 50 mg/ml streptomycin (all from BioWhittaker, Walkersville, MD). After washing, the medium was discarded and 10 10^6 cells were re-suspended in 1 ml of Flash Freeze Buffer containing 10% DMSO and 90% heat inactivated fetal bovine serum before being frozen in liquid nitrogen. Frozen cells from healthy controls were obtained using the same process at a concentration of 10 10^6 per tube.

2.2.2. RNA extraction and cDNA synthesis

PBMC were thawed and washed in PBS. The cells were then resuspended in Trizol® reagent for RNA extraction according to the manufacturer's instructions. The concentration and quality of RNA for each sample was accurately determined using nanoRNA Chips (Agilent®, United

Table 1
Clinical characteristics of the patients at the time of inclusion

Patient number	Gender	Disease type	Age (year)	Disease duration	Date of bleeding	Lesion volume	Gd^{+ve} lesions	New Gd^{+ve} lesions	EDSS
704	M	RR	27		M0	3.1799	0	0	–
					M1	2.8055	0	0	–
					M2	3.3302	0	0	0
					M4	3.9973	0	0	–
					M6	4.0135	0	0	2,5
820	F	RR	45	3	M0	10.4098	1	–	3
					M1	10.9371	3	2	–
					M2	10.7552	1	0,5	–
					M3	11.3906	1,5	1	3,5
					M4	11.3036	1,5	1	–
					M5	10.8158	0	0	–
818	F	RR	31	4	M0	6.4178	4	–	–
					M1	5.6426	1	1	–
					M2	6.5733	5	5	–
					M3	7.5384	5	3	–
					M4	6.1277	2	2	–
					M6	6.2701	0	0	–
808	F	RR	51	6	M0	8.7275	3,5	0,5	–
813	F	RR	42	2	M0	6.2965	0	0	1,5
812	F	RR	37		M1	0.9255	0	0	2
					M6	2.2755	0	0	2
507	M	SP	45	8	M0	9.3973	2	0,5	–
					M1	9.3683	2	2	–
611	F	SP	38		M0	14.4466	0	0	6
617	F	SP	39	12	M0	5.0625	0	0	3
					M2	4.6644	0	0	–
					M5	4.8727	0	0	3

Kingdom). According to these criteria, only 27 of the 65 frozen samples studied, were of good enough quality and were retained for analysis (corresponding to nine MS patients; six RR-MS and three SP-MS patients), the remaining samples showing some RNA degradation, likely due to the long freezing time, were thus discarded. None of the samples from HI showed RNA degradation. 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 100 μl.

2.2.3. TCR repertoire analysis

cDNA was amplified by PCR using a Cβ primer and one of the 26 Vβ specific primers. The amplifications were performed in a 9600 Perkin-Elmer thermocycler (Applied Biosystems, Foster City, CA) as previously described (Gagne et al., 2000). Analysis of CDR3-LD was first performed using Immunoscope® software (Brouard et al., 1999; Douillard et al., 1996; Pannetier et al., 1995). The percentage of 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 (Gorochov et al., 1998). 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 studied and the average control distribution, calculated from 14 healthy individuals. Global CDR3-LD alterations were represented as an alteration "topview" landscape (see below) enabling an easy visual appraisal of the CDR3-LD biases. Topview of Immunoscope alteration landscape will be referred to as "landscape topview" in the rest of the text. In this representation percentages of CDR3-LD alterations are represented as a colour code, from deep blue ($\leq -30\%$) to dark red ($\geq +30\%$). The *X* axis displays the 26 human Vβ families and the *Y* axis gives the CDR3 lengths. Missing data were represented as dark blue lines.

To evaluate changes in CDR3 distributions between two time points of a given patient, two by two comparisons were made of the distributions of all the Vβ families present. Briefly, comparison of two distributions makes it possible to quantify the amount of dissimilarity between the two distributions: a Minkowski distance of order one. Thus, a global distance between two time points could be calculated by summing the differences identified between the CDR3 distributions of all Vβ families present. A large "distance" means that the distributions greatly change from one time point to the next; a small distance means that the distributions do not really change during that time interval. These variations in the CDR3 length distributions were then compared to the variation of a gadolinium score G_i in the same time period (see below).

2.2.4. Assessment of altered Vβ families

To identify Vβ families presenting clonal expansions, we applied the following statistical method (Gorochov et al., 1998; Han et al., 1999). Briefly, a Vβ family was considered as having an altered distribution when it significantly differed from the mean distribution calculated from the 14

healthy controls. For a given Vβ family, a percentage of alteration exceeding the mean percentage of alteration in the control group for the same Vβ family by two standard deviations was considered as significant. Because CDR3-LD were missing in some samples, the proportion of altered Vβ families was used for statistical comparisons.

2.2.5. MR imaging and image evaluation

MRI scans were carried out for each patient at baseline, then every 4 weeks for 6 months. Imaging was performed on a General Electric Sigma 1.5-T unit (General Electric, Milwaukee, WI). Proton-density and T2-weighted images were obtained on the whole brain by contiguous 3 mm thick slices with an in-plane voxel size of 0.94 mm × 0.94 mm (24 cm field of view with a 256 × 192 acquisition matrix). Images were also obtained by applying a T1-weighted spin-echo pulse sequence after administration of an i.v. bolus of 10 ml of 0.5 M gadopentate dimeglumine (Gd-DTPA) (Magnevist, Berlex). Post contrast T1-weighted (Gd+) images in the axial plane resulted from a 600/19/1 (repetition time/echo time/excitations) spin-echo sequence. Slice thickness was 4 mm with 1 mm gap. Proton density- and T2-weighted images were analysed using an automated computerized procedure allowing for a reproducible method of lesional volume assessment. Gd-enhanced T1-weighted images were evaluated by two radiologists for each patient data set. The total number of enhancing lesions and the number of new enhancing lesions at each time point were determined independently by each rater and the average number was used in the analysis.

The following 3 parameters were measured: total number of enhancing lesions, number of new enhancing lesions and total volume of T2 lesions.

The variation of gadolinium positive lesions with time was also assessed by a dedicated score, G_i. This score depends on the appearance, the persistence and the loss of

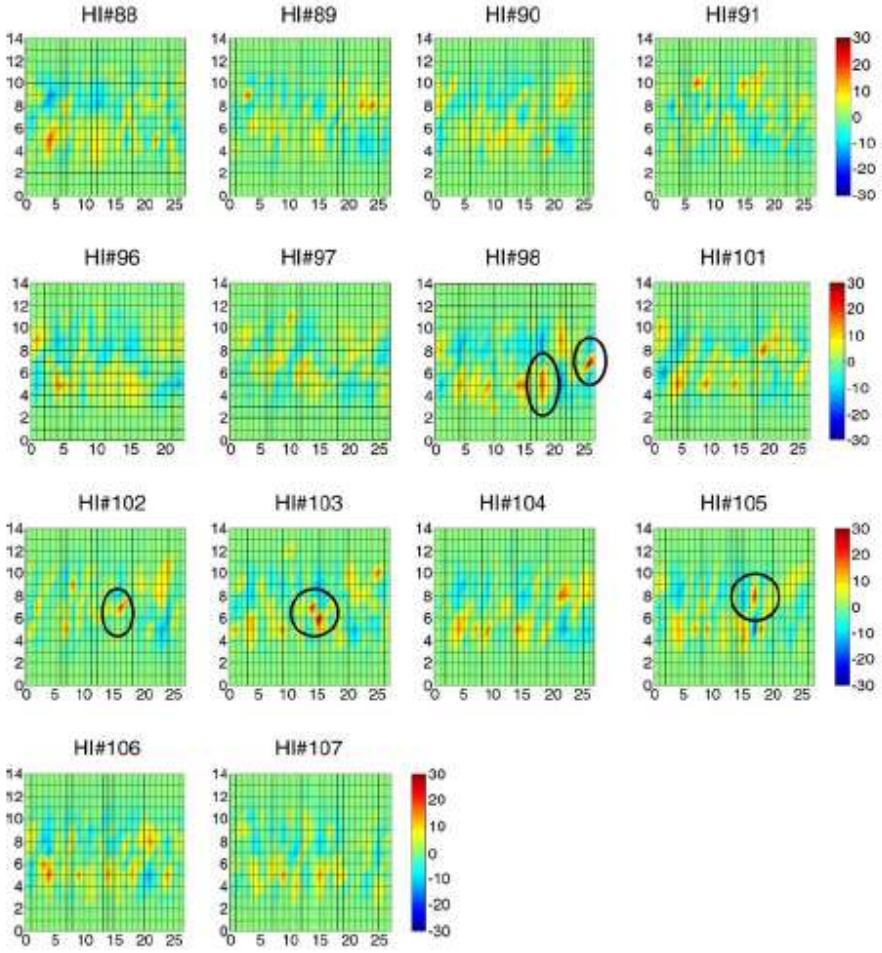


Fig. 1. Landscape Topviews of healthy individuals (HI) for whom PBMC had been frozen for a similar time period as those from MS patients. The *X* axis displays the Vβ families from Vβ1 to Vβ 24. The *Y* axis displays the CDR3 length in amino-acid number for each Vβ family. The colour code represents the TCR skewing for each Vβ family from a deep blue (−30% of alteration) to a dark red (+30% of alteration). The circles indicate the altered Vβ families, as defined by a percentage of alteration exceeding two standard deviation from the mean calculated with the same Vβ family in the Healthy control group. Note that some Vβ families from other healthy individuals seemed to be skewed, appearing as spots on the landscape Topviews (for instance, Vβ 15, HI#101) but did not reach statistical significance.

gadolinium positive lesions and, thus, is related to lesion activity. Special weighting was attributed for each of these 3 events. The evolution of a patient's status between t_i and t_{i+1} could be summarized as follows by the gadolinium positive lesions score, $G_{i,\ i+1}$:

$$G_{i,i+1} = a^*(N_{i+1}) + p^*(S_{i+1} - N_{i+1}) + d^*(S_i + N_{i+1} - S_{i+1})$$

Where:

a is the weight of a lesion appearance;
p is the weight of a persistent lesion;
d is the weight of a lesion loss;
S_i is the total number of lesions at t_i;
S_{i+1} is the total number of lesions at t_{i+1};
N_{i+1} is the number of new lesions at t_{i+1}.

The basic way of scoring these 3 kinds of event leads to the score "+1" for the appearance or persistence of a lesion and "−1" for the loss of a lesion.

We then compared the evolution of the total volume of T2 lesions and the gadolinium positive lesion score. Evolution of the total volume of T2 lesions between t_i and t_{i+n} was calculated by the difference between the lesion volume at t_{i+n} and at t_i. A linear relationship ($r^2 = 0.75$) was found between the evolution of the lesion

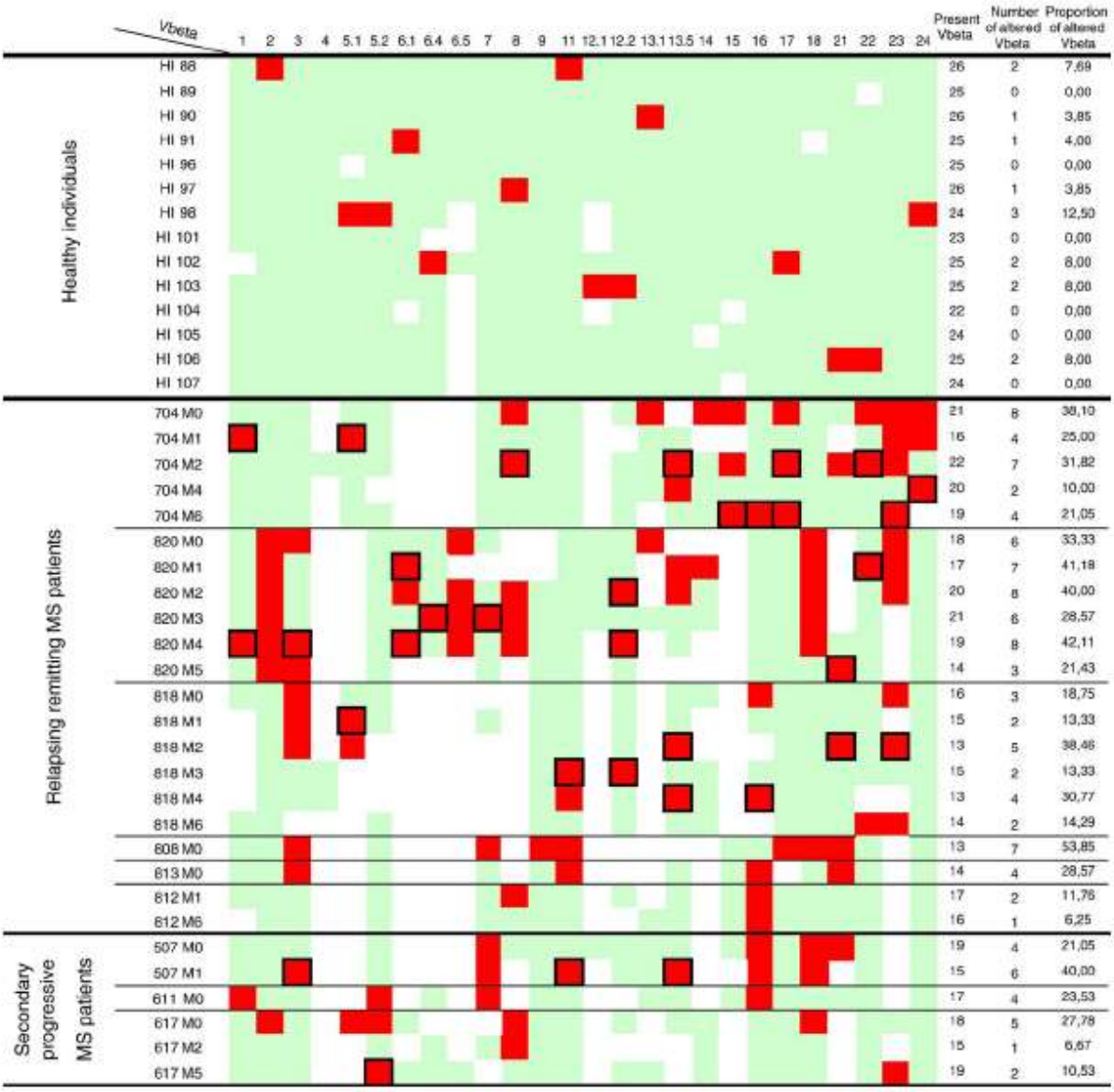


Fig. 2. CDR3 length alterations for all of the multiple sclerosis patients and healthy individuals. A green square indicates a global Gaussian distribution of the expressed Vβ family. A red square indicates an altered Vβ family (percentage of alteration exceeding the mean by two standard deviations). A surrounded red square indicates the appearance of a new altered Vβ family as compared to the preceding sample. A white square corresponds to missing data for the Vβ family.

volume and the gadolinium positive lesion score. The correlation between these two variables was significant at $p<0.01$ (non-parametric Spearman $\rho=0.884$) and bringing confidence on the relevance of the clinical parameters tested.

2.2.6. Statistical procedures

For all usual statistical tests the SPSS v 14.0 and PRISM software were used and a $p<0.05$ was considered significant. A Mann-Whitney test was used to compare the global percentage of CDR3-LD alteration, to compare each Vβ family and to compare the proportion of altered Vβ families in each patient group with the controls. For patients followed over time, the average of the percentage of alteration of each Vβ family was used. Next, six samples from the six RR-MS patients were compared to three samples from three SP-MS patients.

In order to explore the relationship between altered Vβ families and lesion activity, we correlated the number of gadolinium enhancing lesions and the T2 lesion load for each time point with the proportion of altered Vβ families at the same time point. To correlate the variation of gadolinium enhancing lesions with the variation of altered Vβ families, we compared the apparition of new gadolinium enhancing lesions and new altered Vβ families at each time point. The correlation between the T2 lesion load, the number of gadolinium enhancing lesions and the amount of altered Vβ families was calculated using a non-parametric Spearman correlation test, as well as correlation between the variation of the number of altered Vβ families between two time points and the appearance of new gadolinium enhancing lesions.

3. Results

3.1. CDR3 length distribution in normal individuals

First, 14 Healthy individuals (HI) whose PBMC had been frozen for a period of time similar to that of the MS samples (8 years) were used as controls to investigate the normal range of the level of CDR3 length distribution (CDR3-LD) alterations. The global percentage of CDR3-LD alteration in frozen samples was measured of 12.9%±5.7% (ranging from 10.2 to 16%). Nevertheless, clonal expansions (corresponding to altered Vβ families) in PBMC, even following long-term freezing remained rare. Fig. 1 shows the 14 Vβ families with altered patterns of CDR3-LD on the landscape Topview display. Vβ families other than those outlined in Fig. 1 (for example Vβ 15, HI#101) display a borderline colour code but their percentage of alteration did not reach statistical significance (two standard deviations above the mean percentage of alteration in the control group, see Patients and methods). Collectively, eight of the 14 normal individuals tested displayed at least one Vβ family with clonal expansion. These results were summarized in Fig. 2.

3.2. MS patients exhibited higher global percentages of blood T cell CDR3-LD alterations than normal controls

We then compared the level of CDR3-LD alterations in samples from MS and healthy individuals that had been frozen for the same length of time. Several Vβ families were not investigated in all the MS samples because the signal was too weak (white bands for corresponding Vβ families in Fig. 2). The global percentage of CDR3-LD alterations in MS patients and normal individual controls was significantly different with a percentage of alteration of 19.7%±4.04% (ranging from 14% to 24.7% in MS patients) as compared to 12.9%±5.7% in controls ($p<0.001$, Mann-Whitney test, Fig. 3a).

CDR3-LD alterations in MS blood T cells preferentially affected certain Vβ families, as shown by the side by side comparison of each Vβ family from MS patients and normal individuals. Significant skewing involved several Vβ families: Vβ 5.2, 8, 12.2, 16 and Vβ 21 ($p<0.05$, Mann-Whitney test). A comparison of the proportion of altered Vβ families, shown as a red spot on the landscape Topview, revealed significantly higher proportion of altered Vβ families in the MS group than in the controls, with a mean proportion of altered Vβ families of 25.3%

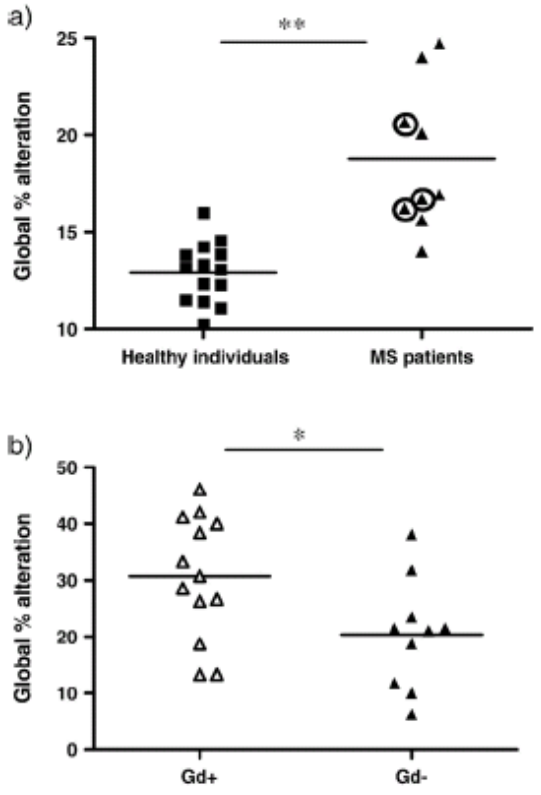


Fig. 3. a) Global percentage of alteration in RR-MS, SP-MS and healthy individuals. b) Global percentage of alteration in patients with gadolinium enhancing lesions according to MRI versus patients without gadolinium enhancing lesions. Mann-Whitney tests. *$p<0.05$, **$p<0.01$.

in MS patients and 3.9% in healthy individuals (Mann-Whitney test, $p<0.001$). As shown in Fig. 2, all patients displayed at least one altered Vβ family, as compared with only eight among the 14 healthy individuals.

Taken collectively, these data confirm a significant skewing of blood T cell repertoire diversity in MS patients.

CDR3-LD was then analysed in the different clinical groups of MS patients. The global percentage of CDR3-LD alterations was compared in relapsing–remitting versus secondary progressive MS patients. The global percentage of CDR3-LD alteration was 20.6± 10.3% (ranging from 14 to 24.7%) in RR-MS patients and 18.1± 7% (ranging from 16.7 to 20.8%) in SP-MS patients (Mann-Whitney test, NS). Furthermore, even when considered independently, RR-MS as well as SP-MS patients were significantly more altered than the controls ($p<0.01$).

3.3. Serial analysis of TCR alterations in MS patients

In MS patients the presence of altered Vβ families was roughly stable over time in a given patient but differed from one patient to another. During the follow-up, some "red spots" corresponding to Vβ family transcripts with strong CDR3-LD alterations appeared and/or disappeared, as shown in the example given in Fig. 4 (patient 820) and summarized in Fig. 2 and in Supplementary figure 2. As shown in the example concerning patient 820, the Vβ 2 family exhibited a strong CD3-LD alteration and this spot was roughly stable over time. Nevertheless, other Vβ families exhibited significant CDR3-LD alterations appearing over the follow-up period and summarized in Fig. 2. As in the example shown in Fig. 4, Vβ 18 appeared significantly altered at M4 but not at M5 (Supplementary figure 3).

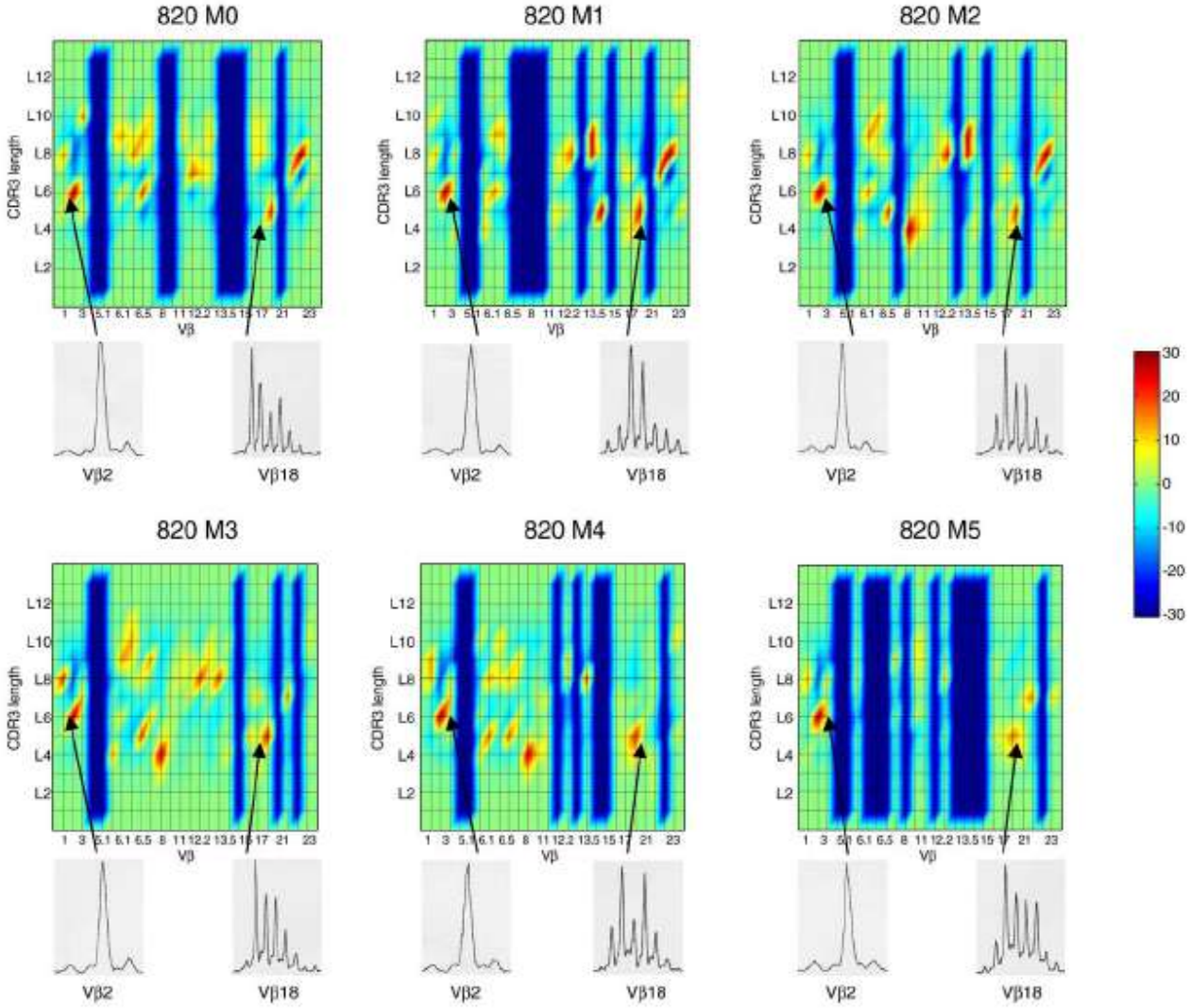


Fig. 4. A significant example of landscape Topviews from patient 820 and their variations with time. Arrows indicate an example of persisting altered Vβ families throughout the follow-up period. The Immunoscope profiles of the persisting altered families and of appearing/disappearing altered Vβ families are shown (Vβ 18).

3.4. Correlation of CDR3-LD alterations with MRI parameters

First, we compared the level of CDR3-LD alterations in patients with and without gadolinium enhancing lesions on their MRI and found a significant difference between the two patient groups (30.6%±10.8 and 20.4%±9.6% respectively, Mann-Whitney test, $p<0.05$, Fig. 3b). In addition, the proportion of altered Vβ families in each group of patients reached significant difference (9% versus 19%, Mann-Whitney test, $p<0.01$). Furthermore, a significant correlation was observed between the proportion of altered Vβ families and the T2 lesion load (Spearman correlation test, $r=0.51$, $p<0.01$, Fig. 5a) and the number of gadolinium enhancing lesions ($r=0.44$, $p<0.05$, Fig. 5b).

3.5. Longitudinal analysis of variations in CDR3-LD alterations and lesion activity

Despite the relatively low number of patients, a significant correlation was also observed between the variation of gadolinium enhancing lesions with time and the appearance of new altered Vβ families (Spearman correlation test, $r=0.36$, $p=0.05$). The correlation was stronger when only patients 818 and 820 were considered, for whom sufficient data was available and gadolinium positive lesions were present ($r=0.74$, $p<0.01$, Fig. 5c).

In addition, we compared the "amount" of T cell repertoire diversity changes that occurred at each time interval (by comparing the variation of the percentage of alteration for each Vβ family present between two samples, see Patients and methods) with the evolution of the gadolinium score G_i for patients 818 and 820. As shown in Fig. 5d, for patient 818, the level of CDR3 length distribution changes roughly paralleled the evolution of the G_i score.

Altogether, these data suggest an association between the lesion activity seen on the MRI and newly altered Vβ families in the blood of MS patients.

4. Discussion

Multiple sclerosis is thought to be an autoimmune T cell driven disease. In this study we serially analysed and monitored the TCR repertoire in the blood of MS patients with different disease types using an analysis of CDR3-LD alteration. In addition, we tested the hypothesis of possible correlations between TCR biases and clinical parameters in patients who had monthly MRI scans during their follow-up.

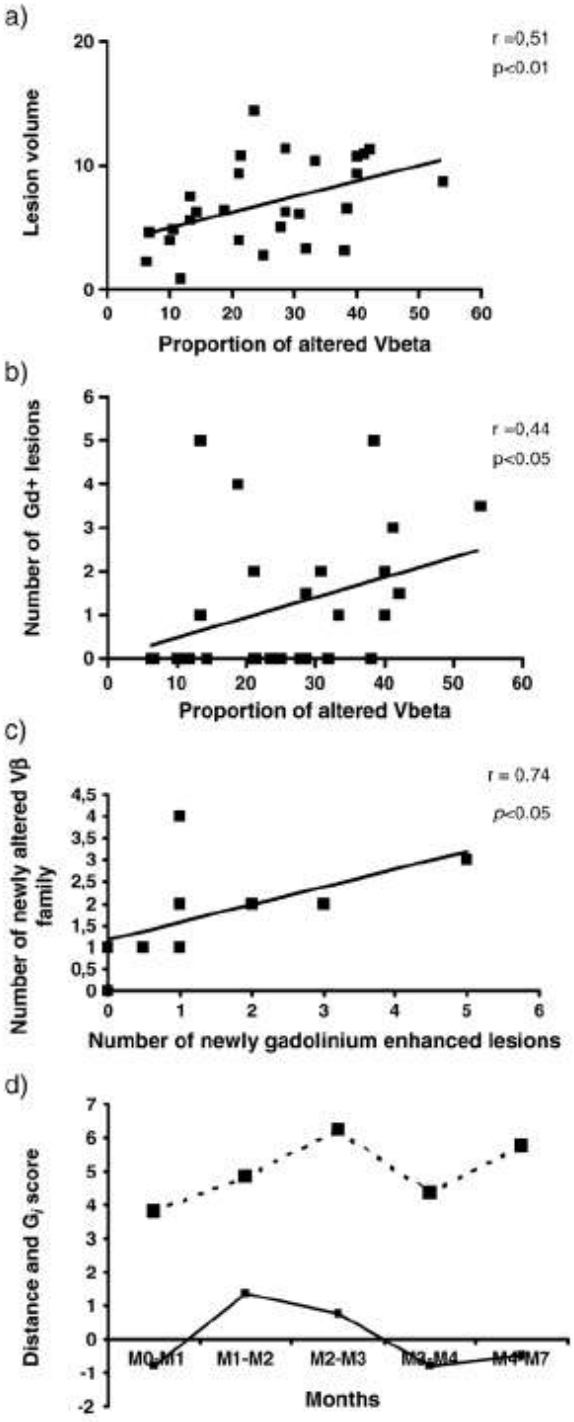


Fig. 5. Correlation of MRI parameters with altered Vβ families. a) Correlation between the T2 lesion load and the proportion of altered Vβ families (Spearman correlation test, $r=0.51$, $p<0.01$). The X axis indicates the T2 lesion volume assessed by MRI and the Y axis indicates the proportion of altered Vβ families. b) Correlation between the number of gadolinium enhancing lesions and the proportion of altered Vβ families ($r=0.44$, $p<0.05$). The X axis indicates the number of gadolinium enhancing lesions and the Y axis indicates the proportion of altered Vβ families. c) Correlation between the newly appearing altered Vβ families with time and the newly gadolinium enhancing lesions in patients 818 and 820 ($r=0.74$, $p<0.01$). d) Correlation with time between gadolinium score G_i and the variation of alterations assessed by a designated distance (see Patients and methods) in patient 818. The X axis indicates time (months) and the Y axis indicates G_i score and variation of distances. The dotted line indicates the variation in G_i score with time for patient 818. The black line indicates the variation of distances with time (see Patients and methods) for patient 818.

The fact that all of the patients in this cohort belonged to the placebo arm of an interrupted therapeutic trial rendered the biological study of this cohort even more informative since this enabled us to analyse patients in the absence of disease modifying therapies. Finally, the MRI data available for each patient at the time of each blood harvesting made it possible for us to perform correlations between the T cell repertoire biases and the different clinical parameters. Only samples giving high quality mRNA (as assessed by Agilent technology) were analysed.

Using appropriate controls (PBMCs from healthy volunteers stored for a same time period than those from MS patients), we confirm and extend the findings of our previous study of the TCR Vβ chain repertoire in fresh blood of MS patients (Laplaud et al., 2004). Although oligoclonal expansions of CD8 T cells have been shown to occur with age, the fact that the patients and healthy individuals were relatively young means that it is unlikely that the skewing observed in our group of patients was due to age (see (Effros et al., 2003) for review). The TCR skewing observed in our study involved several Vβ families, including Vβ 5.2 that has been extensively studied in other works. Vβ 5.2 family has been reported to be frequently used by MBP-specific clones from HLA-DR2 MS patients (Kotzin et al., 1991). We also found other Vβ families with significant skewing such as Vβ 12 that was also reported to be used by MBP specific cells (Hong et al., 1999; Wucherpfennig et al., 1990). Finally, Vβ 8 and Vβ 21 families, significantly altered in the MS group were also mentioned biased by other authors (Matsumoto et al., 2003).

We first studied the stability of TCR alteration over time in three healthy individuals over a period of 3 months. The TCR profiles showed some Vβ families with altered CDR3-LD which are reproducibly found over time (data not shown, see Supplementary file 1). Such private alterations have already been reported in healthy individuals and might correspond to the T cell response occurring during different virus infections such as influenza, CMV or EBV viruses (Bitmansour et al., 2001; Maini et al., 2000; Prevost-Blondel et al., 1997). The presence of persisting peripheral blood TCR CDR3-LD alterations are likely linked to the "immunological history" of each normal individual. It is likely that these patterns are also superimposed in MS patients.

The stability of the TCR alterations over time in MS has been only investigated in a small number of patients (Matsumoto et al., 2003; Muraro et al., 2002). In the latter studies, the global percentage of CDR3-LD alteration was roughly stable over time in a given patient. However, although some Vβ family alteration patterns remained stable throughout the follow-up, we also observed time-related variations, suggesting, as shown by Muraro et al. (2002), that some epitope spreading can also occur in the periphery.

The clinical significance of blood TCR skewing has been rarely investigated before in MS patients. In our previous study of TCR alterations, we showed that the T cells responsible for the skewing were mainly CD8 cells producing Th1 cytokines, and importantly, that some of these cells produced IFNγ when stimulated with human MBP (Laplaud et al., 2004). TCR skewing has also been correlated to clinical symptoms in other diseases, such as AIDS (Gorochov et al., 1998), melanoma (Degauque et al., 2004) or in renal allograft recipients (Brouard et al., 2005; Degauque et al., 2004). Moreover, in this study, we found a positive correlation between the change in blood TCR biases (assessed by the variation of distances and by the apparition of newly altered Vβ families) and lesion activity, especially for two patients. This correlation, which was assessed either by the new appearing gadolinium positive lesions or by a G_i score taking into account the new gadolinium positive lesions, the persisting gadolinium positive lesions and the disappearance of gadolinium positive lesions, may be of potential relevance for the clinical management of MS. More importantly, a positive correlation was also detected between the TCR biases and the number of gadolinium positive lesions, suggesting that the level of TCR biases may be a marker of disease activity. These results are in accordance with those of Muraro et al. who also reported a positive correlation between lesion activity as assessed by MRI and the level of skewing of blood TCR repertoire based on Vβ gene expansions. However, the alteration of the Vβ families were not systematically tested in their work. Even though we found correlations between lesion activity/T2 lesion load and TCR biases by two different methods, our observations are based on a small cohort of patients with serial analysis, and should be interpreted with caution. Taken together, these data suggest a direct involvement of the T cells from altered Vβ families in the pathophysiological process. This work provides new insight into the clinical significance of the potential role of altered peripheral blood T cell repertoire in MS and suggests that the exhaustive study of Vβ transcriptome alteration may be of some value in the management of MS.

Acknowledgments

This work was supported in part by grants from the International Tolerance Network (to C.R.) and the Association pour la Recherche sur la Sclerose en Plaques (to D.A.L.). We thank Dr. Joanna Ashton-Chess for manuscript editing.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2006.05.006.

References

Babbe, H., Roers, A., Waisman, A., Lassmann, H., Goebels, N., Hohlfeld, R., Friese, M., Schroder, R., Deckert, M., Schmidt, S., Ravid, R., Rajewsky, K., 2000. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J. Exp. Med. 192, 393–404.

Battistini, L., Piccio, L., Rossi, B., Bach, S., Galgani, S., Gasperini, C., Ottoboni, L., Ciabini, D., Caramia, M.D., Bernardi, G., Laudanna, C., Scarpini, E., McEver, R.P., Butcher, E.C., Borsellino, G., Constantin, G., 2003. CD8+T cells from patients with acute multiple sclerosis display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1. Blood 101, 4775–4782.

Bitmansour, A.D., Waldrop, S.L., Pitcher, C.J., Khatamzas, E., Kern, F., Maino, V.C., Picker, L.J., 2001. Clonotypic structure of the human CD4+ memory T cell response to cytomegalovirus. J. Immunol. 167, 1151–1163.

Booss, J., Esiri, M.M., Tourtellotte, W.W., Mason, D.Y., 1983. Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. J. Neurol. Sci. 62, 219–232.

Brouard, S., Vanhove, B., Gagne, K., Neumann, A., Douillard, P., Moreau, A., Cuturi, C., Soulillou, J.P., 1999. T cell repertoire alterations of vascularized xenografts. J. Immunol. 162, 3367–3377.

Brouard, S., Dupont, A., Giral, M., Louis, S., Lair, D., Braudeau, C., Degauque, N., Moizant, F., Pallier, A., Ruiz, C., Guillet, M., Laplaud, D., Soulillou, J.P., 2005. Operationally tolerant and minimally immunosuppressed kidney recipients display strongly altered blood T-cell clonal regulation. Am. J. Transplant 5, 330–340.

de Vos, A.F., van Meurs, M., Brok, H.P., Boven, L.A., Hintzen, R.Q., van der Valk, P., Ravid, R., Rensing, S., Boon, L., t Hart, B.A., Laman, J.D., 2002. Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. J. Immunol. 169, 5415–5423.

Degauque, N., Schadendorf, D., Brouard, S., Guillet, M., Sebille, F., Hohn, H., Pallier, A., Ruiz, C., Dupont, A., Chapin, S., Hofmann, U., Maeurer, M., Soulillou, J.P., 2004. Blood T-cell Vbeta transcriptome in melanoma patients. Int. J. Cancer 110, 721–729.

Douillard, P., Pannetier, C., Josien, R., Menoret, S., Kourilsky, P., Soulillou, J.P., Cuturi, M.C., 1996. Donor-specific blood transfusion-induced tolerance in adult rats with a dominant TCR-Vbeta rearrangement in heart allografts. J. Immunol. 157, 1250–1260.

Effros, R.B., Cai, Z., Linton, P.J., 2003. CD8 T cells and aging. Crit. Rev. Immunol. 23, 45–64.

Gagne, K., Brouard, S., Giral, M., Sebille, F., Moreau, A., Guillet, M., Bignon, J.D., Imbert, B.M., Cuturi, M.C., Soulillou, J.P., 2000. Highly altered V beta repertoire of T cells infiltrating long-term rejected kidney allografts. J. Immunol. 164, 1553–1563.

Gorochov, G., Neumann, A.U., Kereveur, A., Parizot, C., Li, T., Katlama, C., Karmochkine, M., Raguin, G., Autran, B., Debre, P., 1998. Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy. Nat. Med. 4, 215–221.

Gran, B., Gestri, D., Sottini, A., Quiros Roldan, E., Bettinardi, A., Signorini, S., Primi, D., Ballerini, C., Taiuti, R., Amaducci, L., Massacesi, L., 1998. Detection of skewed T-cell receptor V-beta gene usage in the peripheral blood of patients with multiple sclerosis. J. Neuroimmunol. 85, 22–32.

Han, M., Harrison, L., Kehn, P., Stevenson, K., Currier, J., Robinson, M.A., 1999. Invariant or highly conserved TCR alpha are expressed on double-negative (CD3+CD4−CD8−) and CD8+ T cells. J. Immunol. 163, 301–311.

Hong, J., Zang, Y.C., Tejada-Simon, M.V., Kozovska, M., Li, S., Singh, R. A., Yang, D., Rivera, V.M., Killian, J.K., Zhang, J.Z., 1999. A common TCR V-D-J sequence in V beta 13.1 T cells recognizing an immunodominant peptide of myelin basic protein in multiple sclerosis. J. Immunol. 163, 3530–3538.

Kotzin, B.L., Karuturi, S., Chou, Y.K., Lafferty, J., Forrester, J.M., Better, M., Nedwin, G.E., Offner, H., Vandenbark, A.A., 1991. Preferential T-cell receptor beta-chain variable gene use in myelin basic protein-reactive T-cell clones from patients with multiple sclerosis. Proc. Natl. Acad. Sci. U. S. A. 88, 9161–9165.

Laplaud, D.A., Ruiz, C., Wiertlewski, S., Brouard, S., Berthelot, L., Guillet, M., Melchior, B., Degauque, N., Edan, G., Brachet, P., Damier, P., Soulillou, J.P., 2004. Blood T-cell receptor beta chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution. Brain 127, 981–995.

Lozeron, P., Chabas, D., Duprey, B., Lyon-Caen, O., Liblau, R., 1998. T cell receptor V beta 5 and V beta 17 clonal diversity in cerebrospinal fluid and peripheral blood lymphocytes of multiple sclerosis patients. Mult. Scler. 4, 154–161.

Maini, M.K., Gudgeon, N., Wedderburn, L.R., Rickinson, A.B., Beverley, P. C., 2000. Clonal expansions in acute EBV infection are detectable in the CD8 and not the CD4 subset and persist with a variable CD45 phenotype. J. Immunol. 165, 5729–5737.

Matsumoto, Y., Yoon, W.K., Jee, Y., Fujihara, K., Misu, T., Sato, S., Nakashima, I., Itoyama, Y., 2003. Complementarity-determining region 3 spectratyping analysis of the TCR repertoire in multiple sclerosis. J. Immunol. 170, 4846–4853.

Melchior, B., Nerriere-Daguin, V., Degauque, N., Brouard, S., Guillet, M., Soulillou, J.P., Brachet, P., 2005. Compartmentalization of TCR repertoire alteration during rejection of an intrabrain xenograft. Exp. Neurol. 192, 373–383.

Miller, D.H., Khan, O.A., Sheremata, W.A., Blumhardt, L.D., Rice, G.P., Libonati, M.A., Willmer-Hulme, A.J., Dalton, C.M., Miszkiel, K.A., O'Connor, P.W., 2003. A controlled trial of natalizumab for relapsing multiple sclerosis. N. Engl. J. Med. 348, 15–23.

Muraro, P.A., Bonanni, L., Mazzanti, B., Pantalone, A., Traggiai, E., Massacesi, L., Vergelli, M., Gambi, D., 2002. Short-term dynamics of circulating T cell receptor V beta repertoire in relapsing–remitting MS. J. Neuroimmunol. 127, 149–159.

Musette, P., Benveniste, O., Lim, A., Bequet, D., Kourilsky, P., Dormont, D., Gachelin, G., 1996. The pattern of production of cytokine mRNAs is markedly altered at the onset of multiple sclerosis. Res. Immunol. 147, 435–441.

Noseworthy, J.H., Lucchinetti, C., Rodriguez, M., Weinshenker, B.G., 2000. Multiple sclerosis. N. Engl. J. Med. 343, 938–952.

Pannetier, C., Even, J., Kourilsky, P., 1995. T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol. Today 16, 176–181.

Polman, C.H., O'Connor, P.W., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D.H., Phillips, J.T., Lublin, F.D., Giovannoni, G., Wajgt, A., Toal, M., Lynn, F., Panzara, M.A., Sandrock, A.W., 2006. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. N. Engl. J. Med. 354, 899–910.

Prat, A., Antel, J., 2005. Pathogenesis of multiple sclerosis. Curr. Opin. Neurol. 18, 225–230.

Prevost-Blondel, A., Lengagne, R., Letourneur, F., Pannetier, C., Gomard, E., Guillet, J.G., 1997. In vivo longitudinal analysis of a dominant TCR repertoire selected in human response to influenza virus. Virology 233, 93–104.

Wucherpfennig, K.W., Ota, K., Endo, N., Seidman, J.G., Rosenzweig, A., Weiner, H.L., Hafler, D.A., 1990. Shared human T cell receptor V beta usage to immunodominant regions of myelin basic protein. Science 248, 1016–1019.

Zamvil, S., Nelson, P., Trotter, J., Mitchell, D., Knobler, R., Fritz, R., Steinman, L., 1985. T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. Nature 317, 355–358.

Etude II : Effets de l'acétate de glatiramère sur le répertoire T de patients atteints de sclérose en plaques

Article en préparation.

No effect of a short Glatiramer acetate therapy on T cell repertoire in multiple sclerosis patients.

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

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

[2] : Centre Hospitalier Universitaire de Nantes, Service de neurologie et Centre d'Investigation Clinique, Nantes, F-44093 France

Corresponding author: Jean-Paul Soulillou

Jean-Paul.Soulillou@univ-nantes.fr, fax : +33 2 40 08 74 11

Abbreviations:

APC : Antigen presenting cell

MS: Multiple Sclerosis

GA: Glatiramer Acetate

RR : Relapsing-Remitting

PCR : Polymerase Chain Reaction

TCR: T-Cell Receptor

CDR3-LD: Complementary Determining Region 3- Length Distribution

Abstract

Glatiramer acetate (GA) is a random copolymer used as an approved drug in relapsing-remitting multiple sclerosis (RR-MS). Its mechanism of action is poorly understood and several hypotheses have been made, a majority lying on in-vitro studies. GA can provide a huge number of different epitopes with possible similarity of sequences with auto-antigens following processing by APC, able to stimulate a large proportion of T cells. Because in a previous study we showed that MS patients present more alterations of the Vβ T-cell receptor (TCR) usage than normal individuals, we raised the hypothesis that GA could affect the potentially auto-reactive T cells. For the first time we analysed by Quantitative-PCR and electrophoresis, the CDR3 length distribution (LD) and amount of Vβ TCR and various Th1 and Th2 cytokines in 10 RR-MS patients before and after treatment with GA without any ex vivo stimulation. After 24 months of GA treatment, the status of responder and non-responder patients was determined using the more recent published criteria. There was no significant modification in cytokine production, in Vβ TCR mRNA accumulation and in CDR3-LD in the patients after 3 or 24 months with GA treatment compared to before treatment. Moreover, there was no difference in term of CDR3-LD in the group of GA responder patients (n=6) compared to the group of non-responder patients (n=4). Focusing our study on responder patients, we performed TCR repertoire analysis in the CD4+ and CD8+ T cell compartment. Alterations of CDR3-LD were predominantly found in CD8+ T cell compartment, but there was also no signification variation after GA treatment. This work suggests that treatment with GA does not induce significant variations T cell repertoire 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 central nervous system white matter (Noseworthy, Lucchinetti et al. 2000). One of the approved drugs used as disease modifying therapy for relapsing-remitting MS is the copolymer glatiramer acetate (GA, Copaxone®) randomly composed of a stable proportion of the four following amino-acids: glutamic acid, leucine, alanine and tyrosine, with various molecular weights from 40 to 100 residues (Teitelbaum, Meshorer et al. 1971).

The mechanisms of action of GA are not still completely understood and different hypotheses have been formulated (Neuhaus, Farina et al. 2001; Farina, Weber et al. 2005). First, as the presence of GA reactive CD4+ T cells with a Th2 type has been shown in the blood of MS patients, a shift of cytokine production was suggested with a bystander suppression induced by the GA reactive CD4+ T cells (Aharoni, Teitelbaum et al. 2000; Duda, Schmied et al. 2000; Wiesemann, Klatt et al. 2001). This modification in the cytokine profile could also be due to a complex action of GA on antigen presenting cells (APCs), as recently suggested (Vieira, Heystek et al. 2003; Kim, Ifergan et al. 2004). Furthermore, GA could also exert as an altered-ligand peptide and induce the apoptosis or anergy of myelin-peptide reactive CD4+ T cells, as suggested from the works from Aharoni et al. (Aharoni, Teitelbaum et al. 1999; Fridkis-Hareli, Stern et al. 2001). In parallel of its immune properties, another potential action of GA was by the way of the production of the neurotrophic factor BDNF from GA selected T cells suggesting a possible role in remyelination processes (Ziemssen, Kumpfel et al. 2002; Chen, Valenzuela et al. 2003). GA seems to act also on CD8+ T cells, inducing suppressive cells and clonal expansion (Biegler, Yan et al. 2006; Tennakoon, Mehta et al. 2006). Nevertheless, the majority of these studies focusing on the mechanisms of action of the drug have been

conducting in vitro with different procedures of cell culture and direct ex-vivo analyses are lacking to strengthen the description of these mechanisms.

In previous studies, using the TcLandscape® methodology (Guillet, Brouard et al. 2002), we showed that the T cell repertoire from MS patients was significantly more altered than from healthy individuals whatever was the disease stage of the patients (from clinically isolated syndrome to clinically definite MS and worsening MS) (Laplaud, Ruiz et al. 2004). Moreover, new altered Vβ families in blood of MS patients correlate with new lesions in the brain of MS patients (Laplaud, Berthelot et al. 2006). As GA is a random copolymer of variable sequences and lengths, its processing by APCs should generate a huge number of peptides with different sequences. Then, it could be hypothesized that it would modify the peripheral T cell repertoire by inducing T cell proliferation from widely various T cell clones. Moreover, GA stimulation seems to induce a focused oligoclonal CD8+ T cell repertoire (Biegler, Yan et al. 2006).

In this regard, the study of ex-vivo peripheral T cell repertoire from GA treated patients compared to before GA treatment, should allow to recognize the in-vivo GA driven T cell clones and then further characterize them. Because not all the patients have a clinical response to treatment, we selected after a 24 months follow-up the responding and non responding patients to GA according to Rio et al criteria (Rio, Nos et al. 2006) and studied the peripheral T cell repertoire at the CDR3 level in both groups.

Material and methods

Patients and healthy subjects

Ten patients with a relapsing-remitting multiple sclerosis (RR-MS) as defined by the McDonald's criteria (McDonald, Compston et al. 2001) and 7 healthy controls (HC) age matched (age mean =35.1±8.8 years) were included in the study and followed during two years. Blood samples were collected at the onset of the treatment, after 3 and 24 months of Glatiramer Acetate (GA) treatment. Informed consent from patients and controls was obtained for participation in this study according to our local ethics committee. The clinical characteristics of the patients were summarized in Table 1. The patients were considered as responding or non-responding to the therapy according to Rio et al (Rio, Nos et al. 2006). Briefly, after a follow-up of two years, patients with no relapse and no exacerbation of the disease (no new lesions at the MRI) were considered responder to GA treatment.

Isolation of cell populations

Peripheral blood mononuclear cells (PBMC) were extracted from blood using Ficoll (Eurobio, Les Ulis, France). A part of PBMC ($10x10^6$) were directly frozen in trizol® at -80°C and the rest was frozen in autologous plasma, 20% DMSO. Cells were thawed for cell sorting and phenotype analysis. Labeled PBMC with antibodies against CD3 coupled with PE-Cy7 and anti-CD8 coupled with APC (all antibodies purchased with BD Biosciences) were used for CD4+ and CD8+ T cell isolation in a speed cell sorter (ARIA, BD Biosciences).

T cell repertoire analysis

PBMC, CD4+ and CD8+ T cells were frozen in trizol® reagent for RNA extraction. RNA from PBMC was extracted according to the manufacturer's instructions. 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 Biosystem). The PCR fragments migration in acrylamide gel gave the distribution of the different CDR3 lengths and analysis were performed using Immunoscope® software (Pannetier, Even et al. 1995; Douillard, Pannetier et al. 1996; Brouard, Vanhove et al. 1999). 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 (Gorochov, Neumann et al. 1998). 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 studied and the average control distribution, calculated from 14 healthy individuals. Data from quantitative PCR and percentages of CDR3-LD alterations were represented in a global three-dimensional representation named TcLandscape®(Sebille, Gagne et al. 2001). 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

In order to assess Th2 biaise induced by glatiramer acetate and production of neurotrophic factors as described previously (Ziemssen, Kumpfel et al. 2002; Chen, Valenzuela et al. 2003), we performed quantitative PCR to measure the level of those molecules mRNA. Briefly, 11µL of cDNA from PBMC, CD4+ or CD8+ T cells were added to 12.5µL Master Mix (Applied Biosystem) and 1.5µL of each primer mix (Applied Biosystem). The tested transcripts were for Th1 cytokines: IFNγ, TNFα, IL2, IL6, IL8, IL12; for Th2 cytokines: IL4, IL5, IL10, IL13, for neurtrophic factors TGFβ; BNDF, NGF, NT3, GNDF. Hypoxanthine-

guanine PhosphoRibosylTransferase (HPRT) was used as an endogenous control gene to normalize the varying starting amounts of RNA. Relative expression between a given sample and a reference sample (RNA from human thymus) was calculated with the $2^{-\Delta\Delta Ct}$ method where the reference represents one fold expression.

Statistical analysis

A Mann-Whitney test (two-tailed) was used to compare MS patient and healthy control group values: percentages of CDR3-LD alterations and quantitative PCR values. For time variation studies in each group, a non parametric paired test (Wilcoxon test) was performed and to study the variation of the two groups during time a two-way Anova was used. For all tests, $p<0.05$ was considered as significant. Analyses were performed using Prism 4.0 software (San Diego, CA, USA).

Results

T cell repertoire analysis after three months of GA treatment: Vβ mRNA accumulation

To determine the specific modifications induced by GA treatment on the level of Vβ family mRNA accumulation, we studied the Vβ/HPRT ratio for the 26 different Vβ families obtained from quantitative PCR. There is no significant modification (increase or decrease) of Vβ mRNA accumulation after GA treatment for the ten MS patients independently for the responding or non responding patients. The global mean of Vβ/HPRT ratio appeared stable (ratio=1.04±0.11 before treatment and ratio=1.11±0.15 after three months with GA treatment). Analysing each Vβ/HPRT ratio for each Vβ family, we observed individual variability but no significant variations (fig 1). This inter-individual variability was also found in the group of healthy controls followed during the same time period (data not shown). So, at the quantitative level, there was no significant modification of Vβ family mRNA accumulation after GA treatment.

Analysis of CDR3 length distribution

After examination of the quantitative component of T cell repertoire, we studied the CDR3 length distribution (CDR3-LD) of the ten MS patients before and after GA treatment. The global percentages of CDR3-LD alterations did not change with GA treatment (16.18±2.85% before GA treatment, 16.79±2.74% after three months of GA treatment and 16.18±5.94% after 24 months of GA treatments) for the ten patients neither for responding patients to GA treatment (15.13±1.95% before, 16.30±2.76% after 3 months with GA treatment and 16.78±6.16% after 24 months with GA treatment) or non responding patients (17.76±3.53% before, 17.52±2.94% after 3 months with treatment and 15.77±6.03% after 24 months with GA treatment) (fig 2). As for the quantitative part, the CDR3-LD showed variability, due to inter-individual diversity, found also in healthy control group.

We then dressed the Tclanscapes® in order to analyse both mRNA accumulation and CDR3-LD. Figure 3 shows a representative example for one MS patient of Tclandscapes® obtained before, 3 months and 24 months after treatment with GA. No peak (combined high mRNA accumulation and CDR3-LD alteration) appeared with GA treatment. So, GA did not induced clonal expansion in the blood of MS patients, neither decreased the CDR3-LD alterations of the MS patients.

T cell repertoire analysis in CD4+ and CD8+ compartment

We previously described that CDR3-LD alterations were predominantly found in the CD8+ T cell compartment for MS patients (Laplaud, Ruiz et al. 2004), so the T cell repertoire was also studied for 5 responding MS patient CD4+ and CD8+ T cell compartment before and after GA treatment. CD8+ T cell compartment appeared significantly more altered in term of CDR3-LD (19.04±4.04%) than CD4+ T cell compartment (15.2±1.66%) before GA treatment ($p<0.05$). Three months of GA therapy did not induce massive modifications of percentages of alterations (22.1±2.51% for CD8+ compartment and 16.1±1.91%. for CD4+ T cells after 3 months with GA treatment) (fig 4). As for total T cells, there is no significant modifications of T repertoire in CD4+ or CD8+ T cell compartment in the blood of MS patients after GA treatment.

Cytokines and neurotrophic factors expression 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 of mRNA amount for all the cytokines (Th1: IL2, IFNγ, TNFα, IL6, IL8 and IL12, Th2: IL4, IL5, IL10, IL13, TGFβ) and neurotrophic factors (BDNF, NT3, NGF, GDNF) after three months with GA treatment. Figure 5 shows 4 representative examples of mRNA amount of

Th1 and Th2 cytokines. mRNA amount of neurotrophic factors were not detectable in PBMC of all MS patients before and after GA treatment. There is also no difference between responding and non responding MS patients.

Discussion

MS patients compared to healthy individuals present more CDR3 length distribution alterations in their blood T cell repertoire (Laplaud, Ruiz et al. 2004). It has been shown that GA treatment acts on T cells and induce a Th2 shift in cytokines production (Farina, Weber et al. 2005). We then assessed the effects of GA treatment on T cell repertoire in blood of MS patients. Despite the promising data obtained after in vitro GA stimulation of T cells (Biegler, Yan et al. 2006), a short therapy during three months as a long therapy (24 months) with GA does not induce spectacular TCR biases in MS patients. There is effectively no difference due to the treatment for MS patients compared to normal evolution of T cell repertoire in healthy individuals. These data showed that GA treatment has no effect on T cell repertoire. We were also unable to detect difference between responding and non responding MS patients.

Analysis of mRNA amount of several cytokines and neurotrophic factors before and after three months of GA treatment did not show differences. Other studies found that IL5, IL13, IL4 and TGFβ were increased in the blood of MS patients treated with GA compared to non treated MS patients (Miller, Shapiro et al. 1998; Wiesemann, Klatt et al. 2001) and these studies examined long term treated patients (more than fifteen months with GA therapy). So, we will analyse blood samples from the same MS patients after 24 months of GA treatment in order to assess the effects of long term GA treatment on cytokine production.

Legends

Table 1: Clinical data of MS patients

GA= Glatiramer acetate. Patient status was evaluated according to the Rio criteria (Rio, Nos et al. 2006) after 24 months following. NR= non responding patients and R= responding patients. Briefly, MS patients were considered NR if they presented during the 24 months of follow-up relapse or increased number of lesions at MRI.

Figure 1: 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 were performed using Cβ and Vβ primers. Here are represented two examples of Vβ/HPRT ratio for the ten MS patients and for the responding and non responding MS patients to GA for two representative Vβ families.

Figure 2: Percentages of CDR3-LD alterations

To analyse CDR3-LD, the percentage of CDR3-LD alterations were calculated for blood samples obtained before and after three months of GA treatment.

Figure 3: Two Tclanscapes® of patient MS-2 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 4: Percentages of CDR3-LD alterations in CD4+ and CD8+ T cells

On this figure are shown the percentage of CDR3-LD alterations in CD4+ and CD8+ T cells obtained after cell sorting from frozen PBMC of MS patients.

Figure 5: mRNA amount of cytokines before and after three month of GA treatment

On these graphics, the ΔΔCt calculated from quantitative PCR raw data are represented for each cytokines.

References

1. Noseworthy, J.H., et al., *Multiple sclerosis.* N Engl J Med, 2000. **343**(13): p. 938-52.
2. Teitelbaum, D., et al., *Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide.* Eur J Immunol, 1971. **1**(4): p. 242-8.
3. Farina, C., et al., *Glatiramer acetate in multiple sclerosis: update on potential mechanisms of action.* Lancet Neurol, 2005. **4**(9): p. 567-75.
4. Neuhaus, O., et al., *Mechanisms of action of glatiramer acetate in multiple sclerosis.* Neurology, 2001. **56**(6): p. 702-8.
5. Aharoni, R., et al., *Specific Th2 cells accumulate in the central nervous system of mice protected against experimental autoimmune encephalomyelitis by copolymer 1.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11472-7.
6. Duda, P.W., et al., *Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis.* J Clin Invest, 2000. **105**(7): p. 967-76.
7. Wiesemann, E., et al., *Glatiramer acetate (GA) induces IL-13/IL-5 secretion in naive T cells.* J Neuroimmunol, 2001. **119**(1): p. 137-44.
8. Vieira, P.L., et al., *Glatiramer acetate (copolymer-1, copaxone) promotes Th2 cell development and increased IL-10 production through modulation of dendritic cells.* J Immunol, 2003. **170**(9): p. 4483-8.
9. Kim, H.J., et al., *Type 2 monocyte and microglia differentiation mediated by glatiramer acetate therapy in patients with multiple sclerosis.* J Immunol, 2004. **172**(11): p. 7144-53.
10. Aharoni, R., et al., *Copolymer 1 acts against the immunodominant epitope 82-100 of myelin basic protein by T cell receptor antagonism in addition to major histocompatibility complex blocking.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 634-9.
11. Fridkis-Hareli, M., et al., *Synthetic peptides that inhibit binding of the myelin basic protein 85-99 epitope to multiple sclerosis-associated HLA-DR2 molecules and MBP-specific T-cell responses.* Hum Immunol, 2001. **62**(8): p. 753-63.
12. Ziemssen, T., et al., *Glatiramer acetate-specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy. Brain-derived neurotrophic factor.* Brain, 2002. **125**(Pt 11): p. 2381-91.
13. Chen, M., R.M. Valenzuela, and S. Dhib-Jalbut, *Glatiramer acetate-reactive T cells produce brain-derived neurotrophic factor.* J Neurol Sci, 2003. **215**(1-2): p. 37-44.
14. Biegler, B.W., et al., *Glatiramer acetate (GA) therapy induces a focused, oligoclonal CD8+ T-cell repertoire in multiple sclerosis.* J Neuroimmunol, 2006. **180**(1-2): p. 159-71.
15. Tennakoon, D.K., et al., *Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis.* J Immunol, 2006. **176**(11): p. 7119-29.
16. Guillet, M., et al., *Different qualitative and quantitative regulation of V beta TCR transcripts during early acute allograft rejection and tolerance induction.* J Immunol, 2002. **168**(10): p. 5088-95.
17. Laplaud, D.A., et al., *Blood T-cell receptor beta chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution.* Brain, 2004. **127**(Pt 5): p. 981-95.
18. Laplaud, D.A., et al., *Serial blood T cell repertoire alterations in multiple sclerosis patients; correlation with clinical and MRI parameters.* J Neuroimmunol, 2006. **177**(1-2): p. 151-60.
19. Rio, J., et al., *Defining the response to interferon-beta in relapsing-remitting multiple sclerosis patients.* Ann Neurol, 2006. **59**(2): p. 344-52.

20. McDonald, W.I., et al., *Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis.* Ann Neurol, 2001. **50**(1): p. 121-7.
21. Brouard, S., et al., *T cell repertoire alterations of vascularized xenografts.* J Immunol, 1999. **162**(6): p. 3367-77.
22. Douillard, P., et al., *Donor-specific blood transfusion-induced tolerance in adult rats with a dominant TCR-Vbeta rearrangement in heart allografts.* J Immunol, 1996. **157**(3): p. 1250-60.
23. Pannetier, C., J. Even, and P. Kourilsky, *T-cell repertoire diversity and clonal expansions in normal and clinical samples.* Immunol Today, 1995. **16**(4): p. 176-81.
24. Gorochov, G., et al., *Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy.* Nat Med, 1998. **4**(2): p. 215-21.
25. Sebille, F., et al., *Direct recognition of foreign MHC determinants by naive T cells mobilizes specific Vbeta families without skewing of the complementarity-determining region 3 length distribution.* J Immunol, 2001. **167**(6): p. 3082-8.
26. Miller, A., et al., *Treatment of multiple sclerosis with copolymer-1 (Copaxone): implicating mechanisms of Th1 to Th2/Th3 immune-deviation.* J Neuroimmunol, 1998. **92**(1-2): p. 113-21.

Figures

Table 1

Patients	Age	Sex	EDSS Score Before GA treatment	EDSS Score After 24 months of GA treatment	Number of relapse during 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	1 (during the first month of study)	R

Figure1

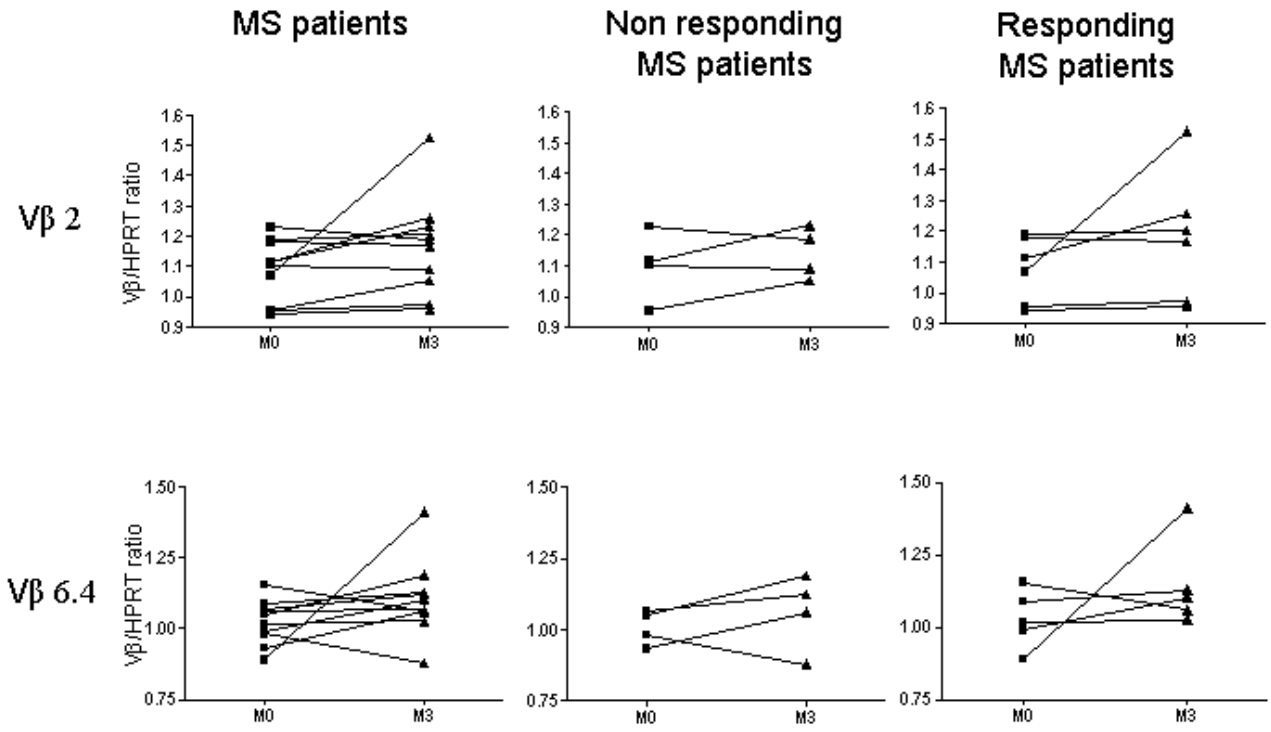

MS patients
Non responding MS patients
Responding MS patients
Vβ 2
Vβ 6.4
Vβ/HPRT ratio
M0
M3

Figure 2

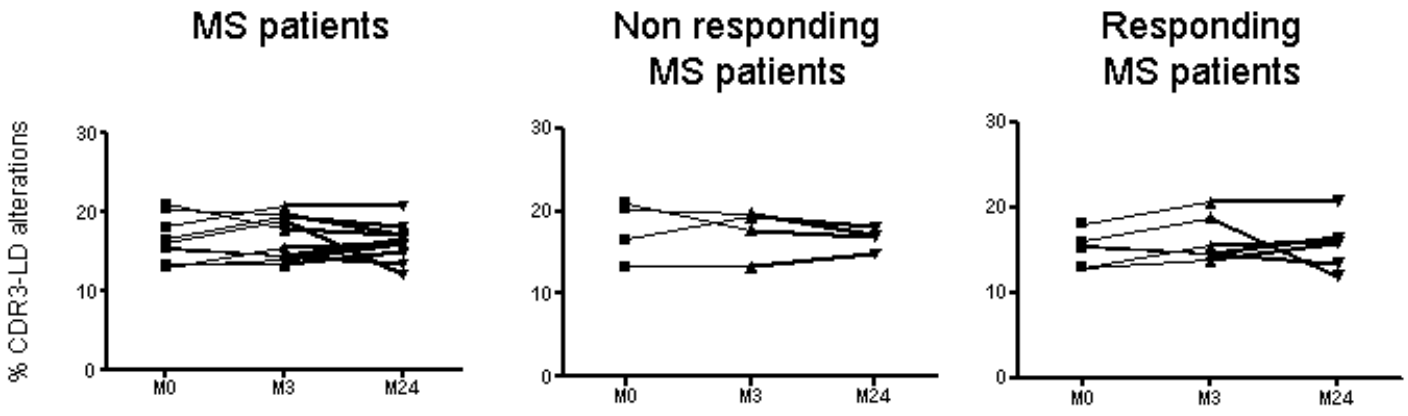
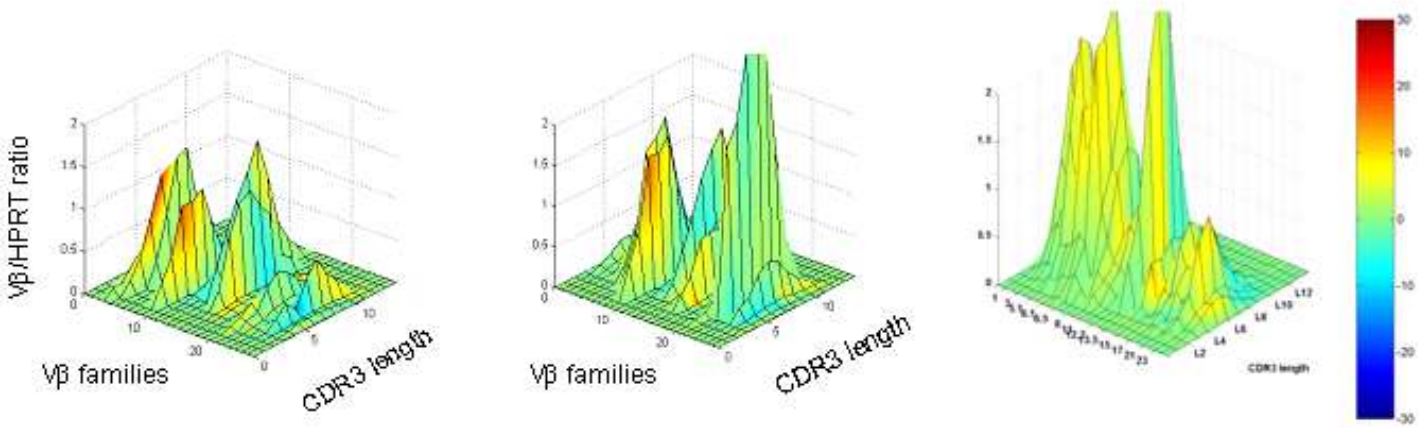

MS patients
Non responding MS patients
Responding MS patients
% CDR3-LD alterations
30
20
10
0
M0
M3
M24

Figure 3



Before GA treatment

After 3 months with GA treatment

After 24 months with GA treatment

Figure 4

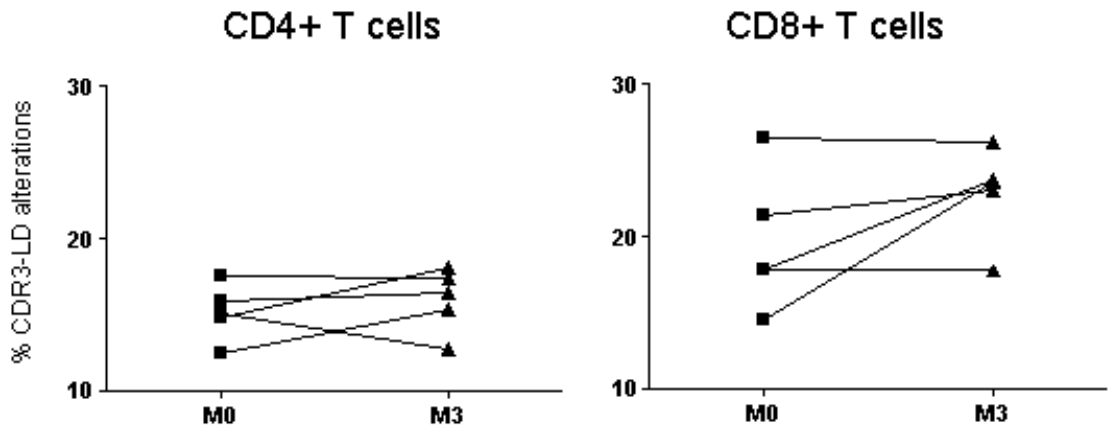
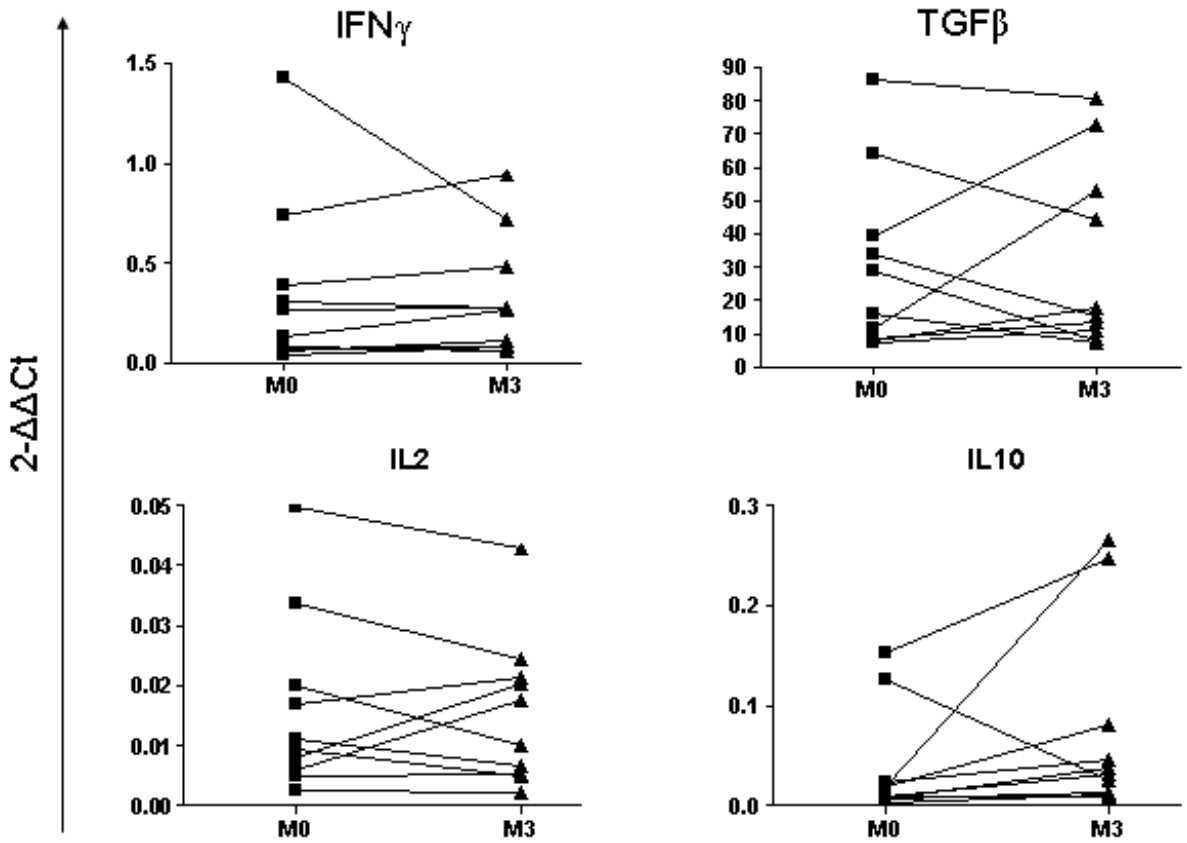

CD4+ T cells
CD8+ T cells
% CDR3-LD alterations
30
20
10
M0
M3
30
20
10
M0
M3

Figure 5


2-ΔΔCt
IFNγ
1.5
1.0
0.5
0.0
M0
M3
TGFβ
90
80
70
60
50
40
30
20
10
0
M0
M3
IL2
0.05
0.04
0.03
0.02
0.01
0.00
M0
M3
IL10
0.3
0.2
0.1
0.0
M0
M3

Etude III: Etude du répertoire T de lymphocytes infiltrant le système nerveux central chez une patiente décédée atteinte de sclérose en plaques

Objectif du travail

En collaboration avec l'équipe de Silva Markovic-Plese (Chapell Hill, Etats-Unis), ma partie de l'étude s'est attachée à analyser le répertoire T de cellules isolées de quatre lésions différentes et de la substance blanche d'apparence normale d'une patiente atteinte de SEP.

Résumé de l'étude menée à Chapel Hill

Title: Characterization Of Oligoclonally Expanded T-cells Derived From Multiple Sclerosis Lesions

M. Montes[a], T. Kondo[b], W. Bruck[c], V. Jewells[d], D. Armao[d], J. Smrtka[a], C. Pinilla[e], S. Markovic-Plese[a],

[a]Department of Neurology, University of North Carolina, Chapel Hill, United States, [b]Department of Clinical Research, Nagasaki Medical Center of Neurology, Nagasaki, Japan, [c]Neuropathology Institute, Georg-August University, Gottingen, Germany, [d]Department of Radiology, University of North Carolina, Chapel Hill, United States, [e]Torrey Pines Institute for Molecular Studies, San Diego, United States

Objective: to characterize T-cell infiltrates and *in vivo* expanded clonotypes derived from multiple sclerosis (MS) lesions.
Background: Oligoclonally expanded T-lymphocytes perpetuate inflammatory response against CNS myelin antigens. This study addresses their representation in the MS lesions at various stages of development, and their antigen specificity.
Methods: Brain and spinal cord tissue was obtained six hours post mortem from patient with aggressive relapsing remitting (RR) MS. Lesions were characterized based on the longitudinal CNS MRI, the last one 4 day prior to death, and histopathological and immunohistochemistry studies. Viable cells from six lesions and normal appearing white matter (NAWM) were expanded *in vitro*. Single-strand conformational polymorphism (SSCP) pattern analysis identified limited number of TCRVB chains expressed in the T-cell infiltrates from the lesions and NAWM, correlating to the *in vivo* expanded clonotypes.
Results: Antigen specificity of MS-lesion derived T-cells was tested against a panel of immunodominant myelin-derived peptides. The highest proliferative response was detected against PLP 190-209 in acute pontine, chronic periventricular and thoracic spine lesions. Cells derived from acute lesion exhibited proliferative response against six myelin peptides, four of which were recognized in chronic active thoracic, and three in chronic periventricular lesion. SSCP analysis revealed distinct patterns of TCR VB family chains in each MS lesion and NAWM. However, TCRVB4, VB5.1, VB8, and VB13.1 were present in all lesions. Antigen specificity of the clones corresponding to the *in vivo* expanded clonotypes is presently characterized using synthetic combinatorial peptide libraries.
Relevance: This is the first study to characterize post-mortem derived T-cell infiltrates from MS lesions at various stages of development.

Matériel et méthode

Les cellules infiltrant quatre lésions différentes (RL : lésion aigüe dans le mésencéphale, OLD : lésion chronique au niveau du nerf optique, CER : lésion dans la région cervicale de la moelle épinière et THO : lésion dans la région thoracique de la moelle épinière) et la substance blanche d'apparence normale (MG, au niveau du lobe frontal) ont été extraites 6h après le décès d'une jeune patiente atteinte de SEP. Ces cellules ont ensuite été amplifiées *in vitro* par une stimulation à la PHA pendant 45 jours. Les ARN ont ensuite été extraits de ces cellules infiltrantes grâce au trizol®. Pour chaque échantillon, 2µg d'ARN ont servi à la rétro-transcription. Les régions CDR3 des ADN complémentaires ont été amplifiées par PCR quantitative grâce aux amorces Cβ et Vβ (décrites précédemment). Les fragments amplifiés ont migrés ensuite sur gel d'acrylamide afin de dresser les profils des distributions des différentes longueurs de CDR3. Les Tclanscapes® ont enfin été dressés.

Résultats

L'analyse du répertoire T des cellules infiltrant des lésions actives ou chroniques et de la substance blanche d'apparence normale a été réalisée par l'étude des répartitions des différentes longueurs de CDR3 de la chaîne β et de l'accumulation des ARNm des différentes familles Vβ. Les Tclandscapes® dressés à partir des cinq échantillons montrent de nombreuses et fortes altérations du répertoire Vβ dans les quatre lésions différentes mais également au niveau de la substance blanche d'apparence normale (fig 16). Toutes les familles Vβ des cinq échantillons présentent des profils extrêmement altérés, tous étant oligoclonaux. La proportion de familles Vβ présentant un profil monoclonal est également élevée : 30,4% pour RL, 20,0% pour OLD, 47,3% pour CER, 20,8% pour THO et 43,4% pour MG. Les pourcentages globaux d'altérations (moyenne des pourcentages d'altérations de toutes les familles Vβ) atteignent pour chaque échantillons des valeurs très hautes : 60,54% pour l'échantillon RL, 58,25% pour OLD, 67,33% pour MG, 64,66% pour CER et 51,75% pour THO. De plus, certaines altérations apparaissent sous la forme de pics sur le Tclanscape, indiquant une grande accumulation d'ARN messager de ces familles Vβ et donc très probablement des expansions clonales des lymphocytes T portant ces TCR particuliers.

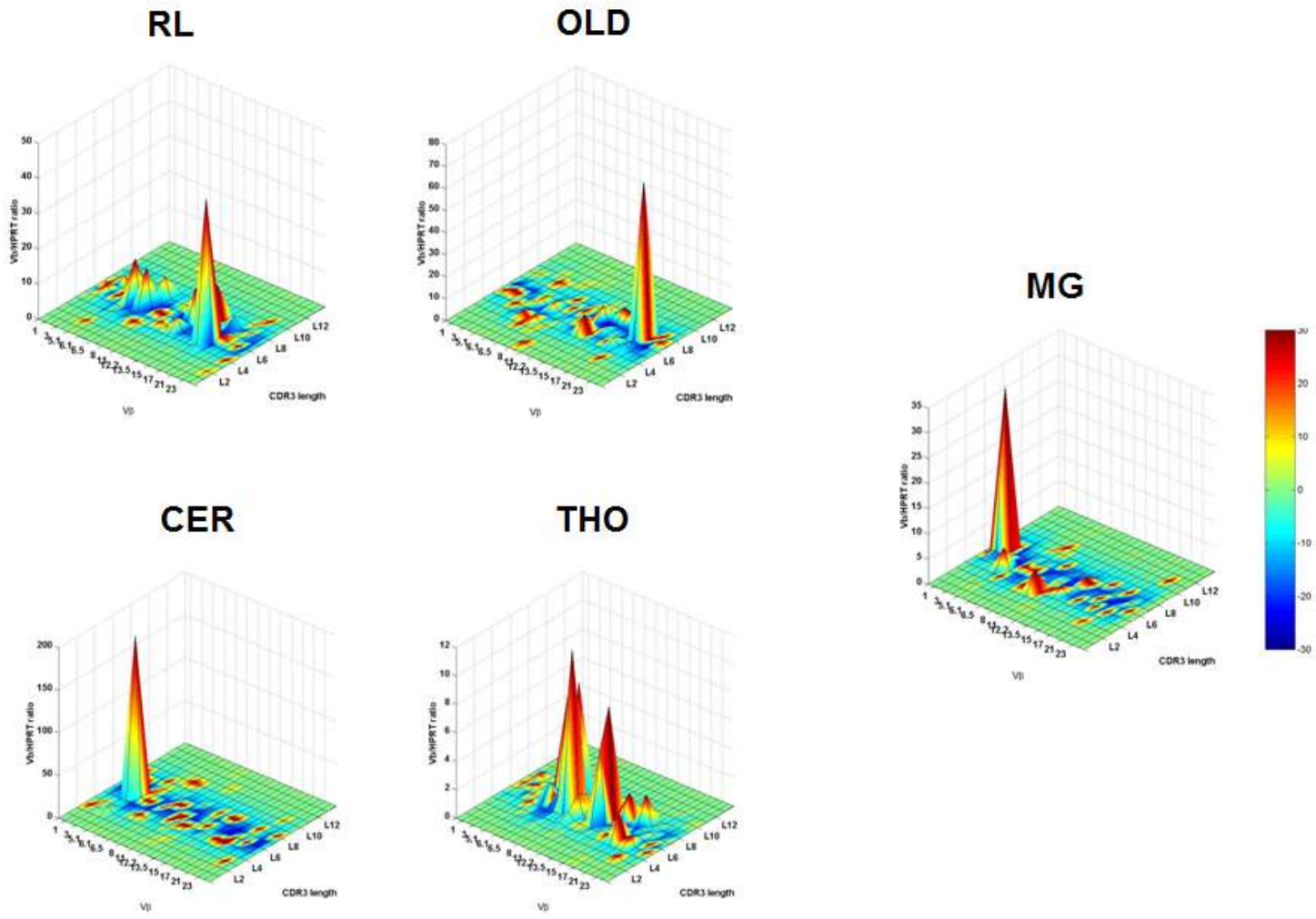


Figure 16: Tclandscapes® des cellules infiltrant le SNC d'une patiente atteinte de SEP
RL : lésion aigüe dans le mésencéphale, OLD : lésion chronique au niveau du nerf optique, CER : lésion dans la région cervicale de la moelle épinière, THO : lésion dans la région thoracique de la moelle épinière et MG : substance blanche d'apparence normale au niveau du lobe frontal

Ces résultats semblent indiquer que des infiltrats de lymphocytes T sont présents au niveau des lésions actives mais également toujours présents au niveau des lésions chroniques. De façon surprenante la substance blanche d'apparence normale contient également des infiltrats de lymphocytes T qui ne présentent apparemment pas de différences avec les cellules infiltrant les lésions.

Discussion

L'étude du répertoire T de cellules infiltrant des régions différentes du système nerveux central chez une patiente décédée atteinte de SEP a montré un fort biais d'utilisation du TCR par ces cellules. En effet, les profils de distribution des différentes longueurs de CDR3 pour toutes les familles Vβ apparaissent extrêmement altérés. Ce phénomène est visible au niveau des lésions actives mais aussi des lésiosn chroniques et également au niveau de la substance blanche d'apparence normale. Ces résultats semblent indiquer que l'ensemble du SNC est en état de forte inflammation sans localisation focale au niveau des lésions actives. Une étude du transcriptôme avait également abouti à cette conclusion (Lindberg, De Groot et al. 2004).

Cependant, la stimulation et l'amplification des cellules infiltrantes *in vitro* par la PHA pourrait également avoir modifié le répertoire T. D'autres études ont vérifié par cytométrie en flux l'expression des différentes familles Vβ à la surface des cellules et semblent indiquer que la stimulation par la PHA n'a pas d'effet sur l'utilisation des différents Vβ (Arenz, Herzog-Hauff et al. 1997; Sospedra, Zhao et al. 2005). Afin de nous assurer que cette stimulation ne modifie pas la répartition des différentes longueurs de CDR3, nous analysons actuellement le répertoire T avant et après 45 jours de stimulation à la PHA d'échantillons provenant du sang de 5 patients atteints de SEP et également de cellules infiltrant le SNC d'un patient opéré pour cause d'épilepsie et de cellules provenant du sang de ce même patient.

Partie II : Etude de la spécificité des lymphocytes T CD8+

Etude IV : Epitopes de la myéline restreints pour les molécules de CMH de Classe I provoquant des réponses des lymphocytes T CD8+ chez des patients atteints de sclérose en plaques

Article en préparation.

Class-I restricted myelin epitopes eliciting blood CD8$^+$ T cell responses in multiple sclerosis patients

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

[1]: INSERM, U643, et ITERT (Institut de Transplantation Et de la Recherche en Transplantation), Nantes, F-44093 France

[2] : CHU de Nantes, Service de neurologie et Centre d'Investigation Clinique, Nantes, F-44093 France

[3]: Institut Cochin, Paris, F-75000 France

Corresponding author: Jean-Paul Soulillou

Jean-Paul.Soulillou@univ-nantes.fr, fax : +33 2 40 08 74 11

Abbreviations used:

MHC: Major Histocompatibility Complex

MS: Multiple Sclerosis

MBP: Myelin Basic Protein

MOG: Myelin Oligodendrocyte Glycoprotein

PLP: Proteolipid Protein

TCR: T Cell Receptor

Abstract

Multiple Sclerosis (MS) is associated with T cells reacting against myelin proteins. $CD8^+$ T cells are a dominant T cell specie in MS. In addition, we previously showed that MS display highly significant blood T cell receptor biases, predominantly found in $CD8^+$ T cell compartment. However, only few Class-I restricted epitopes from myelin have been characterized. Our objective was to identify myelin peptides eliciting $CD8^+$ T cell response in the blood of MS. We focused the analysis on five HLA supertypes: -A3, -A2, -B7, -B27 and –B44 predominantly found in our MS patient cohort. We first screened the sequence of 3 myelin proteins (MBP, PLP, MOG) for the HLA binding motifs and selected peptides were tested for their binding avidity on the 5 HLA alleles. Cellular responses of 25 MS patients and 19 healthy controls were tested every three months during one year in an IFNγ ELISPOT only detecting precommitted $CD8^+$ T cells. Among the 188 possible 8- to 11-mer sequences, 132 peptides actually interacted with their corresponding Class-I MHC molecules. Both MS patients and HV showed auto-reactive responses against the myelin peptides without significant difference in responding cell frequency. Responses against citrullinated peptides from MBP were not detected. A total of 71 epitopes (69 newly characterized) could elicit a significant response in MS patients or healthy controls, the MOG protein being the most immunogenic. Binders on HLA-A3 were the most frequent. Moreover, citrullinated peptides from MBP did not elicit enhanced response. The epitopes inducing the strongest responses were not those with the highest HLA avidity suggesting that high avidity epitopes induce also apoptosis in MS patients. Our data, based on a large set of auto-antigens and patients extend the concept that the frequency of blood T cell reactive against myelin in MS patients is not increased compared to normal controls despite circulating $CD8^+$ T cells exhibiting abnormal selection.

Introduction

Multiple Sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system associated with the presence of autoantibodies and T lymphocytes reacting against myelin determinants including Myelin Basic Protein (MBP), Myelin Oligodendrocyte Glycoprotein (MOG) and Proteolipid Protein (PLP) [1-5]. Individuals with the Class-II Major Histocompatibility Complex (MHC) HLA-DR2 are more susceptible to the disease [6] and most of the studies of auto-reactivity focused on $CD4^+$ Th1 response. However, several groups have recently emphasized a potential role for CD8+ in MS pathology. Actually, $CD8^+$ T cells predominantly infiltrate the white matter lesions and $CD8^+$ clones can persist for several years in the cerebrospinal fluid and blood of MS patients [7-9]. The production of cytokines by $CD8^+$ T cells also correlates to Magnetic Resonance Image (MRI) feature of tissue destruction [10] and disability score [11]. In agreement with these observations, we showed that circulating blood lymphocytes of MS patients present greater biases in Vβ chain T-cell receptor usage than those of normal subjects [12]. T cell repertoire alterations mostly concerned $CD8^+$ T cells and sorted TCR-altered Vβ families contain a high frequency of MBP reactive cells [12]. Moreover, we also showed that new gadolinium positive lesions on successive MRI were correlated to the appearance of oligoclonal patterns of the peripheral T cell repertoire [13]. Taken together, these data suggested that the T cells responsible for the blood biased repertoire could be involved in the pathophysiology of MS. A few auto-reactive $CD8^+$ T-cells have been described in MS [14, 15]. In addition, oligodendrocytes, expressing HLA Class-I and -II MHC molecules during inflammation in vivo [16], can be lysed by $CD8^+$ T-cells in vitro [17]. Finally, passive transfer of MBP-specific or MOG-specific $CD8^+$ lymphocytes from immunized animals can induce a disease resembling MS in rodents, suggesting that such cells may also act as effectors in humans [18, 19]. Collectively, these

results suggest that $CD8^+$ effector eventually transiting in the blood can contribute to the CNS autoimmune process by direct cytotoxicity on oligodendrocytes or by indirectly release proinflammatory factors in oligodendrocyte environment (see [20] for review). In addition, the possibility that cytotoxic T cells could directly contribute to axonal loss is a major subject of investigation. There is thus a need to better understand the fine auto-antigen repertoire of $CD8^+$ T cells and the actual role of those and develop new immunointervention strategies. Only few myelin peptides, able to bind Class-I MHC molecules and thus representing potential epitopes for $CD8^+$ effectors, are currently known. While HLA-A3 is the most common Class-I MHC associated with MS [21, 22], only one HLA-A3 restricted peptide (PLP 43-53) has been described [23]. Most of the myelin epitopes have been described for HLA-A2 allele (MBP87-95, MBP110-118, PLP80-88, MAG556-564 and TAL168-176) [15, 24] and HLA-A24 (MBP14-22 and MBP134-142) [24]. Moreover, MBP110-118 specific clones showed the ability to lyse oligodendrocytes in vitro [17].

A study concerning reactivity to MBP, described an enhanced T cell responses in MS patients to citrulline-containing MBP [25]. Moreover, the citrullinated isoform of MBP is increased in the MS brain tissue. This post-translational modification makes MBP more susceptible to proteolyse and thus could generate epitopes (see for review [26]).

The main objective of our study was to describe the myelin epitopes corresponding to the representative presenting Class-I MHC present in MS patients able to stimulate blood $CD8^+$ T cells. We focused the analysis on the five HLA supertypes: HLA-A3, -A2, -B27, -B44 and -B7 predominantly found in the patients enrolled in our cohort. The search for HLA binding motifs was first oriented by the sequence analysis of three myelin proteins (MBP, PLP and MOG). Then, the selected peptides were tested for their binding avidity on the five HLA molecules and for their capacity to stimulate PBMC of 25 MS patients and 20 healthy controls in IFNγ ELISPOT assay, IFNγ cytokine production being

representative of CD8+ T cell responses and cytotoxicity [27]. To avoid any ex vivo artifact, blood T cells were directly analyzed from fresh blood without selection/amplification procedure and the test used only detected precommited $CD8^+$ T cells. Moreover, nine peptides derived from citrullinated MBP were tested.

Material and methods

Patients and healthy controls

Twenty five patients with a relapsing-remitting multiple sclerosis (RR-MS) and 20 healthy controls (HC) were included in the study and followed during one year, blood samples were collected every 3 months. RR-MS was clinically defined using the McDonald criteria [28]. No patients were under immunosuppressive therapy (Table 1). Typing of human leukocyte antigen (HLA) class-I molecules was performed by PCR (Olerup SSP™ HLA-A, -B and -Cw low resolution). MS patients and healthy controls were sex and age matched (RR-MS age mean: 35.05, range: 21 to 47; HC: 34.1, 25 to 50 years old) and displayed roughly similar frequency of HLA class-I alleles of the supertypes HLA-A2, -A3, -B44 and -B7.

Sequence analysis of myelin antigens and selection of peptides

In the group of MS patients, the most frequent HLA class-I molecule supertypes were HLA-A3, -A2, -B27, -B7 and -B44. To select peptides able to bind to these HLA molecules, a search for HLA binding motifs was performed by the sequence analysis of human MBP [29], MOG [30] and PLP [31] using Sette et al. method [32]. A total number of 188 peptides (8- to 10-mers) containing anchor residues required for binding to the HLA class-I supertypes mentioned above was selected. This number included 39 MBP, 90 MOG and 59 PLP peptides. When indicated, peptides from Epstein-Barr virus (EBV), Cytomegalovirus (CMV) and Influenza virus able to bind HLA-A1, -A2, -A24 and HLA-B8, -B7, B27, -B44, -B18, -B37 were used as positive controls in ELISPOT. All peptides were then synthesized (Mimotopes, Clayton Australia), dissolved in 4% DMSO and stored at –20°C before used. Nine peptides from MBP containing the arginyl (R) residu, site of potential citrullination were selected (citrullinated sequences are described in [25]). These peptides were produced,

arginines being replaced by citrullinyl residus (Cambrigde research biochemicals, United Kingdom).

Peptide/HLA class I binding assay

Wells of microtiter plates (Nune Maxisorp, 96 wells, Taastrop, Denmark) were coated with monoclonal anti-HLA antibodies at a concentration of 10µg/ml in PBS for 2h at 37°C. The following antibodies were used: BB7.2 to detect the HLA-A2 molecule, A11.1M and GAP-A3 for the HLA-A3, B1.23.2 or PA2.6 for HLA-B7/35/51, HLA-B27 and HLA-B44. Most of the antibodies were obtained from American Type Culture Collection (ATCC) [33], excepted for B1.23.2. The plates were washed 3 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 Epstein-Barr virus and denatured as previously described [34]. HLA molecules (2 µg/100 µl) were incubated with 3 dilutions of each peptide (10^{-4}, 10^{-6} and 10^{-8} M) and 2 µg/ml β-2 microglobulin in PBS-Tween, 1% BSA, 1mM PMSF (p-amidino-phenyl-methyl-sulfonyl-fluoride), 10 µg/ml trypsin inhibitor for 2h at room temperature. The HLA molecules preincubated with peptides were then added in duplicate in antibody-coated wells for 90 min at 37°C and the plates were washed 3 times with PBS-Tween. To detect the formation of correctly folded HLA/peptide complexes, the anti-β-2 microglobulin antibody M28, coupled to alkaline phosphatase, was added 1h 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). In each test, the positive control was a viral epitope known to bind on the given MHC class-I molecule: 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 was exceeded at least 30% of fluorescence obtained with viral control

peptide. The percentage of fluorescence obtained with a myelin peptide in comparison with fluorescence of positive control is described as binding index.

IFNγ ELISPOT assay and cytokines detection in culture supernatants

Because the selected 8- to 11-mer peptides are Class-I restricted, fresh unstimulated peripheral blood mononuclear cells (PBMC) from relapsing-remitting (RR-MS) patients and healthy controls (HC) were tested by IFNγ ELISPOT. PBMC were isolated from 100 ml blood on a Ficoll layer (Eurobio, Les Ulis, France) and assayed without intermediate culture step with a human IFNγ ELISPOT kit according to manufacturer recommendation (Endogen, Pierce, Rockford, USA). Briefly, 4 x 10^5 PBMC (or purified cells) per well were incubated with 10 µg/ml peptides during 15h at 37°C, in complete RPMI medium, 10% AB serum in antibody-coated plate. The supernatants were collected and frozen for further studies (see below). Then the plates were washed 6 times and incubated 1h at room temperature with IFNγ antibody. After 6 additional washings, the plates were incubated with streptavidin-AP 1h at room temperature. The plates were again washed 6 times and incubated 15min with the NBT/BCIP substrate solution. The enzymatic reaction was stopped with desionized water and the spots were counted by the ELISPOT reader system (AID, Strassberg Germany). The criteria in ELISPOT assay for epitope selection were: a spot number over 20 spots per 400000 PBMC and over at least three time background spot number.

Cell purification

B cells, monocytes, CD4+ and $CD8^+$ $CD45RO^+$ or $CD45RO^-$ were separated from PBMC on a speed cell sorter ARIA (BD Biosciences). Cells samples (approximately 1 x 10^6) were incubated 15min with monoclonal antibodies: 20µL anti-CD8-PerCP-Cy5.5, 20µL anti-CD45RO-ECD, 20µL anti-CD14-FITC and 20µL anti-CD19-PE-Cy7 (purchased from BD Biosciences) and 5µL anti-CD25-Alexa647 (anti-CD25 from Immunotech and the coupling

was performed in the lab using a molecular prob kit, Invitrogen). Purity was >95% for all sorted subpopulations.

Statistical analyses

Non-parametric tests were used in this study. Mann-Whitney test was performed for the comparison of ELISPOT assay responses and phenotypical analyses between MS patient and HC groups. Spearman correlation was used to determine the link between binding index and ELISPOT responses.

Results

Binding of myelin peptides on HLA class-I molecules:

The most frequent Class-I MHC in MS patient cohort were HLA-A3, -A2, -B7, -B27 and -B44. Peptides potentially able to bind to these MHC Class-I molecules were first selected by sequence analysis of putative myelin auto-antigens, synthesized and tested for their affinity for the corresponding restriction MHC molecules. The binding test was performed in microplates using purified MHC molecules incubated with the synthetic peptides and human β2-microglobulin. When a MHC/peptide complex is formed, it can be recognized by a conformation-dependent anti-MHC antibody [35] (see material and methods). The peptides eliciting binding above the threshold (binding index above 30) were respectively: 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 among the 188 tested peptides (70%) interacted significantly with their corresponding HLA Class-I molecules *in vitro* (fig 1). The method of peptide selection based on anchor binding site [32] appeared effective to determine MHC binders.

IFNγ ELISPOT assay detected only $CD8^+$ memory T cell responses:

The aim of our study was restricted to the analyze of blood $CD8^+$ reactivity against myelin derived peptides, without previous in vitro stimulation step to avoid culture artefacts. Indeed ex vivo generated cell lines may not reflect the actual profile of blood auto-reactive cells. However, it was also important to ensure that the ELISPOT read out only detected precommited $CD8^+$ T cells and could not recruit naïve cells. To discriminate the different cell subpopulations responding to peptide stimulations and to ensure our test indeed did not recruit

and activate naïve cells, monocytes, B cells, $CD4^+$ T cells and $CD8^+$ T cells were sorted from fresh PBMC (ARIA cell sorter) to study their reactivity in the ELISPOT after peptide stimulation. A reference viral pool of peptides (from Influenza, CMV and EBV) was systematically used as positive control [36]. Figure 2A shows a representative experiment indicating that purified monocytes, B lymphocytes, $CD4^+$ T cells and naive CD8+ T cells ($CD45RO^-$) alone do not produce IFNγ against the putative auto-antigen peptides tested in our experimental setting. Only memory $CD8^+$ T cells ($CD45RO^+$) were able to produce IFNγ with the Class-I restricted peptides derived from virus and from myelin. These results confirmed that the ELISPOT assay used in this paper only detected responses of precommitted effectors from blood and did not generate in vitro stimulation of naïve cells. In addition, B cells only added to $CD8^+$ $CD45RO^+$ T cells induced a borderline increased of IFNγ production (p=0.06) (fig 2B).

IFNγ positive $CD8^+$ T cells responding to myelin peptides in blood of MS patients and of healthy controls:

Fresh PBMC from 25 patients with a defined relapsing-remitting multiple sclerosis (RR-MS) and from 20 healthy controls (HC) were isolated from blood every three months during one year and immediately tested without in vitro priming step. There was no difference on $CD8^+$ T cell count as assessed by flow cytometry in the two populations (data not shown). Without peptide stimulation in the ELISPOT assay, IFNγ producing $CD8^+$ T cell frequencies in MS patient and healthy control (HC) PBMC were almost nil (fig 3A). The frequency of responding cells against the pool of viral epitopes was roughly comparable between the two groups (67.7 ±50.7 spots for HC and 63.0 ± 42.8 spots for MS patients, ns) (fig 3B). However, MS patients and HC showed also roughly similar auto-reactive responses against

the myelin peptides and there was no significant difference between the two groups concerning the frequency of responding cells to myelin peptides (fig 3C) and to each tested peptide (an example for peptide MOG243-51 is given on figure 3D). Collectively, the frequency of IFNγ producing cells appeared lower with myelin derived peptides than following stimulation with viral peptides, both in MS patients and healthy controls ($p<0.01$). When independently analyzed per MHC restriction element (HLA-A2, -A3, -B7,-B27 and -B44), there was no significant difference between MS patients and HC. There was also no difference between non treated MS patients and MS patients under immunomodulatory treatment (glatiramer acetate or IFNβ). Thus, our data, based on a large set of auto-antigen derived peptides and a large group of MS patients and normal individuals tested confirm and extend the concept that the frequency of blood peripheral $CD8^+$ T cell reactive to myelin auto-antigens in MS patients is not significantly increased compared to normal individuals.

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

Because avidity could be a false criterion of autoimmunity, we took the option to test the capacity of all the 188 "in silico" selected myelin peptides to induce a cellular response in the IFNγ ELISPOT assay, whatever results of the binding tests. Among the 188 myelin tested peptides, 71 new epitopes elicited a response of PBMC from either MS patients or healthy control (HC), indicating that the human repertoire display a high frequency of TCR reacting against myelin auto-antigen and that human $CD8^+$ T cells interact with a high diversity of these epitopes. It has been suggested that citrullination of MBP, a characteristic of immature myelin could be preferentially recognized by auto-reactive lymphocytes [25]. However, citrullinated peptide stimulation did not induced $CD8^+$ T cell response in tested MS patients and healthy controls (data not shown). Figure 4 shows the overall distribution of the epitopes. Among the various auto-antigens, the MOG protein appeared to be the most immunogenic with 37 epitopes, 24 epitopes derived from MBP and 10 from PLP. Among the PLP sequence,

the epitopes are dispersed and found in intra-cell, extra-cell and membrane domain. The epitopes were also found all along the sequence of MBP and MOG, but some MBP and MOG regions seemed to be more immunogenic. For MBP, the region 151 to 192 seemed to be highly recognized with eight epitopes; unfortunately the exact structure and functions of this protein are unknown. The epitopes on MOG protein were more focused on the extracellular part (1-125) and a part which may be an intracellular domain (218-260) (fig 4). Concerning the HLA restriction of the epitopes, most of epitopes were binders of HLA-A3 (9 from MBP, 6 from MOG, 1 from PLP). Eleven epitopes bound HLA-A2 (2 MBP, 7 MOG, 2 PLP), nine -B27 (5 MBP, 4 MOG), six -B7 (2 MBP, 3 MOG, 1 PLP) and two -B44 (1 MBP, 1 PLP).

Each individual presents a private pattern of responses:

After the epitope characterization, we studied the distribution of the responses to these epitopes in the two clinical groups. There was no general "public" recognition of myelin peptides and each patient (independently MS or healthy individual) had a "private" response pattern (fig 5). Thus, each individual (MS patient or HC) responded to a given combination of epitopes. However, some peptides seemed to have an elevated stimulation potential, combining a relatively high frequency of responding cells and more MS patient and HC responders (as MOG97-105 and PLP202-210 on figure 5). No epitope induced a positive response in all individuals, even in those sharing the same Class-I MHC molecules.

The immunodominant peptides were weak HLA binders:

To assess if the binding of myelin epitopes on the presenting Class-I MHC molecules could be linked to the magnitude of the cellular response of $CD8^+$ T cells, we compared the mean

IFNγ positive spots in ELISPOT tests and the level of fluorescence in binding tests. Figure 6A shows that the epitopes eliciting the strongest ELISPOT response were not the more efficient HLA binder suggesting that high avidity epitopes might have induced apoptosis of auto-reactive cells. Indeed, despite the most immunogenic Class-I restricted peptides were effective Class-I MHC binders, their avidity was low. This is also illustrated in figure 6A for HLA-A3 and was also observed in the other restricting alleles (-A2, -B27, -B7 and -B44). In addition, the myelin derived peptides globally showed a ten fold higher efficient dose to reached fifty percent of the maximal response (ED_{50}=5µg/ml) than viral peptides (ED_{50}= 0.5µg/ml) in both MS patients and healthy controls (figure 6B).

$CD8^+$ T cell responses to myelin derived epitopes fluctuate in time:

To determine the stability of the $CD8^+$ T cell responses to myelin peptides during the evolution of disease, we tested the reactivity of the MS patients and HC every three months during one year and at each clinical relapse using the same ELISPOT assay. Most of the $CD8^+$ T cells responses to myelin peptides were stable during the year follow-up (fig 7). Some peptides never elicited a response in a given patient (for example MBP168-177 and PLP38-45 for the patient MS-14, fig 7) whearas others elicited stable positive responses (as MBP138-147, MOG97-105 and PLP202-210 for the patient MS-14). However, some individual presented new reactivity against some peptides during the follow-up (MOG225-232 and MOG252-260) whereas reactivity disappeared in others (as MOG 178-186). In addition, we did not observe an increased reactivity against myelin auto-antigens in the blood of MS patients during exacerbating phase of disease in the seven tested MS patients who presented a relapse phase during the studied period.

Discussion

The involvement of $CD8^+$ T cells in MS pathology has been documented and $CD8^+$ effectors may be important in CNS lesions [20]. However, few Class-I restricted epitopes were described. We found in this study 69 new epitopes and confirmed CD8 reactivity against MBP220-228 peptides (described as MBP87-95 in Zang YC. et al.) and MBP244-252 one known as MBP110-118 or MBP 111-119, depending on database sequences [15, 24]. We obtained more $CD8^+$ reactivity against MOG derived peptides for MS patients and healthy controls. Testing reactivity to Class-II restricted 20mer peptides derived from MBP, PLP and MOG proteins and also reactivity to these three proteins, Diaz-Villoslada et al. showed the same prevalent response to MOG [37].

The HLA-A2 allele is commonly found in caucasian (about 60% in general population) and epitopes described previously in MS were for their majority restricted for this MHC allele. However, importance of HLA-A3 and –B7 might have been underestimated given that HLA-A3 and –B7 are associated to MS [21, 22, 38, 39]. In this study, we focused on the representative HLA molecules, present in our cohort of MS patients. Interestingly, we found that a majority of epitopes were HLA-A3 restricted, but only six for –B7 suggesting that HLA-A3 restriction plays a role in the context of MS auto-immunity.

Tranquill LR et al. have shown that MS patients presented enhanced T cell responsiveness to citrulline MBP[25]. However, these results were obtained using a different protocol with in vitro stimulation, 17- to 20-mer peptides addressed to CD4+ T cells and a small studied cohort (n=5 in each group). We tested reactivity against citrunillated peptides derived from MBP, but we detected no ex vivo cellular responses with this stimulation in MS patients and healthy controls.

Analysis of the 188 myelin derived peptides in an IFNγ ELISPOT assay detecting only memory $CD8^+$ T cell responses, did not revealed differences in frequency of $CD8^+$ T cells

reactive against myelin derived peptides between MS patients and normal controls. Tsuchida et al. found also that both MS patients and normal individuals present auto-reactive $CD8^+$ T cell precursors [15]. However, Zang et al. described an increased $CD8^+$ T cell precursor frequencies in MS patients compared to normal controls with MBP87-95 and MBP111-119. However, this was observed after a 7 day stimulation culture [24]. Using ELISPOT assay, Moldovan et al. tested MBP- and PLP-derived overlapping 9-mer peptides reactivity and found that MS patients presented higher cumulative responses [40]. However, the test they utilised used a 48 hours incubation which in our hand may recruit naïve cells. Our results based on ex vivo responses with a 15hours stimulation only, seem to confirm that MS patients as healthy individuals have circulating auto-reactive $CD8^+$ T cells. These data mimic what has been reported with $CD4^+$ T cell reactivity to myelin auto-antigens in ELISPOT, there is also no difference between MS patients and controls [41]. Therefore, the presence of auto-reactive T cells in blood may not be a reliable fact to MS pathology. However, in vitro studies, with auto-reactive T cell lines generated against myelin derived auto-antigens, support the concept that auto-reactive T cells from MS patients present different degree of activation [5, 41]. MS patient cells secrete more pro-inflammatory cytokines as TNFα, IFNγ and IL1β [42]. Auto-reactive T cell lines in MS patients are also less sensitive to apoptosis [43, 44] and $CD8^+$ T cell adhesive capacities to endothelium are increased in MS patients [45]. Consequently, auto-reactive T cells are found in MS patients and in normal controls but cells from MS patients unlike normal individuals seem to be involved in an active immune process.

One other hypothesis concerning the "paradoxal" absence of difference in frequencies of auto-reactive $CD8^+$ T cells between MS patients and healthy controls is that although MBP, MOG and PLP are the major components of myelin, they might be not the recognized auto-antigens in the pathological process. In fact, many other antigens compose the myelin sheath and are also specific of CNS as Oligodendrocyte specific protein (OSP) or Myelin associated oligodendrocyte basic protein (MOBP). However, Niland et al. found an increased response in

MS patients compared to normal controls for a HLA-A2 restricted peptide derived from transaldolase (TAL) protein [46], one in agreement with this possibility.

Our data show that each individual present his own pattern of response and no "public" recognition of myelin peptides, suggesting difficulties to eventually apply immunointervention strategy based on the concept of altered signal. This kind of therapy relies on modifying sequence of peptides which are immunodominant and considered to induce a deleterious immune response.

Acknowledgments

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-Plese for preparation of the manuscript.

Legends

Table1: Clinical characteristics of the patients and HLA typing.

GA= glatiramer acetate. IFNβ treatments correspond to Rebif® or Avonex®. Bold HLA types belong to the studied HLA supertypes: HLA-A3 (-A3, -A11, -A31), HLA-A2 (-A2, -A28, -B70), HLA-B7 (-B7, -B35, -B51), -B27 (-B27, -A32, -B14, -B39) and -B44 (-B44, -A29, -B37, -B45, -B50).

Figure 1: Binding of sequence-selected myelin peptides on Class-I MHC molecules

For each HLA supertype, are shown the binding test results obtained for the 188 myelin derived peptides. Peptides were incubated with MHC molecule and β-microglobulin in precoated plate with antibodies against MHC molecules. The detection of complex was performed with adding an antibody against β-microglobulin. For each HLA, a positive control was used (binding index = 100%). Above 30 for binding index, peptide was considered positive for binding.

Figure 2: Peptidic stimulation of PBMC subpopulations.

PBMC were sorted with an ARIA sorter (BD Biosciences) with the antibodies against CD19-PC7 (B lymphocytes), CD14-FITC (monocytes), CD3-PE, CD4- or CD8- APC and CD45RO-ECD (memory and naïve T lymphocytes). The sorted cells (40000cells/ well) and the PBMC (400000cells/well) were incubated 15h in complete RPMI, 10% AB serum, with viral peptide pool (CMV, EBV, Influenza) and a myelin peptide pool (MOG74-81, MOG97-105, MOG225-232, PLP202-210, MBP244-252, MBP138-147) at 10µg/ml in ELISPOT plates. A. Membranes of ELISPOT shown in this figure correspond to the patient MS-9, a representative example of the four MS patients and two HC tested. Only the $CD8^+$ $CD45RO^+$ T cells are able

to produce IFNγ in response to the peptide stimulation. B. Cells from 5 Ms patients and 3 HC were incubated with myelin pool, ratio $CD8^+$ $CD45RO^+$ T cells: other cells 1:2.

Figure 3: IFNγ Elispot assays in 25 multiple sclerosis patients and 20 healthy controls

400000 fresh PBMC per well in duplicate were incubated 15h in complete RPMI medium, 10% AB serum, in the presence of medium (**A**), a pool of viral peptides at 10µg/ml (**B**) and myelin peptides (**C**) or peptide MOG243-251 (**D**) at 10µg/ml. For **C**, the graph represents the mean of IFNγ positive cells obtained with the myelin derived epitopes.

Figure 3: Localization of epitopes on MBP, MOG and PLP proteins.

Each epitope is described with the amino-acid position in the corresponding protein sequence, between parentheses are indicated the restricting HLA Class-I molecule as defined in the binding test (see M and M). For MBP, no 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 (intra-cellular , extra-cellular , membrane associated domain and membrane) for MOG and for PLP described respectively in [47] and in [48] are indicated. Blank domains ( ) correspond to not known conformation.

Figure 4: Reactivity pattern to 18 myelin-derived epitopes and a pool of viral peptides.

400000 fresh PBMC per well in duplicate were incubated 15h in complete RPMI medium, 10% AB serum, in the presence of a pool of viral peptides at 10µg/ml or each myelin epitope at 10µg/ml. the results from four representative individuals (two MS and two HC) tested in ELISPOT against 18 different epitopes and to the positive viral peptide control, are shown on this figure. The line - - - correspond to the threshold of positive response.

Figure 5: Binding efficacity, concentration and cellular responses.

A. **Avidity force and magnitude of ELISPOT responses for HLA-A3.** Peptides which can potentially bind HLA-A3 were tested in binding assay and in ELISPOT assay as described in material and methods. 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 binding test. The mean of spots number obtained for the (HLA-A3 restricted) individuals stimulated with peptides in ELISPOT assay is on the Y axis. Points under the vertical line - - - correspond to myelin peptides which have a low binding affinity for HLA-A3 and their ELISPOT stimulation induce an elevated cellular response. Points under the horizontal line - - - correspond to myelin peptides which have a high binding affinity for HLA-A3 but are unable to induce cellular response. **B. Response in ELISPOT at different peptide concentration.** 400000 fresh PBMC per well in duplicate were incubated 15h in complete RPMI medium, 10% AB serum, in the presence of a pool of viral peptides and a pool of myelin epitopes with dilution: ranging from 200µg/ml to 0.05µg/ml. The graph shows a representative example (patient MS-4) of peptide dilution.

Figure 6: Kinetic of Elispot responses for MS-4 (Months: M0, M3, M6, M9 and M12).

Every three months during one year, 400000 fresh PBMC per well in duplicate were stimulated 15h in the ELISPOT assay with myelin peptides at 10µg/ml. The line - - - correspond to the threshold of positivity. In this figure, is shown the kinetic of reactivity for the patient MS-4.

References

1. Lovett-Racke, A.E., et al., *Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells.* J Clin Invest, 1998. 101(4): p. 725-30.
2. Markovic-Plese, S., et al., *T cell recognition of immunodominant and cryptic proteolipid protein epitopes in humans.* J Immunol, 1995. 155(2): p. 982-92.
3. Ota, K., et al., *T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis.* Nature, 1990. 346(6280): p. 183-7.
4. Scholz, C., et al., *Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation.* J Immunol, 1998. 160(3): p. 1532-8.
5. Zhang, J., et al., *Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis.* J Exp Med, 1994. 179(3): p. 973-84.
6. Haines, J.L., et al., *Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group.* Hum Mol Genet, 1998. 7(8): p. 1229-34.
7. Skulina, C., et al., *Multiple sclerosis: brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood.* Proc Natl Acad Sci U S A, 2004. 101(8): p. 2428-33.
8. Babbe, H., et al., *Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction.* J Exp Med, 2000. 192(3): p. 393-404.
9. Jacobsen, M., et al., *Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients.* Brain, 2002. 125(Pt 3): p. 538-50.
10. Killestein, J., et al., *Cytokine producing CD8+ T cells are correlated to MRI features of tissue destruction in MS.* J Neuroimmunol, 2003. 142(1-2): p. 141-8.
11. Moldovan, I.R., et al., *Interferon gamma responses to myelin peptides in multiple sclerosis correlate with a new clinical measure of disease progression.* J Neuroimmunol, 2003. 141(1-2): p. 132-40.
12. Laplaud, D.A., et al., *Blood T-cell receptor beta chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution.* Brain, 2004. 127(Pt 5): p. 981-95.
13. Laplaud, D.A., et al., *Serial blood T cell repertoire alterations in multiple sclerosis patients; correlation with clinical and MRI parameters.* J Neuroimmunol, 2006. 177(1-2): p. 151-60.
14. Crawford, M.P., et al., *High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay.* Blood, 2004. 103(11): p. 4222-31.
15. Tsuchida, T., et al., *Autoreactive CD8+ T-cell responses to human myelin protein-derived peptides.* Proc Natl Acad Sci U S A, 1994. 91(23): p. 10859-63.
16. Hoftberger, R., et al., *Expression of major histocompatibility complex class I molecules on the different cell types in multiple sclerosis lesions.* Brain Pathol, 2004. 14(1): p. 43-50.
17. Jurewicz, A., W.E. Biddison, and J.P. Antel, *MHC class I-restricted lysis of human oligodendrocytes by myelin basic protein peptide-specific CD8 T lymphocytes.* J Immunol, 1998. 160(6): p. 3056-9.
18. Huseby, E.S., et al., *A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis.* J Exp Med, 2001. 194(5): p. 669-76.
19. Sun, D., et al., *Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice.* J Immunol, 2001. 166(12): p. 7579-87.

20. McDole, J., A.J. Johnson, and I. Pirko, *The role of CD8+ T-cells in lesion formation and axonal dysfunction in multiple sclerosis.* Neurol Res, 2006. 28(3): p. 256-61.
21. Harbo, H.F., et al., *Genes in the HLA class I region may contribute to the HLA class II-associated genetic susceptibility to multiple sclerosis.* Tissue Antigens, 2004. 63(3): p. 237-47.
22. Fogdell-Hahn, A., et al., *Multiple sclerosis: a modifying influence of HLA class I genes in an HLA class II associated autoimmune disease.* Tissue Antigens, 2000. 55(2): p. 140-8.
23. Honma, K., et al., *Identification of an epitope derived from human proteolipid protein that can induce autoreactive CD8+ cytotoxic T lymphocytes restricted by HLA-A3: evidence for cross-reactivity with an environmental microorganism.* J Neuroimmunol, 1997. 73(1-2): p. 7-14.
24. Zang, Y.C., et al., *Increased CD8+ cytotoxic T cell responses to myelin basic protein in multiple sclerosis.* J Immunol, 2004. 172(8): p. 5120-7.
25. Tranquill, L.R., et al., *Enhanced T cell responsiveness to citrulline-containing myelin basic protein in multiple sclerosis patients.* Mult Scler, 2000. 6(4): p. 220-5.
26. Mastronardi, F.G. and M.A. Moscarello, *Molecules affecting myelin stability: a novel hypothesis regarding the pathogenesis of multiple sclerosis.* J Neurosci Res, 2005. 80(3): p. 301-8.
27. van Lier, R.A., I.J. ten Berge, and L.E. Gamadia, *Human CD8(+) T-cell differentiation in response to viruses.* Nat Rev Immunol, 2003. 3(12): p. 931-9.
28. McDonald, W.I., et al., *Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis.* Ann Neurol, 2001. 50(1): p. 121-7.
29. Pribyl, T.M., et al., *The human myelin basic protein gene is included within a 179-kilobase transcription unit: expression in the immune and central nervous systems.* Proc Natl Acad Sci U S A, 1993. 90(22): p. 10695-9.
30. Pham-Dinh, D., et al., *Characterization and expression of the cDNA coding for the human myelin/oligodendrocyte glycoprotein.* J Neurochem, 1994. 63(6): p. 2353-6.
31. Diehl, H.J., et al., *Individual exons encode the integral membrane domains of human myelin proteolipid protein.* Proc Natl Acad Sci U S A, 1986. 83(24): p. 9807-11.
32. Sette, A. and J. Sidney, *Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism.* Immunogenetics, 1999. 50(3-4): p. 201-12.
33. Connan, F., et al., *A simple assay for detection of peptides promoting the assembly of HLA class I molecules.* Eur J Immunol, 1994. 24(3): p. 777-80.
34. Rousseau, A., et al., *T cell response to Hu-D peptides in patients with anti-Hu syndrome.* J Neurooncol, 2005. 71(3): p. 231-6.
35. Couillin, I., et al., *Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 NEF protein.* J Exp Med, 1994. 180(3): p. 1129-34.
36. Toma, A., et al., *Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients.* Proc Natl Acad Sci U S A, 2005. 102(30): p. 10581-6.
37. Diaz-Villoslada, P., et al., *Autoreactivity to myelin antigens: myelin/oligodendrocyte glycoprotein is a prevalent autoantigen.* J Neuroimmunol, 1999. 99(1): p. 36-43.
38. Jersild, C., A. Svejgaard, and T. Fog, *HL-A antigens and multiple sclerosis.* Lancet, 1972. 1(7762): p. 1240-1.
39. Naito, S., et al., *Multiple sclerosis: association with HL-A3.* Tissue Antigens, 1972. 2(1): p. 1-4.

40. Moldovan, I.R., et al., *Longitudinal single-cell cytokine responses reveal recurrent autoimmune myelin reactivity in relapsing--remitting multiple sclerosis patients.* Mult Scler, 2005. 11(3): p. 251-60.
41. Hellings, N., et al., *T-cell reactivity to multiple myelin antigens in multiple sclerosis patients and healthy controls.* J Neurosci Res, 2001. 63(3): p. 290-302.
42. Hollifield, R.D., et al., *Evidence for cytokine dysregulation in multiple sclerosis: peripheral blood mononuclear cell production of pro-inflammatory and anti-inflammatory cytokines during relapse and remission.* Autoimmunity, 2003. 36(3): p. 133-41.
43. Zang, Y.C., et al., *Impaired apoptotic deletion of myelin basic protein-reactive T cells in patients with multiple sclerosis.* Eur J Immunol, 1999. 29(5): p. 1692-700.
44. Saresella, M., et al., *Programmed cell death of myelin basic protein-specific T lymphocytes is reduced in patients with acute multiple sclerosis.* J Neuroimmunol, 2005. 166(1-2): p. 173-9.
45. Battistini, L., et al., *CD8+ T cells from patients with acute multiple sclerosis display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1.* Blood, 2003. 101(12): p. 4775-82.
46. Niland, B., et al., *CD8+ T cell-mediated HLA-A*0201-restricted cytotoxicity to transaldolase peptide 168-176 in patients with multiple sclerosis.* J Immunol, 2005. 175(12): p. 8365-78.
47. Johns, T.G. and C.C. Bernard, *The structure and function of myelin oligodendrocyte glycoprotein.* J Neurochem, 1999. 72(1): p. 1-9.
48. Greer, J.M. and M.B. Lees, *Myelin proteolipid protein--the first 50 years.* Int J Biochem Cell Biol, 2002. 34(3): p. 211-5.

Table1: Clinical characteristics of the patients and HLA typing

Code	***Age (years)***	***Gender***	***HLA Class I***	***Disease duration (years)***	***Relapse during study***	***EDSS Score***	Treatment
MS1	47	F	**A2 A3 B7 B27**	6	2	4	IFNβ
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	IFNβ
MS5	39	F	**A2** A23 B13 B15	14	2	3	IFNβ
MS6	27	F	**A2 B51 B14**	2	0	0	GA
MS7	39	F	**A2 A3 B7** B57	9	2	2	IFNβ
MS8	21	F	**A2 A3** B18 **B51**	2	0	0	none
MS9	25	M	**A3** A74 **B7** B58	1	0	0	IFNβ
MS10	37	F	**A2 A3 B7 B27**	4	0	1.5	IFNβ
MS11	31	M	**A2** A24 **B51** B45	1	1	1	IFNβ
MS12	33	M	**A2 A11 B7 B27**	10	0	1.5	none
MS13	36	F	**A3 A11 B35 B44**	6	0	2.5	IFNβ
MS14	24	F	**A3 A32 B7**	3	1	1	IFNβ
MS15	42	M	**A3 B7**	13	1	3.5	IFNβ
MS16	38	M	A24 A29 **B7 B44**	1	0	0	IFNβ
MS17	31	F	**A2** A26 **B7 B44**	7	0	1.5	IFNβ
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
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**				

Figure1

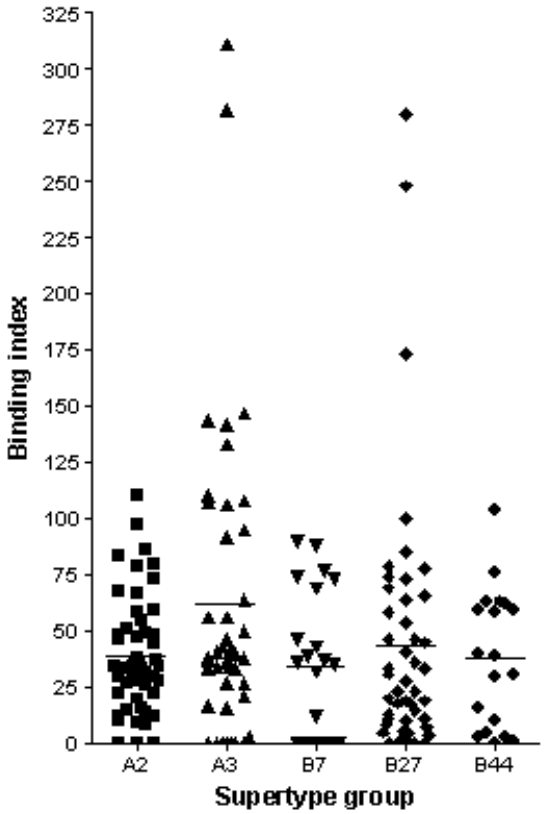

Binding index
325
300
275
250
225
200
175
150
125
100
75
50
25
0
A2
A3
B7
B27
B44
Supertype group

Figure2

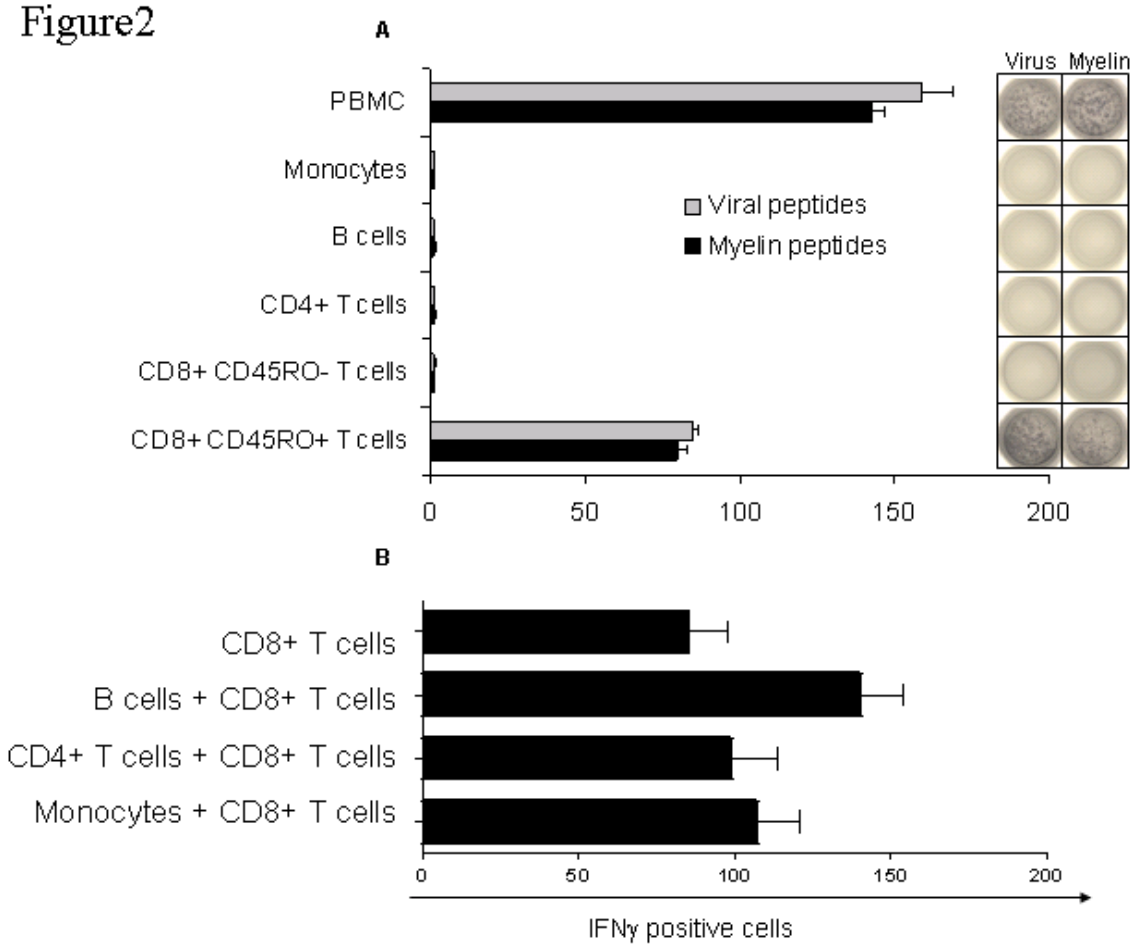

A
Virus
Myelin
PBMC
Monocytes
B cells
CD4+ T cells
CD8+ CD45RO- T cells
CD8+ CD45RO+ T cells
Viral peptides
Myelin peptides
0
50
100
150
200
B
CD8+ T cells
B cells + CD8+ T cells
CD4+ T cells + CD8+ T cells
Monocytes + CD8+ T cells
0
50
100
150
200
IFNγ positive cells

Figure 3

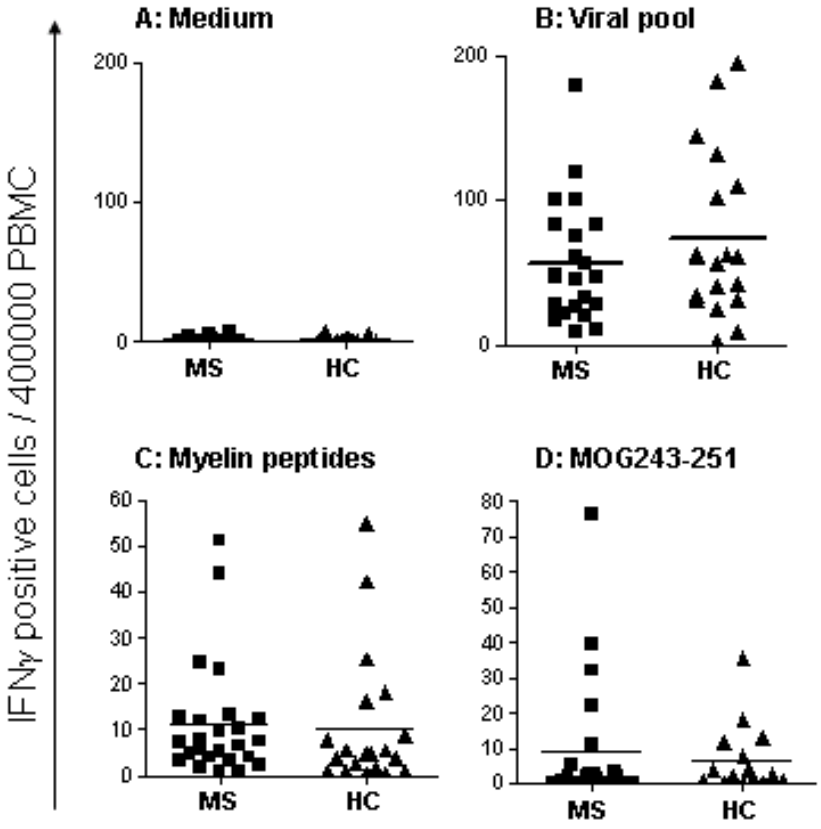
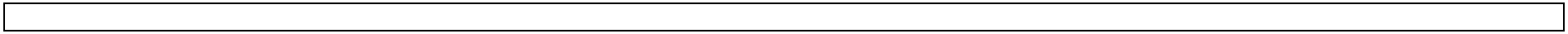

A: Medium
B: Viral pool
C: Myelin peptides
D: MOG243-251
IFNγ positive cells / 400000 PBMC
MS
HC
0
100
200
10
20
30
40
50
60
70
80

Figure4

MBP



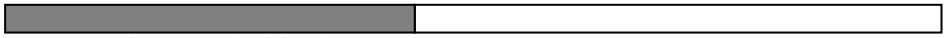
130 **150** **200** **260** **304**

SLDMASQK=130-138 (A3) TASTMDHAR=151-159 (A3) TAHYGSLPQK=200-209 (A3) QDENPVVHF=215-223 (B44) PRTPPPSQGK=230-239 (B27) RASDYKSAHK=262-272 (A3)
MASQKRPSQR=134-143 MDHARHGFL =155-163 SLPQKSHGR=205-214 (A3) NPVVHFFKNI=218-227 (B35) GRGLSLSRF =240-248 (B27) GVDAQGTLSK=274-283 (A3)
KRPSQRHGSK =138-147 (B27) HARHGFLPR=157-165 VVHFFKNIV=220-228 (A2) SLSRFSWGA=244-252 (A2) GTLSKIFK =282-289 (A3)
SQRHGSKYL =141-149 (B27) ARHGFLPRHR=158-167(B27)
LPRHRDTGI=163-171(B7, B35, B51)
DTGILDSIGR=168-177 (A3) SRSGSPMARR=295-304 (B27)
SIGRFFGGDR=174-183 (A3)
GAPKRGSGK=184-192 (A3)

MOG

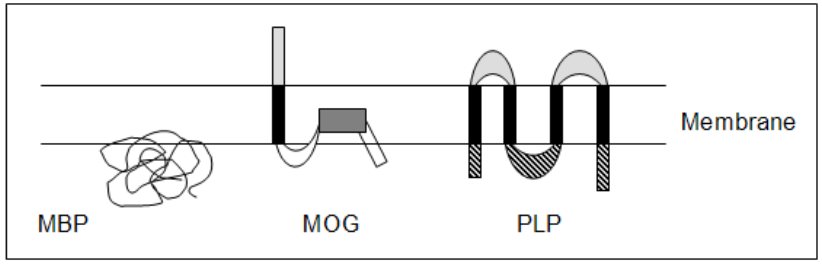
1 Extracellular domain 149 Transmembrane domain 169

VIGPRHPIR =34-42 (A3) ATGMEVGWYR=61-69 YRGTELLK=94-102 (B27) KVEDPFYWV=143-151 (A2) LLAVLPVLLL=157-166 (A2) FLCLQYRLR =175-183 (A3)
GPRHPURAL=36-44 (B7, B35, B51) GMEVGWYR=63-70 RTELLKDAI=97-105 (A2) DPFYWVSPGV=146-155 (B35, B51) VLLLQITV=164-171 (A2) LQYRLRGKL =178-186 (B27)
ALVGDEVEL =43-51(A2) SRVVHLYR=74-81 AIGEGKVTL=104-112 (A2) KLRAEIENL=185-193 (A2)
RVVHLYRNGK=75-84 (A3) VTLRIRNVR=110-118 (A3) LRAEIENLHR =186-195
LRIRNVRF=112-119 (B27)
VRFSDEGGF=117-125 (B27)



205 Membrane associated domain 226 295

FLRVPCWK=201-208 GPLVALIICY=218-228 EATRGRGGL =263-271 (A2)
LRVPCWKI=202-209 (B27) ALIICYNWL=222-230 TRGRGGLLR=265-273 (B27)
RVPCWKITL=203-211 (A2) IICYNWLHR =225-232 (A3) GRGGLLRDHI =267-276
HRRLAGQFL =232-240 LLRDHIPRGK=271-281 (A3)
GQFLEELRKF=237-246 IPRGKEEL=276-283 (B7, B35, B51)
LRKFSSLCY=243-251 (B27)
SLCYKQRI=248-255 (A2)
KQRIKSQER=252-260



PLP

Intracellular Transmembrane Extracellular Transmembrane Intracellular Transmembrane Extracellular Transmembrane Intracellular

LLECCARCL =3-11 (A2) EALTGTEK=38-45 (A3) YEYLINVI=58-65 (B44) GITYALTVV=154-162 ADARMYGV=202-210 FAVLKLMGR =265-273
ALTGTEKLI=39-47 NVIHAFQYVI=63-72 (A2) NLLSICKTA=223-231(A2)
LSICKTAEF=225-233 (B35, B51)

Figure 5

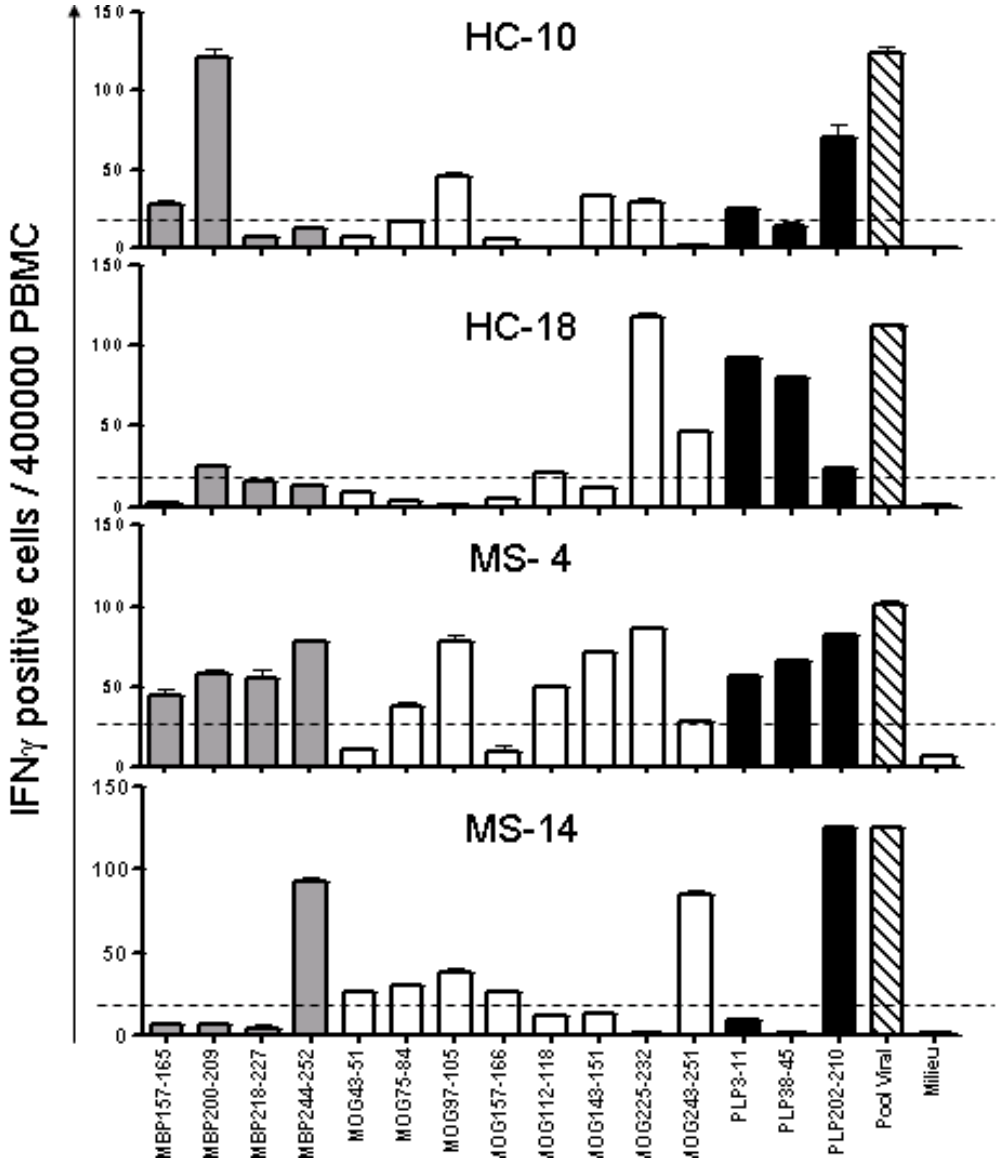

HC-10
HC-18
MS- 4
MS-14
IFNγ positive cells / 400000 PBMC
150
100
50
0
MBP157-165
MBP200-209
MBP218-227
MBP244-252
MOG43-51
MOG75-84
MOG97-105
MOG157-166
MOG112-118
MOG143-151
MOG225-232
MOG243-251
PLP3-11
PLP38-45
PLP202-210
Pool Viral
Milieu

Figure 6

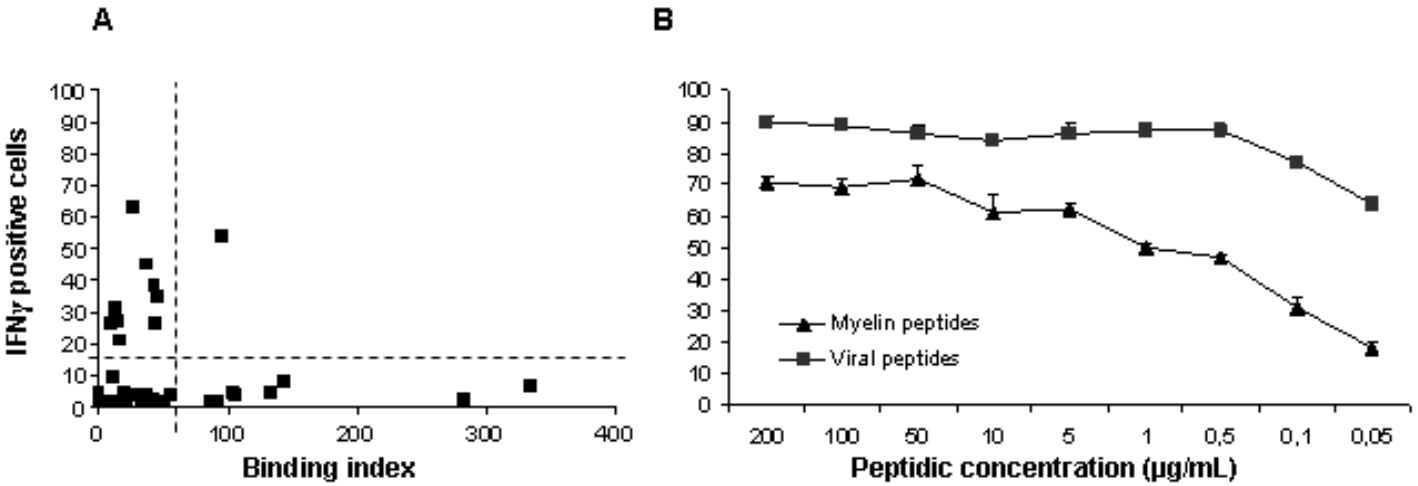
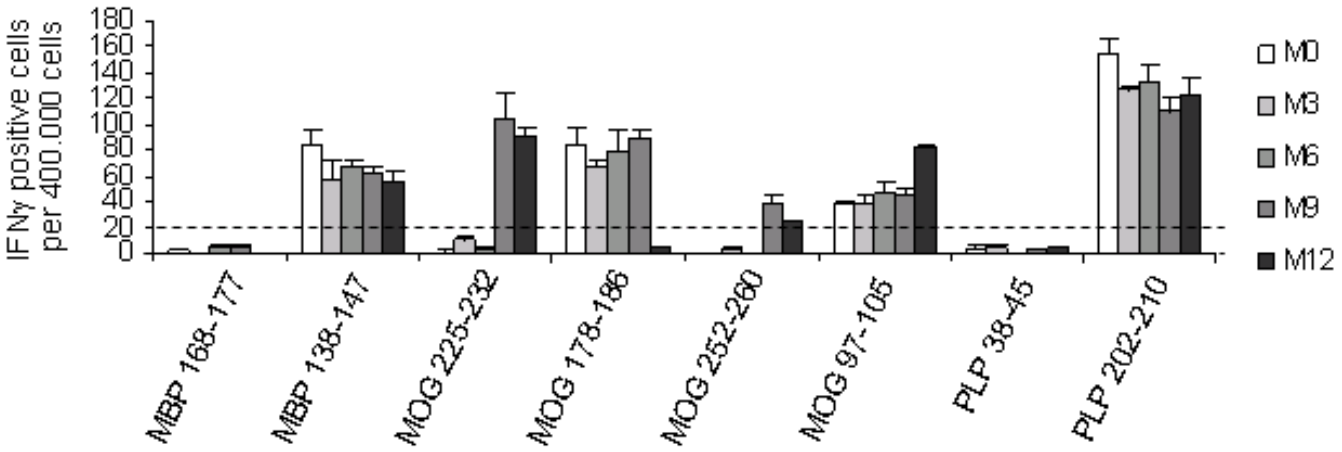

A
B
IFNγ positive cells
Binding index
Peptidic concentration (µg/mL)
Myelin peptides
Viral peptides
0
10
20
30
40
50
60
70
80
90
100
200
300
400
200
100
50
10
5
1
0,5
0,1
0,05

Figure 7


IF Nγ positive cells per 400.000 cells
180
160
140
120
100
80
60
40
20
0
M0
M3
M6
M9
M12
MBP 168-177
MBP 138-147
MOG 225-232
MOG 178-186
MOG 252-260
MOG 97-105
PLP 38-45
PLP 202-210

Discussion et perspectives

La sclérose en plaques (SEP) est une maladie supposée auto-immune dans laquelle les lymphocytes T semblent jouer un rôle crucial. Les analyses du répertoire T ont montré des altérations dans la répartition des différentes longueurs de CDR3 chez les patients atteints de SEP au niveau du sang, du liquide céphalorachidien (LCR) et du système nerveux central (SNC). Dans une première étude (Article I), nous avons voulu savoir s'il y avait un lien entre les altérations du répertoire Vβ observées dans le sang et l'activité de la maladie. Sur une cohorte de 9 patients atteints de SEP de forme rémittente (n=6) ou de forme secondaire progressive (n=3) étudiés de façon sériée sur plusieurs années, l'analyse du répertoire Vβ dans le sang et des données cliniques obtenues par imagerie par résonnance magnétique (IRM) a montré plusieurs corrélations : entre la proportion de familles Vβ altérées et le volume lésionnel ainsi que le nombre de lésions prenant le Gadolinium. De plus, la formation de nouvelles lésions (positives au Gadolinium) est liée à l'apparition de nouvelles familles Vβ altérées dans le sang des patients atteints de SEP. Ces résultats étayent les données existantes sur le lien entre l'activation du système immunitaire et la démyélinisation du SNC. Cette étude étant basée sur une petite cohorte de patients, notre équipe souhaite mettre en place une étude longitudinale de plus grande envergure (avec au minimum 100 patients atteints de SEP) afin de valider ces résultats. Toutes les formes différentes de SEP seront étudiées, le but souhaité à terme étant d'utiliser les analyses du répertoire T dans le sang des patients comme outil de diagnostic fiable, moins traumatisant que l'IRM ou la ponction lombaire.

L'acétate de glatiramère (GA) a montré des effets bénéfiques en tant que thérapie de la SEP (Filippi, Horsfield et al. 1995; Comi, Filippi et al. 2001; Filippi, Rovaris et al. 2001). Les mécanismes d'action de ce traitement décrits précédemment découlent dans leur majorité d'expériences de stimulation avec le GA menées *in vitro*. Nous nous sommes intéressés à l'effet de ce traitement sur le répertoire T *in vivo* chez des patients traités et chez des patients définis comme répondeurs au traitement (Etude II). A 3 et 24 mois après traitement au GA, aucune modification majeure du répertoire T n'a été observée chez les patients atteints de SEP. Comparée aux variations dans le même intervalle de temps des témoins sains, il n'y a pas de variation significative du répertoire T chez les patients traités par le GA. Ce traitement n'induit pas *in vivo* d'altérations du répertoire T et ne diminue pas non plus le niveau d'altérations de la répartition des différentes longueurs de CDR3 pour chaque famille Vβ, plus élevé chez les patients que chez les témoins (montré dans (Laplaud, Ruiz et al. 2004), annexe I). De plus, les

patients décrits rétrospectivement répondeurs au traitement par le GA ne se distinguent pas de façon évidente, au niveau du répertoire T, des patients non répondeurs au GA. Malgré les effets du GA sur les lymphocytes T *in vitro* et en particulier sur le répertoire T des lymphocytes T CD8+ (Biegler, Yan et al. 2006), de façon surprenante aucun effet n'a été détecté sur le répertoire T *in vivo* dans le sang des patients atteints de SEP. De plus, l'analyse sur les sous-popultations lymphocytaires T CD4+ et CD8+ n'a pas non plus montré d'effet du GA sur le répertoire T. L'analyse des transcrits de cytokines et de facteurs de neuroprotection avant et trois mois après traitement n'a révélé aucune surexpression ou diminution d'expression de ces transcrits. Une thérapie relativement courte au GA n'induit pas de modifications majeures *in vivo* au niveau des lymphocytes T circulants des patients traités. Actuellement, nous réalisons ces analyses sur des prélèvements à 24 mois provenant des 10 patients atteints de SEP suivis dans cette étude. Des effets du GA sur l'expression de plusieurs cytokines seront peut-être détectés chez les patients traités à plus long terme.

L'étude du répertoire T des cellules infiltrant le SNC a montré que tous les types de lésions ainsi que la substance blanche d'apparence normale semblent contenir des lymphocytes T portant des altérations du TCR extrêmement élevées, signe que l'inflammation est générale dans le SNC et non focale (Etude III). Cependant, les infiltrats lymphocytaires extraits ont été amplifiés *in vitro* par la PHA pendant 45 jours. Nous effectuons actuellement des expériences contrôles afin d'évaluer l'influence de cette stimulation, même si elle est polyclonale, sur la répartition des différentes longueurs de CDR3 des lymphocytes T. Après extraction des lésions du SNC, certains clones lymphocytaires amplifiés par la PHA ont été testés à Chapell Hill avec des banques de peptides dérivés de la myéline. Nous vérifions maintenant les biais d'usage du répertoire T de ces clones autoréactifs. Cette étude du répertoire T des lésions et de la substance blanche d'apparence normale du SNC, couplée à des études du transcriptome (cytokines et marqueurs d'activation des cellules du système immunitaire en particulier) et des études phénotypiques, va permettre de compléter la caractérisation des infiltrats lymphocytaires du SNC de patients atteints de SEP.

Concernant la spécificité des lymphocytes T CD8+ dans la SEP (représentant la partie la plus importante de mon travail de thèse), mon 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 (Etude IV). Il n'y a cependant pas de différence de fréquences des lymphocytes T CD8+ auto-réactifs entre les patients atteints de SEP et des individus normaux, ni de différence de niveau de réponses. Chaque patient atteint de SEP présente des 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 modifiées et induisant des réponses différentes de celles auto-réatives. 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 des individus normaux pourrait être due à une différence de régulation des réponses de ces lymphocytes ou à un état d'activation différent de ces lymphocytes comme suggéré par plusieurs études (Zhang, Markovic-Plese et al. 1994; Hellings, Baree et al. 2001; Hollifield, Harbige et al. 2003) ou à un défaut de sensibilité à l'apoptose (Zang, Kozovska et al. 1999; Saresella, Marventano et al. 2005). D'autre part, les lymphocytes T CD8+ des patients atteints de SEP présentent des capacités d'adhésion augmentées, permettant un passage de la BHE (Battistini, Piccio et al. 2003). La migration de ces lymphocytes T dans le SNC pourrait diminuer leur nombre dans le sang chez les patients atteints de SEP à la différence d'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 individus sains. Ce test n'est pas exhaustif et la production d'autres cytokines pourraient différencier les patients atteints de SEP des individus sains, comme l'IL17 qui semble jouer un rôle important dans la pathologie. Les cytokines : IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17, TNFα seront doser dans les surnageants congelés des plaques Elispot avec un kit de dosage Luminex et apporteront des informations complémentaires.

La littérature a montré un défaut de fonction régulatrice des cellules T CD4+CD25+ chez les patients atteints de SEP comparés à des individus sains (Viglietta, Baecher-Allan et al. 2004; Haas, Hug et al. 2005; Kumar, Putzki et al. 2006; Venken, Hellings et al. 2006). Ces études ne sont cependant intéressées qu'à la régulation des lymphocytes T CD4+. Nous avons donc entrepris d'étudier la régulation de la prolifération des lymphocytes T CD8+ par les cellules T CD4+CD25+ chez les patients atteints de SEP. La prolifération des lymphocytes T CD8+ induite par l'anticorps anti-CD3 est effectivement inhibée de façon plus faible chez les patients atteints de SEP que chez les individus sains (fig 17). Par conséquent, nos résultats préliminaires montrent

que si tout individu qu'il soit atteint de SEP ou qu'il soit sain, possède des lymphocytes T auto-réactifs circulants, la régulation par les cellules T CD4+CD25+ semble être défectueuse chez les patients atteints de SEP.

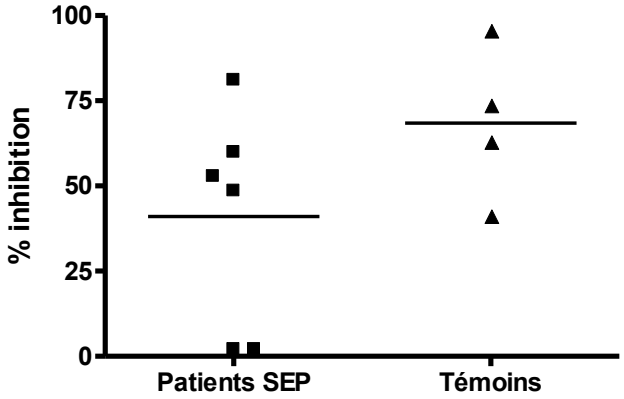


Figure 17: Régulation de la prolifération des lymphocytes T CD8+ par les cellules T CD4+CD25+
Les lymphocytes T CD8+ (20.000 cellules par puits) ont été incubés avec des PBMC irradiés (100.000 cellules par puits) dans des plaques dont les fonds de puits ont préalablement recouvert par l'anticorps OKT3 (anti-CD3) avec ou sans les lymphocytes T CD4+CD25+ (20.000 cellules par puits). Les cocultures ont incubé 5 jours à 37°C dans du milieu RPMI complet, 10% sérum AB. L'incorporation de la thymidine tritiée s'est déroulée ensuite pendant 16 heures et la lecture des coups par minute a été réalisée dans un compteur à scintillation.

D'autre part, l'état d'activation des lymphocytes T semble également être en cause chez les patients atteints de SEP. D'autres personnes dans notre équipe s'attachent à étudier maintenant plus particulièrement l'activation des lymphocytes T CD8+ chez les patients atteints de SEP.

La première partie consiste à étudier l'expression de plusieurs marqueurs phénotypiques d'activation et de recrutement pour le passage de la BHE, exprimés par les lymphocytes T CD8+ auto-réactifs. Dans ce but, nous essayons de marquer les lymphocytes T CD8+ auto-réactifs à l'aide de pentamères. Nous avons actuellement à notre disposition un pentamère HLA-A3 couplé à un peptide de la MOG (IICYNWLHR) et un pentamère HLA-A2 couplé à un peptide de la PLP (LLECCARCL). L'analyse par cytométrie en flux sera également couplée à une analyse par PCR de transcrits cytokiniques exprimés par ces cellules.

Une deuxième étude est entreprise visant également à étudier les marqueurs exprimés par les cellules T CD8+ auto-réactives en utilisant cette fois-ci la technique « TRAP ». Cette nouvelle technique, décrite uniquement chez la souris, consiste à marquer les membranes (par la biotine et un fluorochrome) des cellules présentatrices d'antigènes (CPA) préalablement incubées avec la

protéine d'intérêt (Beadling and Slifka 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/peptide auront capté des morceaux de membranes provenant des CPA et sont facilement identifiables. Cette méthode présente l'avantage de s'affranchir des différents CMH et peptides et d'étudier directement *tous* les lymphocytes auto-réactifs quelque soit les peptides reconnus. Cette technique chez l'Homme est en cours de mise au point dans notre laboratoire. Des résultats préliminaires encourageants ont été obtenus en utilisant la réponse anti-CMV (fig 18).

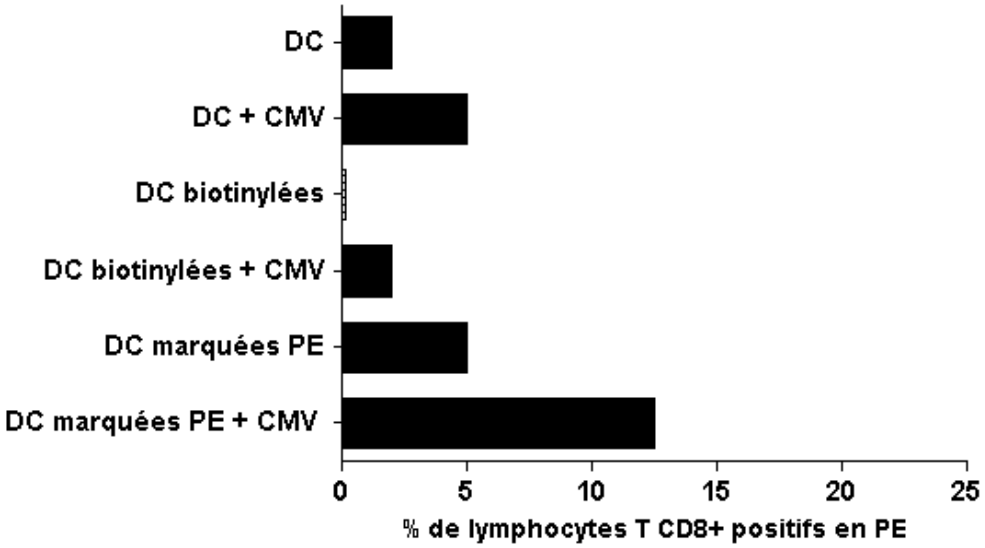


Figure 18: Résultats obtenus en cytométrie en flux après utilisation de la technique TRAP
Des cellules dendritiques (DC) diférenciées *in vitro* ont été incubées avec la biotine puis avec la spreptavidine couplée à la phycoérythrine (PE). Ensuite les cellules dendritiques ont été incubées avec des lymphocytes T CD8+ purifiés avec ou sans CMV. Le transfert de fluorescence a été mesuré en comptant les lymphocytes T CD8+ PE positifs.

La troisième partie consiste à étudier la migration des lymphocytes T CD8+ au travers d'une barrière de cellules endothéliales immortalisées de la BHE ou de cellules fraîchement préparées, issues de prélèvements lors d'interventions chirurgicales dans le cas d'épilepsie. Selon les marqueurs que les cellules expriment et en comparant des cellules circulantes provenant de patients atteints de SEP et de donneurs sains, nous voulons étudier les capacités de transmigration de ces cellules.

Enfin, une quatrième étude tend à déterminer les capacités de cytotoxicité *in vivo* des lymphocytes T CD8+ auto-réactifs présents chez les patients atteints de SEP et les individus sains. Dans ce but, nous élaborons un modèle murin humanisé (porteur du gène humain HLA-A2) dans lequel nous injecterons en périphérie les lymphocytes T CD8+ des patients SEP et des contrôles et étudierons leur migration et les effets potentiellement délétères qu'ils pourraient déclencher chez des souris. Afin d'éviter tout rejet, les souris HHD2 (KO pour les CMH de Classe I murins et KI pour la molécule HLA-A2 humaine directement couplée à la β-2-microglobuline humaine) (Pascolo, Bervas et al. 1997) sont actuellement croisées avec des souris SCID (ne présentant ni lymphocyte T ni lymphocyte B). Cette étude permettra de vérifier le rôle délétère des lymphocytes T CD8+ auto-réactifs au niveau du SNC.

Bibliographie

Bibliographie

-A-

Abraham, M., S. Shapiro, et al. (2005). "Gelatinases (MMP-2 and MMP-9) are preferentially expressed by Th1 vs. Th2 cells." J Neuroimmunol **163**(1-2): 157-64.

Achiron, A., M. Gurevich, et al. (2004). "Blood transcriptional signatures of multiple sclerosis: unique gene expression of disease activity." Ann Neurol **55**(3): 410-7.

Ackerman, A. L. and P. Cresswell (2004). "Cellular mechanisms governing cross-presentation of exogenous antigens." Nat Immunol **5**(7): 678-84.

Agius, M. A., C. A. Kirvan, et al. (1999). "High prevalence of anti-alpha-crystallin antibodies in multiple sclerosis: correlation with severity and activity of disease." Acta Neurol Scand **100**(3): 139-47.

Aharoni, R., B. Kayhan, et al. (2003). "Glatiramer acetate-specific T cells in the brain express T helper 2/3 cytokines and brain-derived neurotrophic factor in situ." Proc Natl Acad Sci U S A **100**(24): 14157-62.

Aharoni, R., D. Teitelbaum, et al. (1999). "Copolymer 1 acts against the immunodominant epitope 82-100 of myelin basic protein by T cell receptor antagonism in addition to major histocompatibility complex blocking." Proc Natl Acad Sci U S A **96**(2): 634-9.

Aharoni, R., D. Teitelbaum, et al. (2000). "Specific Th2 cells accumulate in the central nervous system of mice protected against experimental autoimmune encephalomyelitis by copolymer 1." Proc Natl Acad Sci U S A **97**(21): 11472-7.

Akassoglou, K., J. Bauer, et al. (1998). "Oligodendrocyte apoptosis and primary demyelination induced by local TNF/p55TNF receptor signaling in the central nervous system of transgenic mice: models for multiple sclerosis with primary oligodendrogliopathy." Am J Pathol **153**(3): 801-13.

Albert, M., J. Antel, et al. (2007). "Extensive cortical remyelination in patients with chronic multiple sclerosis." Brain Pathol **17**(2): 129-38.

Aloisi, F. (2001). "Immune function of microglia." Glia **36**(2): 165-79.

Alter, M., U. Leibowitz, et al. (1966). "Risk of multiple sclerosis related to age at immigration to Israel." Arch Neurol **15**(3): 234-7.

Amor, S., M. F. Scallan, et al. (1996). "Role of immune responses in protection and pathogenesis during Semliki Forest virus encephalitis." J Gen Virol **77 (Pt 2)**: 281-91.

Anthony, D. C., B. Ferguson, et al. (1997). "Differential matrix metalloproteinase expression in cases of multiple sclerosis and stroke." Neuropathol Appl Neurobiol **23**(5): 406-15.

Araki, M., T. Kondo, et al. (2003). "Th2 bias of CD4+ NKT cells derived from multiple sclerosis in remission." Int Immunol **15**(2): 279-88.

Arbour, N., A. Holz, et al. (2003). "A new approach for evaluating antigen-specific T cell responses to myelin antigens during the course of multiple sclerosis." J Neuroimmunol **137**(1-2): 197-209.

Arenz, M., S. Herzog-Hauff, et al. (1997). "Antigen-independent in vitro expansion of T cells does not affect the T cell receptor V beta repertoire." J Mol Med **75**(9): 678-86.

Arstila, T. P., A. Casrouge, et al. (1999). "A direct estimate of the human alphabeta T cell receptor diversity." Science **286**(5441): 958-61.

Astier, A. L., G. Meiffren, et al. (2006). "Alterations in CD46-mediated Tr1 regulatory T cells in patients with multiple sclerosis." J Clin Invest **116**(12): 3252-7.

Azoulay, D., V. Vachapova, et al. (2005). "Lower brain-derived neurotrophic factor in serum of relapsing remitting MS: reversal by glatiramer acetate." J Neuroimmunol **167**(1-2): 215-8.

-B-

Babbe, H., A. Roers, et al. (2000). "Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction." J Exp Med **192**(3): 393-404.

Baecher-Allan, C., J. A. Brown, et al. (2001). "CD4+CD25high regulatory cells in human peripheral blood." J Immunol **167**(3): 1245-53.

Baig, S., Y. P. Jiang, et al. (1991). "Cells secreting anti-MAG antibody occur in cerebrospinal fluid and bone marrow in patients with polyneuropathy associated with M component." Brain **114 (Pt 1B)**: 573-83.

Bajramovic, J. J., A. C. Plomp, et al. (2000). "Presentation of alpha B-crystallin to T cells in active multiple sclerosis lesions: an early event following inflammatory demyelination." J Immunol **164**(8): 4359-66.

Balashov, K. E., S. J. Khoury, et al. (1995). "Inhibition of T cell responses by activated human CD8+ T cells is mediated by interferon-gamma and is defective in chronic progressive multiple sclerosis." J Clin Invest **95**(6): 2711-9.

Ballabh, P., A. Braun, et al. (2004). "The blood-brain barrier: an overview: structure, regulation, and clinical implications." Neurobiol Dis **16**(1): 1-13.

Balluz, I. M., G. M. Glasgow, et al. (1993). "Virulent and avirulent strains of Semliki Forest virus show similar cell tropism for the murine central nervous system but differ in the severity and rate of induction of cytolytic damage." Neuropathol Appl Neurobiol **19**(3): 233-9.

Banki, K., E. Colombo, et al. (1994). "Oligodendrocyte-specific expression and autoantigenicity of transaldolase in multiple sclerosis." J Exp Med **180**(5): 1649-63.

Baranzini, S. E., M. C. Jeong, et al. (1999). "B cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions." J Immunol **163**(9): 5133-44.

Barnett, M. H. and J. W. Prineas (2004). "Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion." Ann Neurol **55**(4): 458-68.

Bartholome, E. J., F. Willems, et al. (1999). "IFN-beta interferes with the differentiation of dendritic cells from peripheral blood mononuclear cells: selective inhibition of CD40-dependent interleukin-12 secretion." J Interferon Cytokine Res **19**(5): 471-8.

Basile, E., E. Gibbs, et al. (2006). "During 3 years treatment of primary progressive multiple sclerosis with glatiramer acetate, specific antibodies switch from IgG1 to IgG4." J Neuroimmunol **177**(1-2): 161-6.

Battistini, L., G. Borsellino, et al. (1997). "Phenotypic and cytokine analysis of human peripheral blood gamma delta T cells expressing NK cell receptors." J Immunol **159**(8): 3723-30.

Battistini, L., L. Piccio, et al. (2003). "CD8+ T cells from patients with acute multiple sclerosis display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1." Blood **101**(12): 4775-82.

Beadling, C. and M. K. Slifka (2006). "Quantifying viable virus-specific T cells without a priori knowledge of fine epitope specificity." Nat Med **12**(10): 1208-12.

Becher, B., P. S. Giacomini, et al. (1999). "Interferon-gamma secretion by peripheral blood T-cell subsets in multiple sclerosis: correlation with disease phase and interferon-beta therapy." Ann Neurol **45**(2): 247-50.

Ben-Nun, A., I. Mendel, et al. (1996). "The autoimmune reactivity to myelin oligodendrocyte glycoprotein (MOG) in multiple sclerosis is potentially pathogenic: effect of copolymer 1 on MOG-induced disease." J Neurol **243**(4 Suppl 1): S14-22.

Bendriss-Vermare, N., C. Barthelemy, et al. (2001). "Human thymus contains IFN-alpha-producing CD11c(-), myeloid CD11c(+), and mature interdigitating dendritic cells." J Clin Invest **107**(7): 835-44.

Benvenuto, R., M. Paroli, et al. (1991). "Tumour necrosis factor-alpha synthesis by cerebrospinal-fluid-derived T cell clones from patients with multiple sclerosis." Clin Exp Immunol **84**(1): 97-102.

Berger, T., P. Rubner, et al. (2003). "Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event." N Engl J Med **349**(2): 139-45.

Bettelli, E., D. Baeten, et al. (2006). "Myelin oligodendrocyte glycoprotein-specific T and B cells cooperate to induce a Devic-like disease in mice." J Clin Invest **116**(9): 2393-402.

Biegler, B. W., S. X. Yan, et al. (2006). "Glatiramer acetate (GA) therapy induces a focused, oligoclonal CD8+ T-cell repertoire in multiple sclerosis." J Neuroimmunol **180**(1-2): 159-71.

Bielekova, B., M. Catalfamo, et al. (2006). "Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis." Proc Natl Acad Sci U S A **103**(15): 5941-6.

Bitsch, A., J. Schuchardt, et al. (2000). "Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation." Brain **123 (Pt 6)**: 1174-83.

Blanco, Y., E. A. Moral, et al. (2006). "Effect of glatiramer acetate (Copaxone) on the immunophenotypic and cytokine profile and BDNF production in multiple sclerosis: a longitudinal study." Neurosci Lett **406**(3): 270-5.

Bo, L., J. W. Peterson, et al. (1996). "Distribution of immunoglobulin superfamily members ICAM-1, -2, -3, and the beta 2 integrin LFA-1 in multiple sclerosis lesions." J Neuropathol Exp Neurol **55**(10): 1060-72.

Bogue, M. and D. B. Roth (1996). "Mechanism of V(D)J recombination." Curr Opin Immunol **8**(2): 175-80.

Booss, J., M. M. Esiri, et al. (1983). "Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis." J Neurol Sci **62**(1-3): 219-32.

Bornstein, M. B., A. Miller, et al. (1987). "A pilot trial of Cop 1 in exacerbating-remitting multiple sclerosis." N Engl J Med **317**(7): 408-14.

Brenner, T., R. Arnon, et al. (2001). "Humoral and cellular immune responses to Copolymer 1 in multiple sclerosis patients treated with Copaxone." J Neuroimmunol **115**(1-2): 152-60.

Brochet, B. (2001). "[Therapeutic indications for acute episodes of multiple sclerosis]." Rev Neurol (Paris) **157**(8-9 Pt 2): 988-95.

Bronstein, J. M., R. L. Lallone, et al. (1999). "A humoral response to oligodendrocyte-specific protein in MS: a potential molecular mimic." Neurology **53**(1): 154-61.

Brouard, S., B. Vanhove, et al. (1999). "T cell repertoire alterations of vascularized xenografts." J Immunol **162**(6): 3367-77.

Brousil, J. A., R. J. Roberts, et al. (2006). "Cladribine: an investigational immunomodulatory agent for multiple sclerosis." Ann Pharmacother **40**(10): 1814-21.

Bruck, W., C. Lucchinetti, et al. (2002). "The pathology of primary progressive multiple sclerosis." Mult Scler **8**(2): 93-7.

Burns, J., A. Rosenzweig, et al. (1983). "Isolation of myelin basic protein-reactive T-cell lines from normal human blood." Cell Immunol **81**(2): 435-40.

Burns, J. B., B. D. Bartholomew, et al. (2002). "In vivo activation of myelin oligodendrocyte glycoprotein-specific T cells in healthy control subjects." Clin Immunol **105**(2): 185-91.

Butterfield, R. J., E. P. Blankenhorn, et al. (1999). "Genetic analysis of disease subtypes and sexual dimorphisms in mouse experimental allergic encephalomyelitis (EAE): relapsing/remitting and monophasic remitting/nonrelapsing EAE are immunogenetically distinct." J Immunol **162**(5): 3096-102.

-C-

Cabarrocas, J., C. Cassan, et al. (2006). "Foxp3+ CD25+ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage." Proc Natl Acad Sci U S A **103**(22): 8453-8.

Calabresi, P. A., N. S. Fields, et al. (1998). "ELI-spot of Th-1 cytokine secreting PBMC's in multiple sclerosis: correlation with MRI lesions." J Neuroimmunol **85**(2): 212-9.

Calabresi, P. A., C. M. Pelfrey, et al. (1997). "VLA-4 expression on peripheral blood lymphocytes is downregulated after treatment of multiple sclerosis with interferon beta." Neurology **49**(4): 1111-6.

Camara, N. O., F. Sebille, et al. (2003). "Human CD4+CD25+ regulatory cells have marked and sustained effects on CD8+ T cell activation." Eur J Immunol **33**(12): 3473-83.

Cannella, B. and C. S. Raine (1995). "The adhesion molecule and cytokine profile of multiple sclerosis lesions." Ann Neurol **37**(4): 424-35.

Capello, E. and G. L. Mancardi (2004). "Marburg type and Balo's concentric sclerosis: rare and acute variants of multiple sclerosis." Neurol Sci **25 Suppl 4**: S361-3.

Cepok, S., M. Jacobsen, et al. (2001). "Patterns of cerebrospinal fluid pathology correlate with disease progression in multiple sclerosis." Brain **124**(Pt 11): 2169-76.

Cepok, S., B. Rosche, et al. (2005). "Short-lived plasma blasts are the main B cell effector subset during the course of multiple sclerosis." Brain **128**(Pt 7): 1667-76.

Chabot, S., F. P. Yong, et al. (2002). "Cytokine production in T lymphocyte-microglia interaction is attenuated by glatiramer acetate: a mechanism for therapeutic efficacy in multiple sclerosis." Mult Scler **8**(4): 299-306.

Charcot, J. (1868). Histologie de la sclérose en plaques, Gaz Hosp.

Chen, M., R. M. Valenzuela, et al. (2003). "Glatiramer acetate-reactive T cells produce brain-derived neurotrophic factor." J Neurol Sci **215**(1-2): 37-44.

Chofflon, M., S. Roth, et al. (1997). "Tumor necrosis factor production capacity as a potentially useful parameter to monitor disease activity in multiple sclerosis." Eur Cytokine Netw **8**(3): 253-7.

Chou, Y. K., G. G. Burrows, et al. (2004). "CD4 T-cell epitopes of human alpha B-crystallin." J Neurosci Res **75**(4): 516-23.

Cid, C., J. C. Alvarez-Cermeno, et al. (2004). "Antibodies reactive to heat shock protein 90 induce oligodendrocyte precursor cell death in culture. Implications for demyelination in multiple sclerosis." Faseb J **18**(2): 409-11.

Cifelli, A., M. Arridge, et al. (2002). "Thalamic neurodegeneration in multiple sclerosis." Ann Neurol **52**(5): 650-3.

Coles, A. J., A. Cox, et al. (2006). "The window of therapeutic opportunity in multiple sclerosis: evidence from monoclonal antibody therapy." J Neurol **253**(1): 98-108.

Colombo, M., M. Dono, et al. (2000). "Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients." J Immunol **164**(5): 2782-9.

Comi, G., M. Filippi, et al. (2001). "European/Canadian multicenter, double-blind, randomized, placebo-controlled study of the effects of glatiramer acetate on magnetic resonance imaging--measured disease activity and burden in patients with relapsing multiple sclerosis. European/Canadian Glatiramer Acetate Study Group." Ann Neurol **49**(3): 290-7.

Connan, F., F. Hlavac, et al. (1994). "A simple assay for detection of peptides promoting the assembly of HLA class I molecules." Eur J Immunol **24**(3): 777-80.

Corcione, A., S. Casazza, et al. (2004). "Recapitulation of B cell differentiation in the central nervous system of patients with multiple sclerosis." Proc Natl Acad Sci U S A **101**(30): 11064-9.

Correale, J. and L. Bassani Molinas Mde (2003). "Temporal variations of adhesion molecules and matrix metalloproteinases in the course of MS." J Neuroimmunol **140**(1-2): 198-209.

Correale, J. and M. de los Milagros Bassani Molinas (2002). "Oligoclonal bands and antibody responses in multiple sclerosis." J Neurol **249**(4): 375-89.

Cossins, J. A., J. M. Clements, et al. (1997). "Enhanced expression of MMP-7 and MMP-9 in demyelinating multiple sclerosis lesions." Acta Neuropathol (Berl) **94**(6): 590-8.

Couillin, I., B. Culmann-Penciolelli, et al. (1994). "Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 NEF protein." J Exp Med **180**(3): 1129-34.

Crawford, M. P., S. X. Yan, et al. (2004). "High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay." Blood **103**(11): 4222-31.

Cree, B. A., S. Lamb, et al. (2005). "An open label study of the effects of rituximab in neuromyelitis optica." Neurology **64**(7): 1270-2.

Cua, D. J., H. Groux, et al. (1999). "Transgenic interleukin 10 prevents induction of experimental autoimmune encephalomyelitis." J Exp Med **189**(6): 1005-10.

Cua, D. J., J. Sherlock, et al. (2003). "Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain." Nature **421**(6924): 744-8.

-D-

Dabbert, D., S. Rosner, et al. (2000). "Glatiramer acetate (copolymer-1)-specific, human T cell lines: cytokine profile and suppression of T cell lines reactive against myelin basic protein." Neurosci Lett **289**(3): 205-8.

Davies, S., T. Nicholson, et al. (2005). "Spread of T lymphocyte immune responses to myelin epitopes with duration of multiple sclerosis." J Neuropathol Exp Neurol **64**(5): 371-7.

Davis, M. M., J. J. Boniface, et al. (1998). "Ligand recognition by alpha beta T cell receptors." Annu Rev Immunol **16**: 523-44.

De Jager, P. L. and D. A. Hafler (2007). "New therapeutic approaches for multiple sclerosis." Annu Rev Med **58**: 417-32.

Demoulins, T., G. Gachelin, et al. (2003). "A biased Valpha24+ T-cell repertoire leads to circulating NKT-cell defects in a multiple sclerosis patient at the onset of his disease." Immunol Lett **90**(2-3): 223-8.

Demoulins, T., F. Mouthon, et al. (2003). "The same TCR (N)Dbeta(N)Jbeta junctional region is associated with several different vbeta13 subtypes in a multiple sclerosis patient at the onset of the disease." Neurobiol Dis **14**(3): 470-82.

Diaz-Villoslada, P., A. Shih, et al. (1999). "Autoreactivity to myelin antigens: myelin/oligodendrocyte glycoprotein is a prevalent autoantigen." J Neuroimmunol **99**(1): 36-43.

Diehl, H. J., M. Schaich, et al. (1986). "Individual exons encode the integral membrane domains of human myelin proteolipid protein." Proc Natl Acad Sci U S A **83**(24): 9807-11.

Dolan, B. P., K. D. Gibbs, Jr., et al. (2006). "Dendritic cells cross-dressed with peptide MHC class I complexes prime CD8+ T cells." J Immunol **177**(9): 6018-24.

Douillard, P., C. Pannetier, et al. (1996). "Donor-specific blood transfusion-induced tolerance in adult rats with a dominant TCR-Vbeta rearrangement in heart allografts." J Immunol **157**(3): 1250-60.

Drachman, D. A., P. Y. Paterson, et al. (1975). "Cyclophosphamide in exacerbations of multiple sclerosis. Therapeutic trial and a strategy for pilot drug studies." J Neurol Neurosurg Psychiatry **38**(6): 592-7.

Dressel, A., A. Vogelgesang, et al. (2006). "Glatiramer acetate-specific human CD8(+) T cells: increased IL-4 production in multiple sclerosis is reduced by glatiramer acetate treatment." J Neuroimmunol **181**(1-2): 133-40.

Duda, P. W., J. I. Krieger, et al. (2000). "Human and murine CD4 T cell reactivity to a complex antigen: recognition of the synthetic random polypeptide glatiramer acetate." J Immunol **165**(12): 7300-7.

Duda, P. W., M. C. Schmied, et al. (2000). "Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis." J Clin Invest **105**(7): 967-76.

Dyment, D. A., G. C. Ebers, et al. (2004). "Genetics of multiple sclerosis." Lancet Neurol **3**(2): 104-10.

-E-

Edan, G., D. Miller, et al. (1997). "Therapeutic effect of mitoxantrone combined with methylprednisolone in multiple sclerosis: a randomised multicentre study of active disease using MRI and clinical criteria." J Neurol Neurosurg Psychiatry **62**(2): 112-8.

Ehling, R., A. Lutterotti, et al. (2004). "Increased frequencies of serum antibodies to neurofilament light in patients with primary chronic progressive multiple sclerosis." Mult Scler **10**(6): 601-6.

Engelhardt, B. and R. M. Ransohoff (2005). "The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms." Trends Immunol **26**(9): 485-95.

Evangelou, N., M. M. Esiri, et al. (2000). "Quantitative pathological evidence for axonal loss in normal appearing white matter in multiple sclerosis." Ann Neurol **47**(3): 391-5.

-F-

Fabriek, B. O., J. N. Zwemmer, et al. (2005). "In vivo detection of myelin proteins in cervical lymph nodes of MS patients using ultrasound-guided fine-needle aspiration cytology." J Neuroimmunol **161**(1-2): 190-4.

Farina, C., V. Vargas, et al. (2002). "Treatment with glatiramer acetate induces specific IgG4 antibodies in multiple sclerosis patients." J Neuroimmunol **123**(1-2): 188-92.

Farina, C., M. S. Weber, et al. (2005). "Glatiramer acetate in multiple sclerosis: update on potential mechanisms of action." Lancet Neurol **4**(9): 567-75.

Feger, U., C. Luther, et al. (2007). "Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients." Clin Exp Immunol **147**(3): 412-8.

Fellay, B., M. Chofflon, et al. (2001). "Beneficial effect of co-polymer 1 on cytokine production by CD4 T cells in multiple sclerosis." Immunology **104**(4): 383-91.

Filippi, M., M. A. Horsfield, et al. (1995). "Resolution-dependent estimates of lesion volumes in magnetic resonance imaging studies of the brain in multiple sclerosis." Ann Neurol **38**(5): 749-54.

Filippi, M., M. Rovaris, et al. (2001). "Glatiramer acetate reduces the proportion of new MS lesions evolving into "black holes"." Neurology **57**(4): 731-3.

Fogdell-Hahn, A., A. Ligers, et al. (2000). "Multiple sclerosis: a modifying influence of HLA class I genes in an HLA class II associated autoimmune disease." Tissue Antigens **55**(2): 140-8.

Fogdell, A., J. Hillert, et al. (1995). "The multiple sclerosis- and narcolepsy-associated HLA class II haplotype includes the DRB5*0101 allele." Tissue Antigens **46**(4): 333-6.

Freedman, M. S., E. J. Thompson, et al. (2005). "Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement." Arch Neurol **62**(6): 865-70.

Fridkis-Hareli, M., J. N. Stern, et al. (2001). "Synthetic peptides that inhibit binding of the myelin basic protein 85-99 epitope to multiple sclerosis-associated HLA-DR2 molecules and MBP-specific T-cell responses." Hum Immunol **62**(8): 753-63.

Fridkis-Hareli, M., D. Teitelbaum, et al. (1994). "Direct binding of myelin basic protein and synthetic copolymer 1 to class II major histocompatibility complex molecules on living antigen-presenting cells--specificity and promiscuity." Proc Natl Acad Sci U S A **91**(11): 4872-6.

Fritzsching, B., M. Korporal, et al. (2006). "Similar sensitivity of regulatory T cells towards CD95L-mediated apoptosis in patients with multiple sclerosis and healthy individuals." J Neurol Sci **251**(1-2): 91-7.

Frohman, E. M., M. K. Racke, et al. (2006). "Multiple sclerosis--the plaque and its pathogenesis." N Engl J Med **354**(9): 942-55.

-G-

Galboiz, Y., S. Shapiro, et al. (2001). "Matrix metalloproteinases and their tissue inhibitors as markers of disease subtype and response to interferon-beta therapy in relapsing and secondary-progressive multiple sclerosis patients." Ann Neurol **50**(4): 443-51.

Gay, F. W., T. J. Drye, et al. (1997). "The application of multifactorial cluster analysis in the staging of plaques in early multiple sclerosis. Identification and characterization of the primary demyelinating lesion." Brain **120 (Pt 8)**: 1461-83.

Genain, C. P., B. Cannella, et al. (1999). "Identification of autoantibodies associated with myelin damage in multiple sclerosis." Nat Med **5**(2): 170-5.

Gerritse, K., C. Deen, et al. (1994). "The involvement of specific anti myelin basic protein antibody-forming cells in multiple sclerosis immunopathology." J Neuroimmunol **49**(1-2): 153-9.

Giegerich, G., M. Pette, et al. (1992). "Diversity of T cell receptor alpha and beta chain genes expressed by human T cells specific for similar myelin basic protein peptide/major histocompatibility complexes." Eur J Immunol **22**(3): 753-8.

Godfrey, D. I., K. J. Hammond, et al. (2000). "NKT cells: facts, functions and fallacies." Immunol Today **21**(11): 573-83.

Goebels, N., H. Hofstetter, et al. (2000). "Repertoire dynamics of autoreactive T cells in multiple sclerosis patients and healthy subjects: epitope spreading versus clonal persistence." Brain **123 Pt 3**: 508-18.

Goldsby RA, K. T., Osbrone BA (2001). Immunologie, le cours de Janis Kuby.

Gonsette (1986). Immunosuppresion with cyclophosphamide in multiple sclerosis.

Goodin, D. S., B. G. Arnason, et al. (2003). "The use of mitoxantrone (Novantrone) for the treatment of multiple sclerosis: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology." Neurology **61**(10): 1332-8.

Gorochov, G., A. U. Neumann, et al. (1998). "Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy." Nat Med **4**(2): 215-21.

Goverman, J. (1999). "Tolerance and autoimmunity in TCR transgenic mice specific for myelin basic protein." Immunol Rev **169**: 147-59.

Goverman, J., A. Woods, et al. (1993). "Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity." Cell **72**(4): 551-60.

Gran, B., D. Gestri, et al. (1998). "Detection of skewed T-cell receptor V-beta gene usage in the peripheral blood of patients with multiple sclerosis." J Neuroimmunol **85**(1): 22-32.

Gran, B., L. R. Tranquill, et al. (2000). "Mechanisms of immunomodulation by glatiramer acetate." Neurology **55**(11): 1704-14.

Greer, J. M. and M. B. Lees (2002). "Myelin proteolipid protein--the first 50 years." Int J Biochem Cell Biol **34**(3): 211-5.

Greter, M., F. L. Heppner, et al. (2005). "Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis." Nat Med **11**(3): 328-34.

Group, T. I. M. S. S. (1993). "Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial." Neurology **43**(4): 655-61.

Group, T. L. M. S. S. G. a. T. U. o. B. C. M. M. A. (1999). "TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study." Neurology **53**(3): 457-65.

Groux, H., A. O'Garra, et al. (1997). "A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis." Nature **389**(6652): 737-42.

Guillet, M., S. Brouard, et al. (2002). "Different qualitative and quantitative regulation of V beta TCR transcripts during early acute allograft rejection and tolerance induction." J Immunol **168**(10): 5088-95.

Guillet, M., F. Sebille, et al. (2001). "TCR usage in naive and committed alloreactive cells: implications for the understanding of TCR biases in transplantation." Curr Opin Immunol **13**(5): 566-71.

-H-

Haas, J., A. Hug, et al. (2005). "Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis." Eur J Immunol **35**(11): 3343-52.

Haegert, D. G., T. Cowan, et al. (1999). "Does a shift in the T-cell receptor repertoire precede the onset of MS?" Neurology **53**(3): 485-90.

Hafler, D. A., A. D. Duby, et al. (1988). "Oligoclonal T lymphocytes in the cerebrospinal fluid of patients with multiple sclerosis." J Exp Med **167**(4): 1313-22.

Haines, J. L., H. A. Terwedow, et al. (1998). "Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group." Hum Mol Genet **7**(8): 1229-34.

Hammond, S. R., D. R. English, et al. (2000). "The age-range of risk of developing multiple sclerosis: evidence from a migrant population in Australia." Brain **123 (Pt 5)**: 968-74.

Harbo, H. F., B. A. Lie, et al. (2004). "Genes in the HLA class I region may contribute to the HLA class II-associated genetic susceptibility to multiple sclerosis." Tissue Antigens **63**(3): 237-47.

Hellings, N., M. Baree, et al. (2001). "T-cell reactivity to multiple myelin antigens in multiple sclerosis patients and healthy controls." J Neurosci Res **63**(3): 290-302.

Hofman, F. M., D. R. Hinton, et al. (1989). "Tumor necrosis factor identified in multiple sclerosis brain." J Exp Med **170**(2): 607-12.

Hoftberger, R., F. Aboul-Enein, et al. (2004). "Expression of major histocompatibility complex class I molecules on the different cell types in multiple sclerosis lesions." Brain Pathol **14**(1): 43-50.

Hogquist, K. A., T. A. Baldwin, et al. (2005). "Central tolerance: learning self-control in the thymus." Nat Rev Immunol **5**(10): 772-82.

Hollifield, R. D., L. S. Harbige, et al. (2003). "Evidence for cytokine dysregulation in multiple sclerosis: peripheral blood mononuclear cell production of pro-inflammatory and anti-inflammatory cytokines during relapse and remission." Autoimmunity **36**(3): 133-41.

Hong, J., N. Li, et al. (2005). "Induction of CD4+CD25+ regulatory T cells by copolymer-I through activation of transcription factor Foxp3." Proc Natl Acad Sci U S A **102**(18): 6449-54.

Hong, J., Y. C. Zang, et al. (1999). "A common TCR V-D-J sequence in V beta 13.1 T cells recognizing an immunodominant peptide of myelin basic protein in multiple sclerosis." J Immunol **163**(6): 3530-8.

Honma, K., K. C. Parker, et al. (1997). "Identification of an epitope derived from human proteolipid protein that can induce autoreactive CD8+ cytotoxic T lymphocytes restricted by HLA-A3: evidence for cross-reactivity with an environmental microorganism." J Neuroimmunol **73**(1-2): 7-14.

Huan, J., N. Culbertson, et al. (2005). "Decreased FOXP3 levels in multiple sclerosis patients." J Neurosci Res **81**(1): 45-52.

Huang, Y. M., B. G. Xiao, et al. (1999). "Multiple sclerosis is associated with high levels of circulating dendritic cells secreting pro-inflammatory cytokines." J Neuroimmunol **99**(1): 82-90.

Hunter, C. A. and S. L. Reiner (2000). "Cytokines and T cells in host defense." Curr Opin Immunol **12**(4): 413-8.

Huseby, E. S., D. Liggitt, et al. (2001). "A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis." J Exp Med **194**(5): 669-76.

Hussien, Y., A. Sanna, et al. (2001). "Glatiramer acetate and IFN-beta act on dendritic cells in multiple sclerosis." J Neuroimmunol **121**(1-2): 102-10.

Hvas, J., J. R. Oksenberg, et al. (1993). "Gamma delta T cell receptor repertoire in brain lesions of patients with multiple sclerosis." J Neuroimmunol **46**(1-2): 225-34.

-I-

Ibrahim, M. Z., A. T. Reder, et al. (1996). "The mast cells of the multiple sclerosis brain." J Neuroimmunol **70**(2): 131-8.

Illes, Z., T. Kondo, et al. (2000). "Differential expression of NK T cell V alpha 24J alpha Q invariant TCR chain in the lesions of multiple sclerosis and chronic inflammatory demyelinating polyneuropathy." J Immunol **164**(8): 4375-81.

Illes, Z., M. Shimamura, et al. (2004). "Accumulation of Valpha7.2-Jalpha33 invariant T cells in human autoimmune inflammatory lesions in the nervous system." Int Immunol **16**(2): 223-30.

-J-

Jacobsen, M., S. Cepok, et al. (2002). "Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients." Brain **125**(Pt 3): 538-50.

Jersild, C., A. Svejgaard, et al. (1972). "HL-A antigens and multiple sclerosis." Lancet **1**(7762): 1240-1.

Jingwu, Z., R. Medaer, et al. (1992). "Myelin basic protein-specific T lymphocytes in multiple sclerosis and controls: precursor frequency, fine specificity, and cytotoxicity." Ann Neurol **32**(3): 330-8.

John, G. R., S. C. Lee, et al. (2003). "Cytokines: powerful regulators of glial cell activation." Neuroscientist **9**(1): 10-22.

Johns, T. G. and C. C. Bernard (1999). "The structure and function of myelin oligodendrocyte glycoprotein." J Neurochem **72**(1): 1-9.

Johnson, D., P. A. Seeldrayers, et al. (1988). "The role of mast cells in demyelination. 1. Myelin proteins are degraded by mast cell proteases and myelin basic protein and P2 can stimulate mast cell degranulation." Brain Res **444**(1): 195-8.

Johnson, K. P., B. R. Brooks, et al. (1995). "Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group." Neurology **45**(7): 1268-76.

Johnson, K. P., B. R. Brooks, et al. (1998). "Extended use of glatiramer acetate (Copaxone) is well tolerated and maintains its clinical effect on multiple sclerosis relapse rate and degree of disability. Copolymer 1 Multiple Sclerosis Study Group." Neurology **50**(3): 701-8.

Johnson, K. P., B. R. Brooks, et al. (2000). "Sustained clinical benefits of glatiramer acetate in relapsing multiple sclerosis patients observed for 6 years. Copolymer 1 Multiple Sclerosis Study Group." Mult Scler **6**(4): 255-66.

Johnson, K. P., C. C. Ford, et al. (2005). "Neurologic consequence of delaying glatiramer acetate therapy for multiple sclerosis: 8-year data." Acta Neurol Scand **111**(1): 42-7.

Joseph, J., C. D'Imperio, et al. (1988). "Down-regulation of gamma-interferon-induced class II expression of human glioma cells by recombinant beta-interferon." Ann N Y Acad Sci **540**: 475-6.

Jurewicz, A., W. E. Biddison, et al. (1998). "MHC class I-restricted lysis of human oligodendrocytes by myelin basic protein peptide-specific CD8 T lymphocytes." J Immunol **160**(6): 3056-9.

-K-

Kalkers, N. F., F. Barkhof, et al. (2002). "The effect of the neuroprotective agent riluzole on MRI parameters in primary progressive multiple sclerosis: a pilot study." Mult Scler **8**(6): 532-3.

Karandikar, N. J., M. P. Crawford, et al. (2002). "Glatiramer acetate (Copaxone) therapy induces CD8(+) T cell responses in patients with multiple sclerosis." J Clin Invest **109**(5): 641-9.

Karni, A., M. Abraham, et al. (2006). "Innate immunity in multiple sclerosis: myeloid dendritic cells in secondary progressive multiple sclerosis are activated and drive a proinflammatory immune response." J Immunol **177**(6): 4196-202.

Katz, D., J. K. Taubenberger, et al. (1993). "Correlation between magnetic resonance imaging findings and lesion development in chronic, active multiple sclerosis." Ann Neurol **34**(5): 661-9.

Kerlero de Rosbo, N., R. Milo, et al. (1993). "Reactivity to myelin antigens in multiple sclerosis. Peripheral blood lymphocytes respond predominantly to myelin oligodendrocyte glycoprotein." J Clin Invest **92**(6): 2602-8.

Khan, O., Y. Shen, et al. (2005). "Axonal metabolic recovery and potential neuroprotective effect of glatiramer acetate in relapsing-remitting multiple sclerosis." Mult Scler **11**(6): 646-51.

Khattri, R., T. Cox, et al. (2003). "An essential role for Scurfin in CD4+CD25+ T regulatory cells." Nat Immunol **4**(4): 337-42.

Khoury, S. J., C. R. Guttmann, et al. (1994). "Longitudinal MRI in multiple sclerosis: correlation between disability and lesion burden." Neurology **44**(11): 2120-4.

Kidd, D., F. Barkhof, et al. (1999). "Cortical lesions in multiple sclerosis." Brain **122 (Pt 1)**: 17-26.

Killestein, J., M. J. Eikelenboom, et al. (2003). "Cytokine producing CD8+ T cells are correlated to MRI features of tissue destruction in MS." J Neuroimmunol **142**(1-2): 141-8.

Killestein, J., N. F. Kalkers, et al. (2001). "TNFalpha production by CD4(+) T cells predicts long-term increase in lesion load on MRI in MS." Neurology **57**(6): 1129-31.

Killestein, J., T. Olsson, et al. (2002). "Antibody-mediated suppression of Vbeta5.2/5.3(+) T cells in multiple sclerosis: results from an MRI-monitored phase II clinical trial." Ann Neurol **51**(4): 467-74.

Killestein, J., M. H. Rep, et al. (2001). "Active MRI lesion appearance in MS patients is preceded by fluctuations in circulating T-helper 1 and 2 cells." J Neuroimmunol **118**(2): 286-94.

Kim, H. J., K. Biernacki, et al. (2004). "Inflammatory potential and migratory capacities across human brain endothelial cells of distinct glatiramer acetate-reactive T cells generated in treated multiple sclerosis patients." Clin Immunol **111**(1): 38-46.

Kim, H. J., I. Ifergan, et al. (2004). "Type 2 monocyte and microglia differentiation mediated by glatiramer acetate therapy in patients with multiple sclerosis." J Immunol **172**(11): 7144-53.

Kivisakk, P., D. J. Mahad, et al. (2003). "Human cerebrospinal fluid central memory CD4+ T cells: evidence for trafficking through choroid plexus and meninges via P-selectin." Proc Natl Acad Sci U S A **100**(14): 8389-94.

Komiyama, Y., S. Nakae, et al. (2006). "IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis." J Immunol **177**(1): 566-73.

Kornek, B., M. K. Storch, et al. (2000). "Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions." Am J Pathol **157**(1): 267-76.

Kouwenhoven, M., V. Ozenci, et al. (2001). "Multiple sclerosis: elevated expression of matrix metalloproteinases in blood monocytes." J Autoimmun **16**(4): 463-70.

Kouwenhoven, M., N. Teleshova, et al. (2001). "Monocytes in multiple sclerosis: phenotype and cytokine profile." J Neuroimmunol **112**(1-2): 197-205.

Krakauer, M., P. S. Sorensen, et al. (2006). "CD4(+) memory T cells with high CD26 surface expression are enriched for Th1 markers and correlate with clinical severity of multiple sclerosis." J Neuroimmunol **181**(1-2): 157-64.

Krupp, L. B., C. Christodoulou, et al. (2004). "Donepezil improved memory in multiple sclerosis in a randomized clinical trial." Neurology **63**(9): 1579-85.

Kuhle, J., C. Pohl, et al. (2007). "Lack of association between antimyelin antibodies and progression to multiple sclerosis." N Engl J Med **356**(4): 371-8.

Kuhlmann, T., G. Lingfeld, et al. (2002). "Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time." Brain **125**(Pt 10): 2202-12.

Kumar, M., N. Putzki, et al. (2006). "CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis." J Neuroimmunol **180**(1-2): 178-84.

Kutzelnigg, A., C. F. Lucchinetti, et al. (2005). "Cortical demyelination and diffuse white matter injury in multiple sclerosis." Brain **128**(Pt 11): 2705-12.

-L-

Laman, J. D., L. Visser, et al. (2001). "Novel monoclonal antibodies against proteolipid protein peptide 139-151 demonstrate demyelination and myelin uptake by macrophages in MS and marmoset EAE lesions." J Neuroimmunol **119**(1): 124-30.

Lanier, L. L. (1998). "NK cell receptors." Annu Rev Immunol **16**: 359-93.

Laplaud, D. A., L. Berthelot, et al. (2006). "Serial blood T cell repertoire alterations in multiple sclerosis patients; correlation with clinical and MRI parameters." J Neuroimmunol **177**(1-2): 151-60.

Laplaud, D. A., C. Ruiz, et al. (2004). "Blood T-cell receptor beta chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution." Brain **127**(Pt 5): 981-95.

Lassmann, H. (2007). "Multiple sclerosis: Is there neurodegeneration independent from inflammation?" J Neurol Sci.

Lassmann, H., W. Bruck, et al. (2001). "Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy." Trends Mol Med **7**(3): 115-21.

Lavi, E., D. H. Gilden, et al. (1984). "Experimental demyelination produced by the A59 strain of mouse hepatitis virus." Neurology **34**(5): 597-603.

Lee, M. A., J. Palace, et al. (1999). "Serum gelatinase B, TIMP-1 and TIMP-2 levels in multiple sclerosis. A longitudinal clinical and MRI study." Brain **122 (Pt 2)**: 191-7.

Li, Y., N. Chu, et al. (2007). "Increased IL-23p19 expression in multiple sclerosis lesions and its induction in microglia." Brain **130**(Pt 2): 490-501.

Lichtinghagen, R., T. Seifert, et al. (1999). "Expression of matrix metalloproteinase-9 and its inhibitors in mononuclear blood cells of patients with multiple sclerosis." J Neuroimmunol **99**(1): 19-26.

Lindberg, R. L., C. J. De Groot, et al. (2004). "Multiple sclerosis as a generalized CNS disease--comparative microarray analysis of normal appearing white matter and lesions in secondary progressive MS." J Neuroimmunol **152**(1-2): 154-67.

Lindberg, R. L., C. J. De Groot, et al. (2001). "The expression profile of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in lesions and normal appearing white matter of multiple sclerosis." Brain **124**(Pt 9): 1743-53.

Lindert, R. B., C. G. Haase, et al. (1999). "Multiple sclerosis: B- and T-cell responses to the extracellular domain of the myelin oligodendrocyte glycoprotein." Brain **122 (Pt 11)**: 2089-100.

Lock, C., G. Hermans, et al. (2002). "Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis." Nat Med **8**(5): 500-8.

Lopez, C., M. Comabella, et al. (2006). "Altered maturation of circulating dendritic cells in primary progressive MS patients." J Neuroimmunol **175**(1-2): 183-91.

Lou, J., M. Chofflon, et al. (1997). "Brain microvascular endothelial cells and leukocytes derived from patients with multiple sclerosis exhibit increased adhesion capacity." Neuroreport **8**(3): 629-33.

Lovett-Racke, A. E., J. L. Trotter, et al. (1998). "Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells." J Clin Invest **101**(4): 725-30.

Lozeron, P., D. Chabas, et al. (1998). "T cell receptor V beta 5 and V beta 17 clonal diversity in cerebrospinal fluid and peripheral blood lymphocytes of multiple sclerosis patients." Mult Scler **4**(3): 154-61.

Lu, H. T., J. L. Riley, et al. (1995). "Interferon (IFN) beta acts downstream of IFN-gamma-induced class II transactivator messenger RNA accumulation to block major

histocompatibility complex class II gene expression and requires the 48-kD DNA-binding protein, ISGF3-gamma." J Exp Med **182**(5): 1517-25.
Lublin, F. D. (2005). "Clinical features and diagnosis of multiple sclerosis." Neurol Clin **23**(1): 1-15, v.
Lucchinetti, C., W. Bruck, et al. (1999). "A quantitative analysis of oligodendrocytes in multiple sclerosis lesions. A study of 113 cases." Brain **122 (Pt 12)**: 2279-95.
Lucchinetti, C., W. Bruck, et al. (2000). "Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination." Ann Neurol **47**(6): 707-17.
Lucchinetti, C. F., W. Bruck, et al. (1996). "Distinct patterns of multiple sclerosis pathology indicates heterogeneity on pathogenesis." Brain Pathol **6**(3): 259-74.

-M-

Markovic-Plese, S., H. Fukaura, et al. (1995). "T cell recognition of immunodominant and cryptic proteolipid protein epitopes in humans." J Immunol **155**(2): 982-92.
Marrie, R. A. (2004). "Environmental risk factors in multiple sclerosis aetiology." Lancet Neurol **3**(12): 709-18.
Martin, R., D. Jaraquemada, et al. (1990). "Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals." J Immunol **145**(2): 540-8.
Mastronardi, F. G. and M. A. Moscarello (2005). "Molecules affecting myelin stability: a novel hypothesis regarding the pathogenesis of multiple sclerosis." J Neurosci Res **80**(3): 301-8.
Matarese, G., P. B. Carrieri, et al. (2005). "Leptin increase in multiple sclerosis associates with reduced number of CD4(+)CD25+ regulatory T cells." Proc Natl Acad Sci U S A **102**(14): 5150-5.
Matsumoto, Y., W. K. Yoon, et al. (2003). "Complementarity-determining region 3 spectratyping analysis of the TCR repertoire in multiple sclerosis." J Immunol **170**(9): 4846-53.
Matusevicius, D., P. Kivisakk, et al. (1999). "Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis." Mult Scler **5**(2): 101-4.
Mayo, I., J. Arribas, et al. (2002). "The proteasome is a major autoantigen in multiple sclerosis." Brain **125**(Pt 12): 2658-67.
McDole, J., A. J. Johnson, et al. (2006). "The role of CD8+ T-cells in lesion formation and axonal dysfunction in multiple sclerosis." Neurol Res **28**(3): 256-61.
McDonald, W. I., A. Compston, et al. (2001). "Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis." Ann Neurol **50**(1): 121-7.
Merrill, J., M. Jondal, et al. (1982). "Decreased NK killing in patients with multiple sclerosis: an analysis on the level of the single effector cell in peripheral blood and cerebrospinal fluid in relation to the activity in the disease." Clin Exp Immunol **47**(2): 419-30.
Miller, A., S. Shapiro, et al. (1998). "Treatment of multiple sclerosis with copolymer-1 (Copaxone): implicating mechanisms of Th1 to Th2/Th3 immune-deviation." J Neuroimmunol **92**(1-2): 113-21.
Miller, S. D., J. K. Olson, et al. (2001). "Multiple pathways to induction of virus-induced autoimmune demyelination: lessons from Theiler's virus infection." J Autoimmun **16**(3): 219-27.
Milligan, N. M., R. Newcombe, et al. (1987). "A double-blind controlled trial of high dose methylprednisolone in patients with multiple sclerosis: 1. Clinical effects." J Neurol Neurosurg Psychiatry **50**(5): 511-6.

Moldovan, I. R., R. A. Rudick, et al. (2003). "Interferon gamma responses to myelin peptides in multiple sclerosis correlate with a new clinical measure of disease progression." J Neuroimmunol **141**(1-2): 132-40.

Moldovan, I. R., R. A. Rudick, et al. (2005). "Longitudinal single-cell cytokine responses reveal recurrent autoimmune myelin reactivity in relapsing--remitting multiple sclerosis patients." Mult Scler **11**(3): 251-60.

Morse, R. H., R. Seguin, et al. (2001). "NK cell-mediated lysis of autologous human oligodendrocytes." J Neuroimmunol **116**(1): 107-15.

Munschauer, F. E., L. A. Hartrich, et al. (1995). "Circulating natural killer cells but not cytotoxic T lymphocytes are reduced in patients with active relapsing multiple sclerosis and little clinical disability as compared to controls." J Neuroimmunol **62**(2): 177-81.

Muraro, P. A., L. Bonanni, et al. (2002). "Short-term dynamics of circulating T cell receptor V beta repertoire in relapsing-remitting MS." J Neuroimmunol **127**(1-2): 149-59.

Muraro, P. A., R. Cassiani-Ingoni, et al. (2006). "Clonotypic analysis of cerebrospinal fluid T cells during disease exacerbation and remission in a patient with multiple sclerosis." J Neuroimmunol **171**(1-2): 177-83.

Muraro, P. A., K. P. Wandinger, et al. (2003). "Molecular tracking of antigen-specific T cell clones in neurological immune-mediated disorders." Brain **126**(Pt 1): 20-31.

Musette, P., D. Bequet, et al. (1996). "Expansion of a recurrent V beta 5.3+ T-cell population in newly diagnosed and untreated HLA-DR2 multiple sclerosis patients." Proc Natl Acad Sci U S A **93**(22): 12461-6.

-N-

Naito, S., N. Namerow, et al. (1972). "Multiple sclerosis: association with HL-A3." Tissue Antigens **2**(1): 1-4.

Neuhaus, O., C. Farina, et al. (2001). "Mechanisms of action of glatiramer acetate in multiple sclerosis." Neurology **56**(6): 702-8.

Neuhaus, O., C. Farina, et al. (2000). "Multiple sclerosis: comparison of copolymer-1- reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells." Proc Natl Acad Sci U S A **97**(13): 7452-7.

Neumann, H., I. M. Medana, et al. (2002). "Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases." Trends Neurosci **25**(6): 313-9.

Niland, B., K. Banki, et al. (2005). "CD8+ T cell-mediated HLA-A*0201-restricted cytotoxicity to transaldolase peptide 168-176 in patients with multiple sclerosis." J Immunol **175**(12): 8365-78.

Noseworthy, J. H., C. Lucchinetti, et al. (2000). "Multiple sclerosis." N Engl J Med **343**(13): 938-52.

-O-

Offner, H. and A. A. Vandenbark (1999). "T cell receptor V genes in multiple sclerosis: increased use of TCRAV8 and TCRBV5 in MBP-specific clones." Int Rev Immunol **18**(1-2): 9-36.

Oksenberg, J. R. and S. L. Hauser (2005). "Genetics of multiple sclerosis." Neurol Clin **23**(1): 61-75, vi.

Oksenberg, J. R., M. A. Panzara, et al. (1993). "Selection for T-cell receptor V beta-D beta-J beta gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis." Nature **362**(6415): 68-70.

Oksenberg, J. R., S. Stuart, et al. (1990). "Limited heterogeneity of rearranged T-cell receptor V alpha transcripts in brains of multiple sclerosis patients." Nature **345**(6273): 344-6.
Olerup, O. and J. Hillert (1991). "HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation." Tissue Antigens **38**(1): 1-15.
Ota, K., M. Matsui, et al. (1990). "T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis." Nature **346**(6280): 183-7.
Owens, G. P., H. Kraus, et al. (1998). "Restricted use of VH4 germline segments in an acute multiple sclerosis brain." Ann Neurol **43**(2): 236-43.

-P-

Palace, J. and P. Rothwell (1997). "New treatments and azathioprine in multiple sclerosis." Lancet **350**(9073): 261.
Panitch, H. S., R. L. Hirsch, et al. (1987). "Exacerbations of multiple sclerosis in patients treated with gamma interferon." Lancet **1**(8538): 893-5.
Panitch, H. S., R. L. Hirsch, et al. (1987). "Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system." Neurology **37**(7): 1097-102.
Pannetier, C., J. Even, et al. (1995). "T-cell repertoire diversity and clonal expansions in normal and clinical samples." Immunol Today **16**(4): 176-81.
Pascolo, S., N. Bervas, et al. (1997). "HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice." J Exp Med **185**(12): 2043-51.
Pashenkov, M., Y. M. Huang, et al. (2001). "Two subsets of dendritic cells are present in human cerebrospinal fluid." Brain **124**(Pt 3): 480-92.
Peterson, J. W., L. Bo, et al. (2001). "Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions." Ann Neurol **50**(3): 389-400.
Pette, M., K. Fujita, et al. (1990). "Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals." Neurology **40**(11): 1770-6.
Pette, M., D. F. Pette, et al. (1997). "Interferon-beta interferes with the proliferation but not with the cytokine secretion of myelin basic protein-specific, T-helper type 1 lymphocytes." Neurology **49**(2): 385-92.
Pham-Dinh, D., B. Allinquant, et al. (1994). "Characterization and expression of the cDNA coding for the human myelin/oligodendrocyte glycoprotein." J Neurochem **63**(6): 2353-6.
Polman, C. H., P. W. O'Connor, et al. (2006). "A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis." N Engl J Med **354**(9): 899-910.
Polman, C. H., S. C. Reingold, et al. (2005). "Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria"." Ann Neurol **58**(6): 840-6.
Porcel, J. and X. Montalban (2006). "Anticholinesterasics in the treatment of cognitive impairment in multiple sclerosis." J Neurol Sci **245**(1-2): 177-81.
Pouly, S., B. Becher, et al. (2000). "Interferon-gamma modulates human oligodendrocyte susceptibility to Fas-mediated apoptosis." J Neuropathol Exp Neurol **59**(4): 280-6.
Powell, H. C. and P. W. Lampert (1975). "Oligodendrocytes and their myelin-plasma membrane connections in JHM mouse hepatitis virus encephalomyelitis." Lab Invest **33**(4): 440-5.
Prat, A., A. Al-Asmi, et al. (1999). "Lymphocyte migration and multiple sclerosis: relation with disease course and therapy." Ann Neurol **46**(2): 253-6.
Prat, A., K. Biernacki, et al. (2005). "Th1 and Th2 lymphocyte migration across the human BBB is specifically regulated by interferon beta and copolymer-1." J Autoimmun **24**(2): 119-24.

Pribyl, T. M., C. W. Campagnoni, et al. (1993). "The human myelin basic protein gene is included within a 179-kilobase transcription unit: expression in the immune and central nervous systems." Proc Natl Acad Sci U S A **90**(22): 10695-9.

Prineas, J. W. and R. G. Wright (1978). "Macrophages, lymphocytes, and plasma cells in the perivascular compartment in chronic multiple sclerosis." Lab Invest **38**(4): 409-21.

Putheti, P., A. Pettersson, et al. (2004). "Circulating CD4+CD25+ T regulatory cells are not altered in multiple sclerosis and unaffected by disease-modulating drugs." J Clin Immunol **24**(2): 155-61.

Putheti, P., M. Soderstrom, et al. (2003). "Effect of glatiramer acetate (Copaxone) on CD4+CD25high T regulatory cells and their IL-10 production in multiple sclerosis." J Neuroimmunol **144**(1-2): 125-31.

-Q-

Qin, Y., P. Duquette, et al. (1998). "Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis." J Clin Invest **102**(5): 1045-50.

Qin, Y., D. Q. Zhang, et al. (2000). "Characterization of T cell lines derived from glatiramer-acetate-treated multiple sclerosis patients." J Neuroimmunol **108**(1-2): 201-6.

-R-

Ragheb, S., S. Abramczyk, et al. (2001). "Long-term therapy with glatiramer acetate in multiple sclerosis: effect on T-cells." Mult Scler **7**(1): 43-7.

Ransohoff, R. M. (1999). "Mechanisms of inflammation in MS tissue: adhesion molecules and chemokines." J Neuroimmunol **98**(1): 57-68.

Ratts, R. B., A. E. Lovett-Racke, et al. (2006). "CD28-CD57+ T cells predominate in CD8 responses to glatiramer acetate." J Neuroimmunol **178**(1-2): 117-29.

Reindl, M., M. Khalil, et al. (2006). "Antibodies as biological markers for pathophysiological processes in MS." J Neuroimmunol **180**(1-2): 50-62.

Rep, M. H., R. Q. Hintzen, et al. (1996). "Recombinant interferon-beta blocks proliferation but enhances interleukin-10 secretion by activated human T-cells." J Neuroimmunol **67**(2): 111-8.

Rep, M. H., H. M. Schrijver, et al. (1999). "Interferon (IFN)-beta treatment enhances CD95 and interleukin 10 expression but reduces interferon-gamma producing T cells in MS patients." J Neuroimmunol **96**(1): 92-100.

Rieks, M., V. Hoffmann, et al. (2003). "Induction of apoptosis of CD4+ T cells by immunomodulatory therapy of multiple sclerosis with glatiramer acetate." Eur Neurol **50**(4): 200-6.

Rio, J., C. Nos, et al. (2006). "Defining the response to interferon-beta in relapsing-remitting multiple sclerosis patients." Ann Neurol **59**(2): 344-52.

Rivers, T. S., DH; Berry, GP (1933). "Observations on attempts to produce acute disseminated encephalomyelitis in monkeys." J Exp Med **58**(1): 39-53.

Rosati, G. (2001). "The prevalence of multiple sclerosis in the world: an update." Neurol Sci **22**(2): 117-39.

Rousseau, A., B. Benyahia, et al. (2005). "T cell response to Hu-D peptides in patients with anti-Hu syndrome." J Neurooncol **71**(3): 231-6.

Rovaris, M., M. Codella, et al. (2002). "Effect of glatiramer acetate on MS lesions enhancing at different gadolinium doses." Neurology **59**(9): 1429-32.

Rozniecki, J. J., S. L. Hauser, et al. (1995). "Elevated mast cell tryptase in cerebrospinal fluid of multiple sclerosis patients." Ann Neurol **37**(1): 63-6.

Rudick, R. A., R. M. Ransohoff, et al. (1996). "Interferon beta induces interleukin-10 expression: relevance to multiple sclerosis." Ann Neurol **40**(4): 618-27.

Rudick, R. A., W. H. Stuart, et al. (2006). "Natalizumab plus interferon beta-1a for relapsing multiple sclerosis." N Engl J Med **354**(9): 911-23.

Ruggieri, M., C. Avolio, et al. (2006). "Glatiramer acetate induces pro-apoptotic mechanisms involving Bcl-2, Bax and Cyt-c in peripheral lymphocytes from multiple sclerosis patients." J Neurol **253**(2): 231-6.

-S-

Sadovnick, A. D., H. Armstrong, et al. (1993). "A population-based study of multiple sclerosis in twins: update." Ann Neurol **33**(3): 281-5.

Salama, H. H., J. Hong, et al. (2003). "Blocking effects of serum reactive antibodies induced by glatiramer acetate treatment in multiple sclerosis." Brain **126**(Pt 12): 2638-47.

Saresella, M., I. Marventano, et al. (2005). "Programmed cell death of myelin basic protein-specific T lymphocytes is reduced in patients with acute multiple sclerosis." J Neuroimmunol **166**(1-2): 173-9.

Schmied, M., P. W. Duda, et al. (2003). "In vitro evidence that subcutaneous administration of glatiramer acetate induces hyporesponsive T cells in patients with multiple sclerosis." Clin Immunol **106**(3): 163-74.

Scholz, C., K. T. Patton, et al. (1998). "Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation." J Immunol **160**(3): 1532-8.

Sebille, F., K. Gagne, et al. (2001). "Direct recognition of foreign MHC determinants by naive T cells mobilizes specific Vbeta families without skewing of the complementarity-determining region 3 length distribution." J Immunol **167**(6): 3082-8.

Sepulcre, J., A. Sanchez-Ibarrola, et al. (2005). "Association between peripheral IFN-gamma producing CD8+ T-cells and disability score in relapsing-remitting multiple sclerosis." Cytokine **32**(2): 111-6.

Serafini, B., B. Rosicarelli, et al. (2006). "Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells." J Neuropathol Exp Neurol **65**(2): 124-41.

Sette, A. and J. Sidney (1999). "Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism." Immunogenetics **50**(3-4): 201-12.

Shimonkevitz, R., C. Colburn, et al. (1993). "Clonal expansions of activated gamma/delta T cells in recent-onset multiple sclerosis." Proc Natl Acad Sci U S A **90**(3): 923-7.

Skulina, C., S. Schmidt, et al. (2004). "Multiple sclerosis: brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood." Proc Natl Acad Sci U S A **101**(8): 2428-33.

Sobel, R. A., M. E. Mitchell, et al. (1990). "Intercellular adhesion molecule-1 (ICAM-1) in cellular immune reactions in the human central nervous system." Am J Pathol **136**(6): 1309-16.

Sormani, M. P., P. Bruzzi, et al. (2005). "The distribution of the magnetic resonance imaging response to glatiramer acetate in multiple sclerosis." Mult Scler **11**(4): 447-9.

Sospedra, M., Y. Zhao, et al. (2005). "Recognition of conserved amino acid motifs of common viruses and its role in autoimmunity." PLoS Pathog **1**(4): e41.

Sotgiu, S., M. Pugliatti, et al. (2002). "Multiple sclerosis complexity in selected populations: the challenge of Sardinia, insular Italy." Eur J Neurol **9**(4): 329-41.

Spits, H. (2002). "Development of alphabeta T cells in the human thymus." Nat Rev Immunol **2**(10): 760-72.

Stasiolek, M., A. Bayas, et al. (2006). "Impaired maturation and altered regulatory function of plasmacytoid dendritic cells in multiple sclerosis." Brain **129**(Pt 5): 1293-305.

Steinman, L. (2007). "A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage." Nat Med **13**(2): 139-45.

Steinman, L. and S. S. Zamvil (2006). "How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis." Ann Neurol **60**(1): 12-21.

Streit, W. J. (2002). "Microglia as neuroprotective, immunocompetent cells of the CNS." Glia **40**(2): 133-9.

Stuve, O., S. Chabot, et al. (1997). "Chemokine-enhanced migration of human peripheral blood mononuclear cells is antagonized by interferon beta-1b through an effect on matrix metalloproteinase-9." J Neuroimmunol **80**(1-2): 38-46.

Sun, D., J. N. Whitaker, et al. (2001). "Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice." J Immunol **166**(12): 7579-87.

Sun, J. B., T. Olsson, et al. (1991). "Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls." Eur J Immunol **21**(6): 1461-8.

Suvas, S., U. Kumaraguru, et al. (2003). "CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses." J Exp Med **198**(6): 889-901.

-T-

Takahashi, K., S. Miyake, et al. (2001). "Natural killer type 2 bias in remission of multiple sclerosis." J Clin Invest **107**(5): R23-9.

Teitelbaum, D., R. Aharoni, et al. (1991). "Cross-reactions and specificities of monoclonal antibodies against myelin basic protein and against the synthetic copolymer 1." Proc Natl Acad Sci U S A **88**(21): 9528-32.

Teitelbaum, D., R. Arnon, et al. (1997). "Copolymer 1: from basic research to clinical application." Cell Mol Life Sci **53**(1): 24-8.

Teitelbaum, D., M. Fridkis-Hareli, et al. (1996). "Copolymer 1 inhibits chronic relapsing experimental allergic encephalomyelitis induced by proteolipid protein (PLP) peptides in mice and interferes with PLP-specific T cell responses." J Neuroimmunol **64**(2): 209-17.

Teitelbaum, D., A. Meshorer, et al. (1971). "Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide." Eur J Immunol **1**(4): 242-8.

Tennakoon, D. K., R. S. Mehta, et al. (2006). "Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis." J Immunol **176**(11): 7119-29.

Theoharides, T. C., V. Dimitriadou, et al. (1993). "Synergistic action of estradiol and myelin basic protein on mast cell secretion and brain myelin changes resembling early stages of demyelination." Neuroscience **57**(3): 861-71.

Toma, A., S. Haddouk, et al. (2005). "Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients." Proc Natl Acad Sci U S A **102**(30): 10581-6.

Tranquill, L. R., L. Cao, et al. (2000). "Enhanced T cell responsiveness to citrulline-containing myelin basic protein in multiple sclerosis patients." Mult Scler **6**(4): 220-5.

Trapani, J. A. and M. J. Smyth (2002). "Functional significance of the perforin/granzyme cell death pathway." Nat Rev Immunol **2**(10): 735-47.

Trapp, B. D., J. Peterson, et al. (1998). "Axonal transection in the lesions of multiple sclerosis." N Engl J Med **338**(5): 278-85.

Tsuchida, T., K. C. Parker, et al. (1994). "Autoreactive CD8+ T-cell responses to human myelin protein-derived peptides." Proc Natl Acad Sci U S A **91**(23): 10859-63.

Tuomisto, L., H. Kilpelainen, et al. (1983). "Histamine and histamine-N-methyltransferase in the CSF of patients with multiple sclerosis." Agents Actions **13**(2-3): 255-7.

-U-

Utz, U., W. E. Biddison, et al. (1993). "Skewed T-cell receptor repertoire in genetically identical twins correlates with multiple sclerosis." Nature **364**(6434): 243-7.

-V-

Vaknin-Dembinsky, A., K. Balashov, et al. (2006). "IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production." J Immunol **176**(12): 7768-74.

van Berlo, M. F., R. Warringa, et al. (1989). "Vulnerability of rat and mouse brain cells to murine hepatitis virus (JHM-strain): studies in vivo and in vitro." Glia **2**(2): 85-93.

van Horssen, J., C. M. Vos, et al. (2006). "Matrix metalloproteinase-19 is highly expressed in active multiple sclerosis lesions." Neuropathol Appl Neurobiol **32**(6): 585-93.

van Lier, R. A., I. J. ten Berge, et al. (2003). "Human CD8(+) T-cell differentiation in response to viruses." Nat Rev Immunol **3**(12): 931-9.

Van Weyenbergh, J., P. Lipinski, et al. (1998). "Antagonistic action of IFN-beta and IFN-gamma on high affinity Fc gamma receptor expression in healthy controls and multiple sclerosis patients." J Immunol **161**(3): 1568-74.

Vanderlugt, C. L. and S. D. Miller (2002). "Epitope spreading in immune-mediated diseases: implications for immunotherapy." Nat Rev Immunol **2**(2): 85-95.

Vandevyver, C., N. Mertens, et al. (1995). "Clonal expansion of myelin basic protein-reactive T cells in patients with multiple sclerosis: restricted T cell receptor V gene rearrangements and CDR3 sequence." Eur J Immunol **25**(4): 958-68.

Venken, K., N. Hellings, et al. (2006). "Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression." J Neurosci Res **83**(8): 1432-46.

Vieira, P. L., H. C. Heystek, et al. (2003). "Glatiramer acetate (copolymer-1, copaxone) promotes Th2 cell development and increased IL-10 production through modulation of dendritic cells." J Immunol **170**(9): 4483-8.

Viglietta, V., C. Baecher-Allan, et al. (2004). "Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis." J Exp Med **199**(7): 971-9.

Vollmer, T., L. Key, et al. (2004). "Oral simvastatin treatment in relapsing-remitting multiple sclerosis." Lancet **363**(9421): 1607-8.

Vos, C. M., E. S. van Haastert, et al. (2003). "Matrix metalloproteinase-12 is expressed in phagocytotic macrophages in active multiple sclerosis lesions." J Neuroimmunol **138**(1-2): 106-14.

Vranes, Z., Z. Poljakovic, et al. (1989). "Natural killer cell number and activity in multiple sclerosis." J Neurol Sci **94**(1-3): 115-23.

-W-

Washington, R., J. Burton, et al. (1994). "Expression of immunologically relevant endothelial cell activation antigens on isolated central nervous system microvessels from patients with multiple sclerosis." Ann Neurol **35**(1): 89-97.

Waubant, E., D. E. Goodkin, et al. (1999). "Serum MMP-9 and TIMP-1 levels are related to MRI activity in relapsing multiple sclerosis." Neurology **53**(7): 1397-401.

Webb, C., D. Teitelbaum, et al. (1973). "In vivo and in vitro immunological cross-reactions between basic encephalitogen and synthetic basic polypeptides capable of suppressing experimental allergic encephalomyelitis." Eur J Immunol **3**(5): 279-86.

Webb, C., D. Teitelbaum, et al. (1976). "Molecular requirements involved in suppression of EAE by synthetic basic copolymers of amino acids." Immunochemistry **13**(4): 333-7.

Weinshenker, B. G., D. M. Wingerchuk, et al. (2006). "NMO-IgG: a specific biomarker for neuromyelitis optica." Dis Markers **22**(4): 197-206.

Wiesemann, E., J. Klatt, et al. (2001). "Glatiramer acetate (GA) induces IL-13/IL-5 secretion in naive T cells." J Neuroimmunol **119**(1): 137-44.

Wiesemann, E., J. Klatt, et al. (2003). "Correlation of serum IL-13 and IL-5 levels with clinical response to Glatiramer acetate in patients with multiple sclerosis." Clin Exp Immunol **133**(3): 454-60.

Willer, C. J., D. A. Dyment, et al. (2003). "Twin concordance and sibling recurrence rates in multiple sclerosis." Proc Natl Acad Sci U S A **100**(22): 12877-82.

Wucherpfennig, K. W., J. Newcombe, et al. (1992). "Gamma delta T-cell receptor repertoire in acute multiple sclerosis lesions." Proc Natl Acad Sci U S A **89**(10): 4588-92.

Wucherpfennig, K. W., J. Zhang, et al. (1994). "Clonal expansion and persistence of human T cells specific for an immunodominant myelin basic protein peptide." J Immunol **152**(11): 5581-92.

-Y-

Yeo, T. W., P. L. De Jager, et al. (2007). "A second major histocompatibility complex susceptibility locus for multiple sclerosis." Ann Neurol **61**(3): 228-36.

Yewdell, J. W., E. Reits, et al. (2003). "Making sense of mass destruction: quantitating MHC class I antigen presentation." Nat Rev Immunol **3**(12): 952-61.

Yousry, T. A., E. O. Major, et al. (2006). "Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy." N Engl J Med **354**(9): 924-33.

Yudkin, P. L., G. W. Ellison, et al. (1991). "Overview of azathioprine treatment in multiple sclerosis." Lancet **338**(8774): 1051-5.

-Z-

Zamvil, S. S. and L. Steinman (1990). "The T lymphocyte in experimental allergic encephalomyelitis." Annu Rev Immunol **8**: 579-621.

Zang, Y. C., M. Kozovska, et al. (1998). "Restricted TCR Valpha gene rearrangements in T cells recognizing an immunodominant peptide of myelin basic protein in DR2 patients with multiple sclerosis." Int Immunol **10**(7): 991-8.

Zang, Y. C., M. M. Kozovska, et al. (1999). "Impaired apoptotic deletion of myelin basic protein-reactive T cells in patients with multiple sclerosis." Eur J Immunol **29**(5): 1692-700.

Zang, Y. C., S. Li, et al. (2004). "Increased CD8+ cytotoxic T cell responses to myelin basic protein in multiple sclerosis." J Immunol **172**(8): 5120-7.

Zeine, R., R. Pon, et al. (1998). "Mechanism of gammadelta T cell-induced human oligodendrocyte cytotoxicity: relevance to multiple sclerosis." J Neuroimmunol **87**(1-2): 49-61.

Zhang, J., S. Markovic-Plese, et al. (1994). "Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis." J Exp Med **179**(3): 973-84.

Zhou, D., R. Srivastava, et al. (2006). "Identification of a pathogenic antibody response to native myelin oligodendrocyte glycoprotein in multiple sclerosis." Proc Natl Acad Sci U S A **103**(50): 19057-62.

Zhuang, X., A. J. Silverman, et al. (1996). "Brain mast cell degranulation regulates blood-brain barrier." J Neurobiol **31**(4): 393-403.

Ziemssen, T., T. Kumpfel, et al. (2002). "Glatiramer acetate-specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy. Brain-derived neurotrophic factor." Brain **125**(Pt 11): 2381-91.

Ziemssen, T., T. Kumpfel, et al. (2005). "Secretion of brain-derived neurotrophic factor by glatiramer acetate-reactive T-helper cell lines: Implications for multiple sclerosis therapy." J Neurol Sci **233**(1-2): 109-12.

Annexes

Annexe I : Transcriptome de la chaîne β du récepteur des cellules T dans le sang de patients atteints de sclérose en plaques. Caractérisation des cellules T présentant des altérations au niveau de la répartition des différentes longueurs de CDR3.

DOI: 10.1093/brain/awh119 *Brain* (2004), **127**, 981–995

Blood T-cell receptor β chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution

David-Axel Laplaud,[1,2] Catherine Ruiz,[1] Sandrine Wiertlewski,[2] Sophie Brouard,[1] Laureline Berthelot,[1] Marina Guillet,[3] Benoît Melchior,[1] Nicolas Degauque,[1] Gilles Edan,[4] Philippe Brachet,[1] Philippe Damier[1,2] and Jean-Paul Soulillou[1]

[1]*Institut National de la Santé et de la Recherche Médicale (INSERM) Unité 437: 'Immunointervention dans les Allo- et Xénotransplantations', Institut de Transplantation et de Recherche en Transplantation (ITERT), CHU Hôtel Dieu, 30 Bd Jean Monnet,* [2]*Clinique Neurologique and Centre d'Investigation Clinique, Hôpital G. et R. Laennec, Bd J. Monod,* [3]*TcLand S.A., 30 Bd Jean Monnet, 44093 Nantes Cedex and* [4]*Service de Neurologie, Hôpital Pontchaillou, 35000, Rennes, France*

Correspondence to: Jean-Paul Soulillou, INSERM U437, 30 Bd Jean-Monnet, 44093 Nantes Cedex 01, France
E-mail: jps@nantes.inserm.fr

Summary

Multiple sclerosis is an inflammatory demyelinating disease of the CNS associated with T cells autoreactive for myelin components. In this study, we analysed the T-cell receptor (TCR) usage of the variable β (Vβ) chain transcriptome in the blood of multiple sclerosis patients at various stages of the disease using a global and quantitative comparison of the complementarity-determining region 3 length distribution (CDR3-LD) of transcripts of the 26 Vβ genes. We investigated 35 patients: 12 with a high risk of multiple sclerosis, 10 with clinically definite multiple sclerosis, 13 with a relapsing–remitting worsening and active multiple sclerosis and 13 healthy individuals. Cells bearing the TCR transcripts with altered CDR3-LD were sorted and studied for CD4 or CD8 phenotype, cytokine transcript accumulation and response to human myelin basic protein (MBP). We show that patients from all the groups have a significantly skewed blood T-cell repertoire. Vβ transcriptome patterns were more altered in patients from the clinically definite multiple sclerosis group and the worsening and active multiple sclerosis group than in the high risk group. The T cells sorted from Vβ families with altered CDR3-LD concerned both CD4 and CD8 T cells, with a more pronounced skewing in the CD8 compartment. These cells displayed a significantly increased level of interferon-γ, interleukin-2 and tumour necrosis factor-α transcripts compared with their counterparts from the healthy individual group. Furthermore, using interferon-γ enzyme-linked immunospot (ELISPOT) assays, T cells from four out of seven altered Vβ families tested from multiple sclerosis patients responded to human MBP, whereas no response was observed with human albumin or with altered Vβ families from healthy individuals. Our data support the concept of an early autoimmune component in the disease and emphasize the possible involvement of CD8-positive T cells in multiple sclerosis.

Keywords: CD4/CD8; CDR3; cytokines; multiple sclerosis; T cells; Vβ genes

Abbreviations: APC = antigen-presenting cell; CDMS = clinically definite multiple sclerosis; CDR3 = complementarity-determining region 3; CDR3-LD = complementarity-determining region 3 length distribution; ELISPOT = enzyme-linked immunospot; HI = healthy individuals; HLA = histocompatibility leukocyte antigen; HPRT = hypoxanthine phosphorylribosyl transferase; HRMS = high risk of multiple sclerosis; IFN-γ = interferon-γ; IL = interleukin; MBP = myelin basic protein; PBL = peripheral blood lymphocyte; PBMC = peripheral blood mononuclear cell; TCR = T-cell receptor; TNF-α = tumour necrosis factor-α; Vβ = variable β; WMS = worsening multiple sclerosis

Received October 1, 2003. Revised December 10, 2003. Accepted December 14, 2003. Advance Access publication February 25, 2004

Introduction

Multiple sclerosis is a chronic inflammatory and demyelinating disease of the CNS whose aetiology remains unknown. Autoimmunity seems to play a key role in multiple sclerosis pathogenesis, as suggested by the presence of autoreactive T cells for myelin components in the peripheral blood and CNS of patients (Noseworthy *et al*., 2000). A better characterization of the peripheral blood T lymphocytes in multiple sclerosis would probably serve to evaluate the disease severity, to forecast its evolution and to adopt new therapeutic strategies. One way to characterize the peripheral lymphocytes possibly implicated in multiple sclerosis is to analyse the skewing of the complementarity-determining region 3 length distribution (CDR3-LD) of the T-cell receptor (TCR) variable β chain (Vβ). Such an analysis was developed initially on the basis of a semi-quantitative assessment of CDR3-LD by reverse transcription–polymerase chain reaction (RT–PCR) technology (spectratype/Immunoscope® methods) (Pannetier *et al*., 1995). Several studies have addressed the question of CDR3-LD in unmanipulated T cells collected from the blood of multiple sclerosis patients and detected skewed β gene usage, a fact that supports the possibility of oligoclonal T-cell expansions in the blood (Musette *et al*., 1996; Gran *et al*., 1998; Lozeron *et al*., 1998; Muraro *et al*., 2002; Matsumoto *et al*., 2003). In addition, some studies have focused on myelin basic protein (MBP)-specific T lymphocytes, showing a preferential use of certain Vβ genes (Wucherpfennig *et al*., 1990; Kotzin *et al*., 1991; Oksenberg *et al*., 1993). However, these latter studies were performed following *in vitro* T-cell clone stimulation with myelin peptides, a procedure that may select and promote the growth of low frequency committed T cells and therefore may not reflect the actual autoreactive T-cell pool size involved.

Recently, we developed an approach which integrates both the analysis of CDR3-LD alterations (Immunoscope®/spectratype) and quantitative real-time PCR-based measurements of Vβ/hypoxanthine phosphoribosyl transferase (HPRT) transcript ratios, for all the possible CDR3 lengths. The data are displayed as a global 'T-cell landscape' of the whole β chain transcriptome (Sebille *et al*., 2001; Guillet *et al*., 2002). In this study, we used this approach to investigate the Vβ CDR3-LD and, more specifically, to estimate the magnitude of the skewed T-cell repertoire at various stages of the disease, including at the appearance of the first clinical symptoms. Thirty-five patients suffering from different stages of multiple sclerosis [patients with a high risk of multiple sclerosis (HRMS), patients with clinically definite multiple sclerosis (CDMS) or patients with a relapsing–remitting worsening and active multiple sclerosis (WMS)] and 13 healthy individuals (HI) were compared. Our data show that there is a detectable T-cell immune component in the blood of multiple sclerosis patients at all stages of the disease. The blood T-cell CDR3-LD alterations concerned the CD4- and CD8-positive cells but were prominent in the $CD8^+$ fraction, involving numerous β chain families in CDMS and WMS compared with patients with HRMS. In addition, the T cells from multiple sclerosis patients with a selected TCR accumulated more proinflammatory cytokine transcripts than their normal HI counterparts. Furthermore, using an interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay, we show that most of the T cells from altered Vβ families of the three groups of patients respond to human MBP, suggesting their possible role in the disease process.

Material and methods

Patients

Thirty-five patients divided into two groups were studied (their clinical characteristics are summarized in Table 1). Group I was composed of 22 patients at the onset of the disease. This group was divided into two subgroups. The HRMS group (n = 12) was composed of patients presenting a first demyelinating event clinically well defined and confirmed by neurological or ophthalmological examination and the presence of at least three Barkhof's criteria on a spinal or cerebral MRI (Barkhof *et al*., 1997). Patients in this group were referred to as HRMS patients (CHAMPS Study Group, 2002). The CDMS group (n = 10) was composed of patients undergoing a second or third relapse, reaching a clinically definite multiple sclerosis according to Poser's criteria (Poser *et al*., 1983). Blood from group I patients was collected at the time of a relapse. None of the patients were under immunosuppressive or immunoregulatory drug treatment at the time of or before the study. All patients were interviewed to confirm the absence of infectious illness or other autoimmune diseases, and all had a blood cell count within the normal range. Group II or the WMS group (n = 13) was composed of patients with a clinically definite relapsing–remitting multiple sclerosis (according to Poser's criteria) considered as requiring an immunosuppressive treatment with mitoxantrone (Edan *et al*., 1997). Patients from the WMS group were referred to as patients with a worsening and active multiple sclerosis. The criteria for mitoxantrone treatment were the loss of at least 1 point on the Expanded Disability Status Score during the previous 6 months and/or the occurrence of several relapses despite treatment compliance and the presence of at least one gadolinium-enhanced T1 lesion according to MRI. None of the patients suffered from other detectable autoimmune, inflammatory or infectious diseases. Results from laboratory tests performed at the time of blood sampling were within the normal ranges. All WMS group patients had undergone an immunomodulatory treatment that had been stopped at least 1 month before testing. Histocompatibility leukocyte antigen (HLA)-DR typing was performed for 32 patients. HLA class I typing was available for 14 patients.

HI group

Blood from 13 HI (mean age: 31.8, eight females, five males), who had been interviewed previously to rule out autoimmune or inflammatory disease, was taken for comparison. All multiple sclerosis patients and HI gave their informed consent for this study according to French legislation.

Table 1 *Main clinical characteristics of the patients*

	Age (years)	Gender	Gadolinium+ lesions	HLA	Disease duration (years)	Immunosuppressive drugs
HRMS1	21	F	0	DR2–DR4		
HRMS2	42	F	1	A3–A11–B44–B51–DR2–DR5		
HRMS3	29	F	9	DR1–DR6		
HRMS4	41	M	0	DR1–DR7		
HRMS5	30	F	1	NA		
HRMS6	35	M	0	DR2–DR4		
HRMS7	22	F	11	DR5–DR6		
HRMS8	41	F	2	A2–A3–B27–B51–DR1–DR8		
HRMS9	20	M	9	DR5–DR6		
HRMS10	24	M	0	NA		
HRMS11	31	M	0	DR4–DR7		
HRMS12	19	F	9	DR6–DR8		
Mean	29.5					
CDMS1	30	M	2	A1–A31–B44–B40–DR2–DR4	1	
CDMS2	46	F	3	NA	6 months	
CDMS3	34	F	2	A2–A3–B7–B27–DR1–DR3	7	
CDMS4	27	F	1	A2–A23–B15–B43–DR2	1	
CDMS5	37	M	3	DR2–DR4	2	
CDMS6	48	F	1	DR2	4	
CDMS7	38	F	0	A1–A3–B8–B1402–DR3–DR6	NA	
CDMS8	19	F	15	DR2–DR5	3 months	
CDMS9	39	M	0	A3–B7–B8–DR2–DR3	1	
CDMS10	38	M	0	DR4–DR5	1.5	
Mean	35.6					
WMS1	22	F	8	DR2–DR4	5	–
WMS2	36	F	8	A2–A23–B13–B15–DR4–DR5	10	Azathioprine
WMS3	30	F	3	A1–A2–B8–B18–DR3–DR4	3	–
WMS4	41	F	1	DR3	5	IFN β
WMS5	32	F	1	A1–A2–B37–B51–DR5–DR6	3	–
WMS6	38	F	1	DR2–DR7	13	IFN β
WMS7	32	F	1	A3–A11–B37–B51–DR2–DR4	1	–
WMS8	32	F	1	A1–A29–B8–B56–DR3–DR8	3	IFN β
WMS9	27	M	1	DR3–DR6	2	IFN β
WMS10	33	M	2	DR5–DR6	3	–
WMS11	44	F	1	A2–A3–B7–B27–DR2–DR4	5	IFN β
WMS12	46	F	2	A2–A28–B15–B37–DR4–DR6	3	IFN β
WMS13	32	F	8	DR4–DR6	7	Azathioprine
Mean	34.2					

F = female; M = male; NA = not available.

Blood harvesting, RNA extraction and cDNA synthesis

A 100 ml aliquot of blood was collected by venopuncture. Peripheral blood lymphocytes (PBLs) were recovered after a Ficoll gradient (Eurobio, Les Ulis, France). After washing, 2×10^7 cells were frozen in Trizol® reagent (Invitrogen™, Life Technologies, CA) for RNA extraction according to the manufacturer's instructions. The rest of the cells were frozen at –80°C in AB serum containing 7.5% dimethylsulfoxide. One day later, cells were transferred to a liquid nitrogen tank. The RNA concentration for each sample was determined by optical density measurement, and a quality control on a 1% agarose gel was performed. A 2 µg aliquot of RNA was reverse transcribed using an Invitrogen cDNA synthesis kit (Boeringher Mannheim, Indianapolis, IN) and diluted to a final volume of 100 µl.

TCR repertoire analysis

cDNA was amplified by PCR using a Cβ primer and one of the 26 Vβ-specific primers. The amplifications were performed in a 9600 Perkin-Elmer thermocycler (Applied Biosystems, Foster City, CA) as previously described (Gagne *et al*., 2000). Analysis of CDR3-LD was performed using Immunoscope® software (Pannetier *et al*., 1995; Douillard *et al*., 1996; Brouard *et al*., 1999). The percentage of CDR3-LD alteration for each Vβ family and a global percentage of CDR3-LD alteration for each individual or each group was obtained as described in Gorochov *et al*. (1998). 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 studied and the control distribution, calculated from the 13 age- and sex-matched HI. The global CDR3-LD alteration is represented as a Topview TcLandscape® (see below) enabling an easy appraisal of

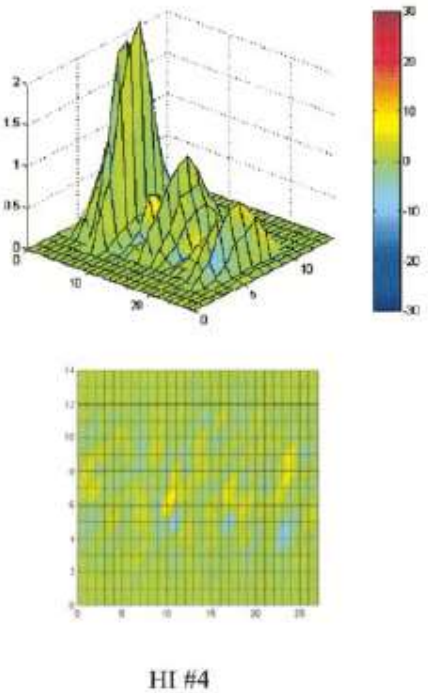
the 'qualitative' measurement of the CDR3-LD bias. Only CDR3 lengths with an alteration >25% were taken into account. The level of Vβ RNA was measured by real-time quantitative PCR and expressed as a ratio of a non- or minimally regulated gene, the HPRT gene, in order to normalize the values, and the primers used were especially designed for quantitative PCR as previously described (Gagne *et al.*, 2000). The data were displayed as a three-dimensional TcLandscape® (Guillet *et al.*, 2001; Sebille *et al.*, 2001). Percentages of CDR3-LD alterations are represented as a colour code, from deep blue (–30%) to dark red (+30%). The *x*-axis displays the 26 human Vβ families, the *y*-axis gives the Vβ/HPRT ratios, and the *z*-axis gives the CDR3 lengths. The colour code is the same for the three-dimensional TcLandscape® and the corresponding Topview.

Cytokine transcript quantification

Cytokine transcript measurement was performed on RNA from sorted Vβ families. The cells were sorted using phycoerythrin-coupled Vβ monoclonal antibodies (Immunotech, Marseille, France) on a FACSvantage (Becton-Dickinson, Mountain View, CA). The purity of sorted cells was >95%. RNA was extracted as described above and cDNA was obtained using a Boehringer SMART kit (Boehringer, IN) according to the manufacturer's recommendations. Briefly, RNA samples were mixed with cDNA synthesis primer and SMART II oligonucleotide, incubated at 72°C for 2 min and chilled on ice. First-strand buffer, dithiothreitol, deoxynucleotide triphosphate (dNTP) and PowerScript Reverse Transcriptase were then added and the tubes incubated at 42°C for 1 h, before being put on ice. A non-specific PCR was then performed, allowing a linear amplification. Pure water, Advantage 2 PCR buffer, dNTP, PCR primers and Advantage Polymerase Mix were added to the first strand cDNA obtained previously. The tubes were then placed in a Perkin-Elmer 9600 automater, and thermal cycling was performed using the following program: 1 min at 95°C, *n* cycles with 5 s at 95°C, 5 s at 65°C, and 6 min at 68°C (the number of cycles was determined by the quantity of RNA as assessed by optical density measurement). Real-time quantitative PCR was performed subsequently using interleukin (IL)-2, IFN-γ, tumour necrosis factor-α (TNF-α), IL-10, IL-13 and IL-2-Rα (CD25) chain primers (IL-2, 5′-AAACACAGCTACAACTGGAGCA-3′ and 3′-GCTGATTAAGTCCCTGGGTCTT-5′; IFN-γ, 5′-TGTCCAACGCAAAGCAATACA-3′ and 3′-TTCGCTTCCCTGTTTTAGCTG-5′; TNF-α, 5′-TTAAGCAACAAGACCACCACT-3′ and 3′-TCAAGGAAGTCTGGAAACATCT-5′; IL-10, 5′-CTGCCTAACATGCTTCGAGATC-3′ and 3′-AACCCTTAAAGTCCTCCAGCAA-5′; IL-13, 5′-GGCAGCATGG TATGGAGCA-3′ and 3′-TTCAGTTGAACCGTCCCTCG-5′; IL-2-Rα, 5′-CAAGGGTCAGGAAGATGGATTC-3′ and 3′-CCAGGACGAGTGGCTAGAGTTT-5′) and normalized against HPRT transcript levels (Guillet *et al.*, 2001).

CD4/CD8 T cell characterization

CD4+ or CD8+ T-cell selection was performed using MACS microbeads according to the manufacturer's recommendations (Miltenyi Biotec, Germany). The kit consists of an indirect magnetic labelling system composed of a hapten–monoclonal antibody cocktail (anti-CD8/anti-CD4, CD16, CD56, CD11b, CD19 and CD36) and iron microbeads coupled with an anti-hapten antibody which enables the magnetic depletion of non-T cells. The magnetic bead-labelled cells are depleted by passing the cells through a MACS column in the magnetic field of an autoMACS. Purity was >90%. The cells were mixed with Trizol® and the RNA extracted and retrotranscribed using the SMART procedure as explained above. The cDNA was then amplified and the CDR3-LD analysis performed as described above.



HI #4

Fig. 1 TcLandscape® of blood T cells from one representative HI. The data are displayed for each individual as a three-dimensional TcLandscape® and as a Topview for easier assessment of global CDR3-LD alterations. The percentages of CDR3-LD alterations are represented as a colour code. The *x*-axis displays the 26 human Vβ families, the *y*-axis gives the Vβ/HPRT ratios, and the *z*-axis gives the 13 possible CDR3 lengths.

In order to assign a selected CDR3 length to a population of CD4+ or CD8+ cells, the Immunoscope profiles of unselected PBL, CD4+ and CD8+ fractions were compared. The percentage corresponding to the frequency of a given selected CDR3 length in a Vβ family was calculated and compared in the PBL, CD4+ and CD8+ fractions. The skewing was assigned to the CD8+ cells when the calculated percentage was higher for CD8 than for CD4+ cells.

ELISPOT assay protocol

T cells from seven Vβ families with an altered CDR3 length were sorted from six patients (HRMS4, Vβ 17; HRMS7, Vβ 3; HRMS12, Vβ 8 and Vβ 14; CDMS1, Vβ 23; WMS7, Vβ 3; and WMS8, Vβ 4), and three Vβ families with an altered CDR3 length were sorted from two HI (HI9, Vβ 21 and Vβ 22; and HI10, Vβ 2) and studied for human MBP reactivity. Additionnally, three other Vβ families with a Gaussian-like CDR3-LD were sorted from three multiple sclerosis patients (WMS2, Vβ 8, WMS12, Vβ 3; and HRMS10, Vβ 23) and also studied for MBP reactivity, in the same conditions. Peripheral blood mononuclear cells (PBMCs) were thawed and washed in phosphate-buffered saline. T cells with a Vβ family with an altered CDR3 length were sorted using the corresponding phycoerythrin-

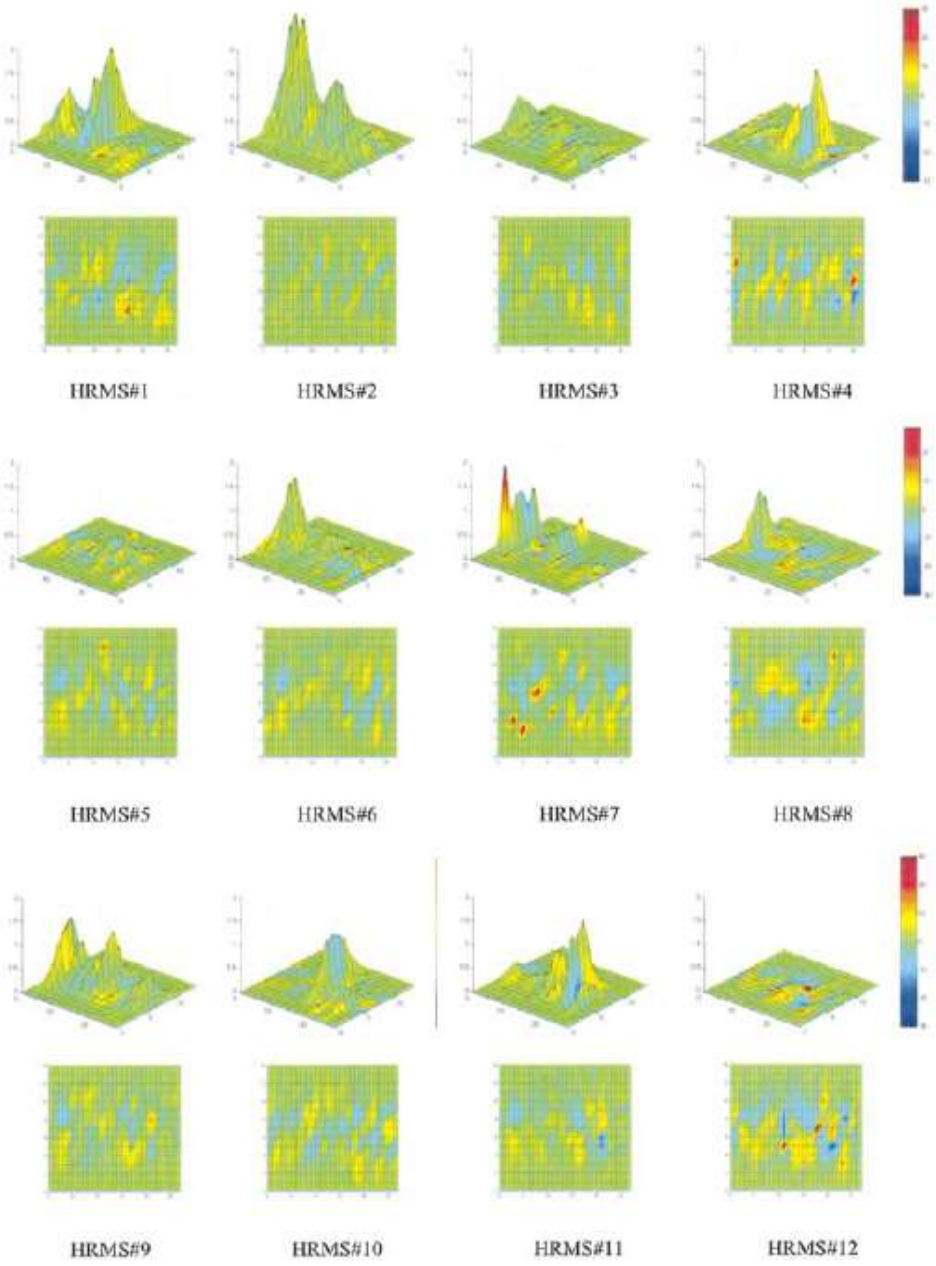


Fig. 2 TcLandscape® and Topview representation of blood T cells from the HRMS group. CDR3 length alterations >25% appear in red.

labelled anti-Vβ antibody and anti-phycoerythrin microbeads in an autoMACS (Miltenyi Biotech, Germany). The purity of the sorted Vβ families was measured by flow cytometry and was >75%. The remaining fraction of the PBMCs was irradiated for 315 s at 15 Gy and they were used as autologous antigen-presenting cells (APCs). First, human MBP (Sigma, France) at a concentration of 20 µg/ml, or human albumin (Sigma, France) at the same concentration was mixed with irradiated PBMCs. Next, 4×10^4 PBMCs were added to a 96-well anti-IFN-γ-coated ELISPOT plate (AID, Germany) and 4×10^4 of the T cells from sorted Vβ families were added to the plate. A control with irradiated PBMCs alone at the same concentration was performed systematically. The cells were incubated for 24 h at 37°C in 5% CO_2 and washed three times with washing buffer according to the manufacturer's instructions. A secondary biotinylated anti-IFN-γ antibody was then added at 100 µl/well for 2.5 h. Plates were washed three times with buffer, and streptavidin–horseradish peroxidase was added for 2 h at room temperature. Plates were washed in buffer and spot colour was developed for a maximum of 1 h by adding 3-amino-9-ethylcarbazol substrate diluted in acetate buffer containing H_2O_2. Plates were then washed with distilled water to stop the reaction. After drying, images of the wells were acquired using the AID ELISPOT software. All the

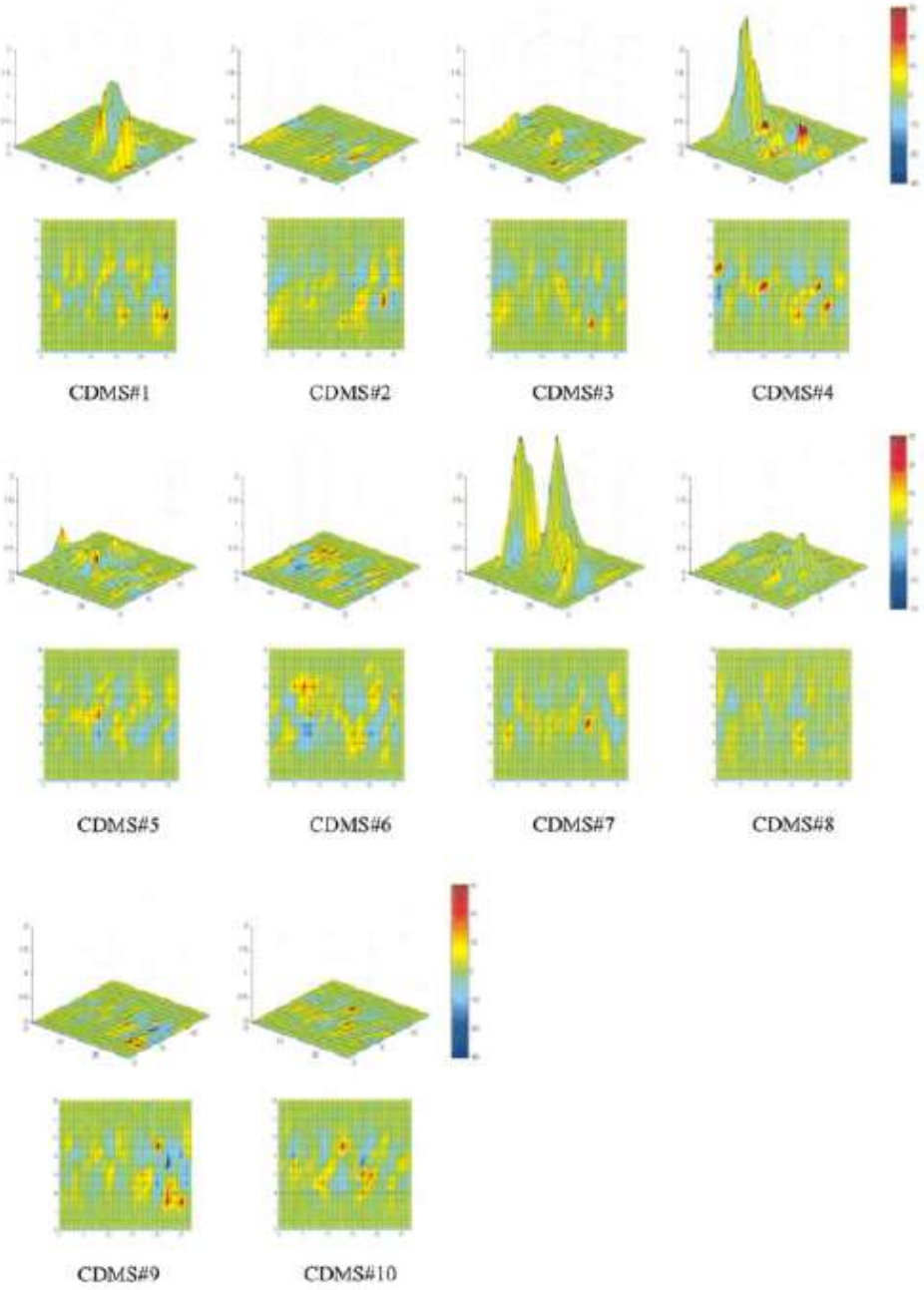


Fig. 3 TcLandscape® and Topview representation of blood T cells from the CDMS group. CDR3 length alterations >25% appear in red.

experiments were run at least in duplicate or triplicate depending on the number of cells available. The results were expressed as means of the du(tri)plicates.

Statistical analysis

A χ^2 test, a Kruskal–Wallis test and a Dunn's multiple comparison test were performed between each group of multiple sclerosis patients (HRMS, CDMS and WMS) and HI for comparison of the Vβ/HPRT transcript ratios and the global CDR3-LD profiles. A Kruskal–Wallis test was performed on cytokine transcript values. A Mann–Whitney test was performed for the different ELISPOT assays. Differences were defined as statistically significant when $P < 0.05$.

Results

HI display few Vβ families with significant CDR3-LD alterations with low Vβ/HPRT transcript ratios

Figure 1 shows one representative example of the CDR3-LD profiles from the HI group. The TcLandscape pattern of every normal individual tested is available online

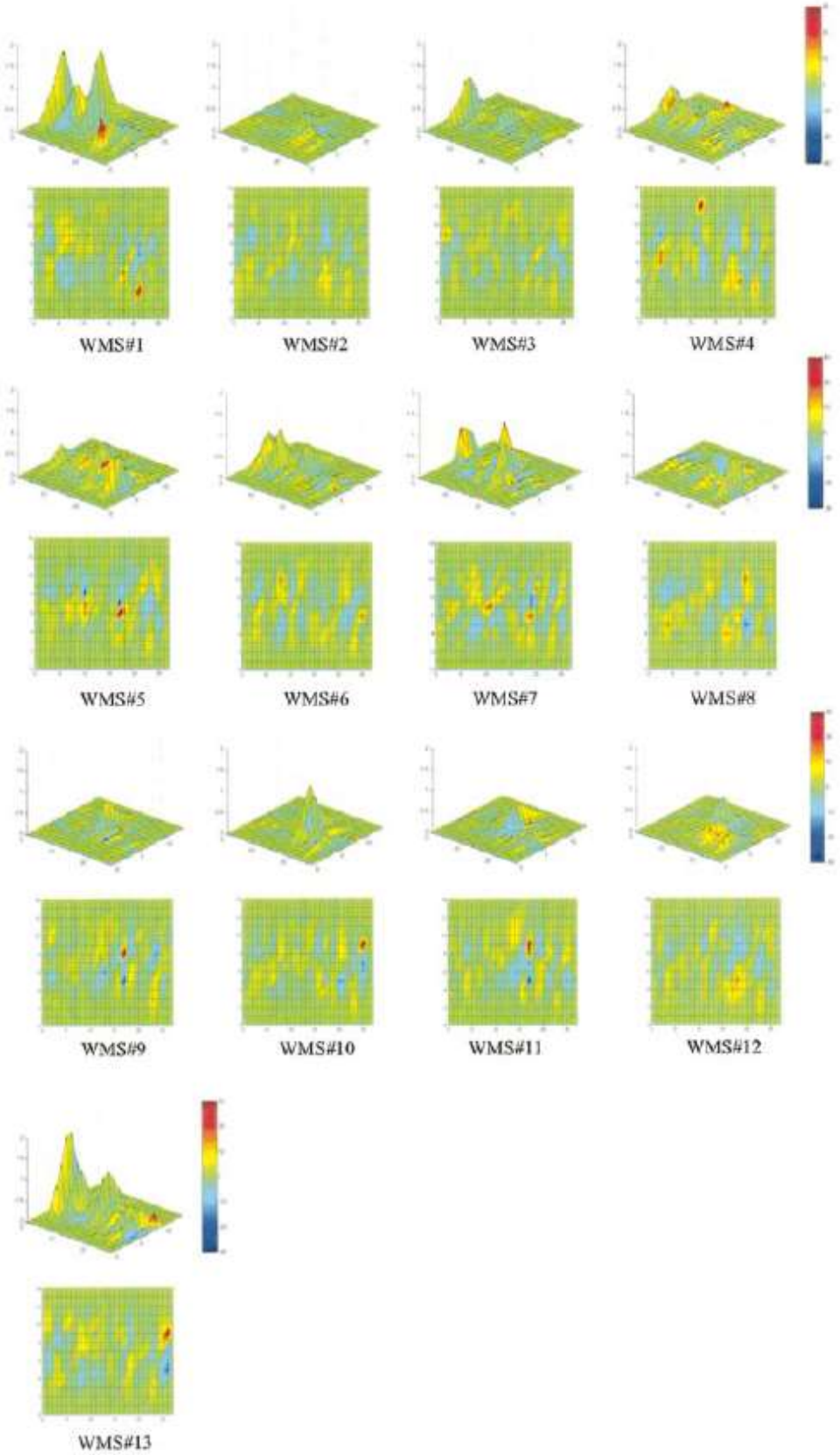


Fig. 4 TcLandscape® and Topview representation of blood T cells from the WMS group. CDR3 length alterations >25% appear in red.

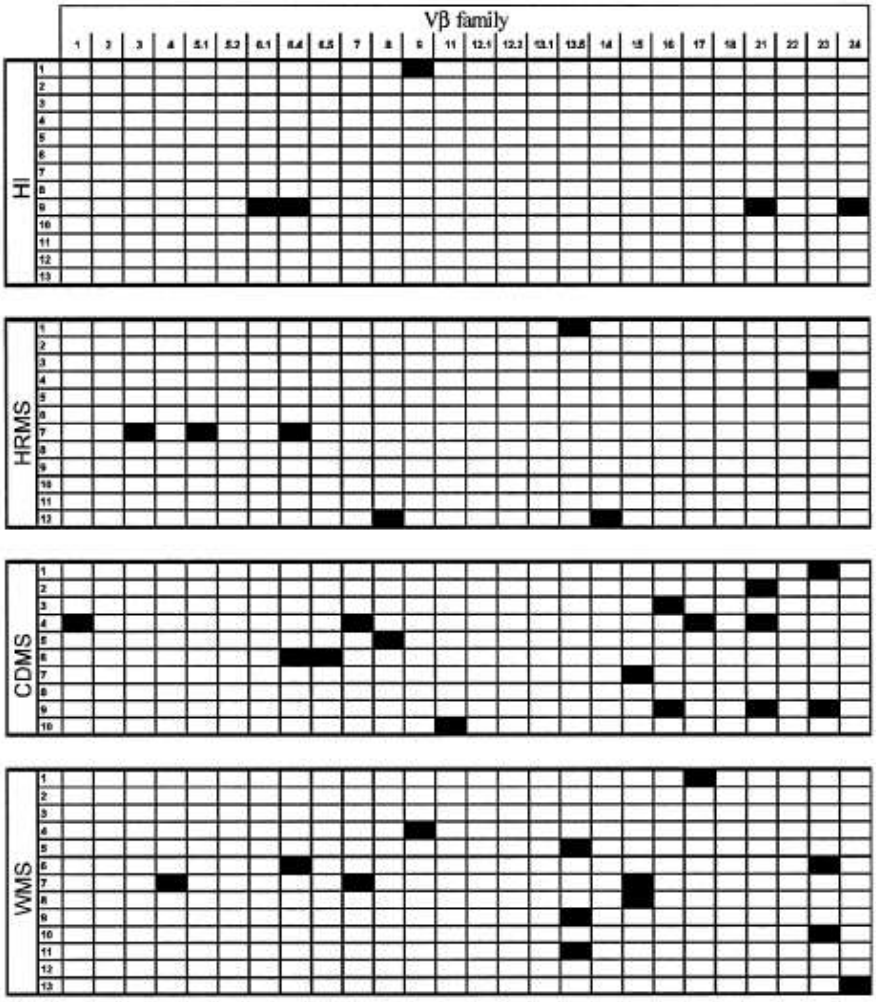


Fig. 5 CDR3 length alterations for all the patients and HI. CDR3 length alterations >25% appear in black.

(www.nantes.inserm.fr/u437/sitesconnexes.html). Only two of the 13 normal individuals expressed Vβ families with a significantly altered CDR3 length (>25%). All details concerning the alteration of Vβ families in the HI group are given in Fig. 5. Furthermore, the mean global percentage of CDR3-LD alteration in this group was 10.5 ± 1.9% (Fig. 6A). The mean Vβ/HPRT transcript ratio in the HI group was 2.3 ± 0.9. In addition, 67% of the Vβ families of HI had a Vβ/HPRT transcript ratio between 0 and 2, while 23% of the Vβ families had a ratio between 2 and 5, and 10% a ratio >5 (Fig. 6C).

HRMS patients express a significantly higher mean CDR3-LD alteration than HI

Figure 2 displays the CDR3-LD of the 12 HRMS patients. Four of the 12 patients displayed a CDR3 length with an alteration >25%. Details of the CDR3 length alterations are given in Fig. 5. Neither the number of patients with significantly altered Vβ (presence of a CDR3 length alteration >25%) nor the number of Vβ families with a significantly altered CDR3 length were different from the HI group. However, the mean percentage of CDR3-LD alterations (16.5 ± 2.9%, Fig. 6A) was significantly higher than that of the HI group (10.58 ± 1.9%, $P < 0.01$).

The blood T cells from CDMS patients display more extensive CDR3-LD alterations than those from HRMS patients and HI

Nine of the 10 CDMS patients displayed a CDR3 length with an alteration >25% (TcLandscapes® and Topviews, Fig. 3). Details of the Vβ families concerned are given in Fig. 5. The number of significant CDR3 length alterations in the CDMS group was significantly different from that of the HI and HRMS groups ($P < 0.01$). The number of patients displaying at least one altered CDR3 length in the CDMS group was also significantly different from the HI and HRMS groups ($P < 0.01$). In addition, the mean percentage of CDR3-LD

alterations (17.8 ± 1.6%, Fig. 6A) was significantly different from that observed in HI (10.58 ± 1.98%, $P < 0.001$) and in the HRMS group (16.5 ± 2.9%, $P < 0.03$).

The blood T cells from WMS patients display significantly more CDR3-LD alterations than those from HI and HRMS patients

Ten of the 13 WMS patients displayed CDR3 lengths with alterations >25% (TcLandscapes® and Topviews, Fig. 4). Details concerning the Vβ families with significant CDR3 length alterations are summarized in Fig. 5. Patients with at least one significantly altered CDR3 length (alteration >25%) were significantly more numerous than in the HI and HRMS groups ($P < 0.01$ and $P < 0.05$, respectively). Furthermore, the number of alterations >25% in the WMS group was significantly different from the HI group ($P < 0.05$). Additionally, the mean percentage of CDR3-LD alterations in WMS patients (15.5 ± 1.5%, Fig. 6A) was significantly higher than that of the HI group (10.58 ± 1.9%, $P < 0.01$) but not that of HRMS patients (16.5 ± 2.9%).

Vβ/HPRT transcript ratios

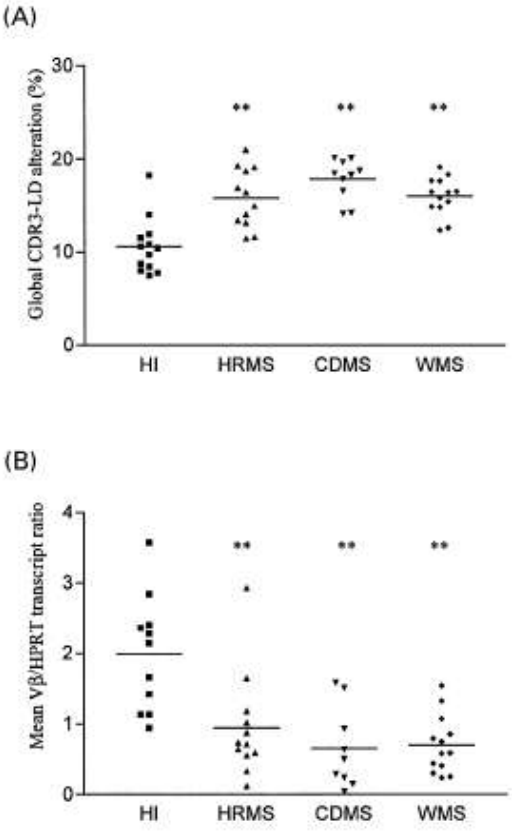
Vβ/HPRT transcript ratio data are an indirect reflection of the pool size of T-cell populations with different CDR3-LD. The mean Vβ/HPRT transcript ratios in the HRMS (0.94 ± 0.5), CDMS (0.6 ± 0.4) and WMS groups (0.7 ± 0.3) were significantly lower than those in the HI group (2.3 ± 0.9, $P < 0.001$, Fig. 6B). Furthermore, when Vβ/HPRT transcript ratios were compared according to a grading scale of 0–2, 2–5 and >5, their distribution was significantly different compared with the HI group (Fig. 6C, $P < 0.01$).

Vβ transcript CDR3-LD alterations according to HLA class I and class II typing and MRI activity

Distribution of CDR3-LD alterations according to HLA-DR typing was analysed in 32 patients, 13 of them being DR2 positive. Ten of these 13 patients displayed significant alterations versus 12 of the 18 negative for DR2 (NS). No public skewed CDR3-LD was observed in HLA-DR2 patients. No correlation between Vβ family alterations and HLA class I typing was found. Correlation of CDR3-LD alterations with T1 gadolinium-enhanced lesions was also studied. For the patients of group I, 14 exhibited at least one gadolinium-enhanced lesion. Eight of them exhibited at least an altered CDR3 length >25% versus four out of eight with no gadolinium-enhanced lesion (NS).

T cells from multiple sclerosis patients with altered CDR3-LD Vβ accumulate proinflammatory cytokine transcripts

In order to better understand the role played by the altered T cells identified by TcLandscape®, five Vβ families with highly altered CDR3-LD were sorted from three different patients (one patient per group: HRMS8, Vβ 3; CDMS4, Vβ7.1, Vβ7.2 and Vβ17; WMS1, Vβ17). These families were compared with sorted Vβ families with Gaussian-like CDR3-LD (HI10, Vβ 3 and Vβ 7; HI11, Vβ 17; HI12, Vβ 4 and Vβ 17; HI13, Vβ 1 and Vβ 16) and three Vβ families with altered CDR3-LD from HI (HI9 and HI12, Vβ 21, Vβ 22 and Vβ 13.1, respectively) for accumulation of different cytokine



(C)

Vβ/HPRT ratio	[0;2[	[2;5]	>5	p
HI	67.13%	22.37%	10.48%	-
HRMS	84.93%	10.89%	4.16%	<0.01
CDMS	92.30%	5.38%	2.30%	<0.01
WMS	90.23%	8.87%	0.88%	<0.01

Fig. 6 (A) Values of global CDR3-LD for each patient and HI. The mean value for each group is indicated by a bar. A Kruskal–Wallis test and a Dunn's multiple comparison test were performed, $^{**}P < 0.01$. (B) Distribution of the mean Vβ/HPRT transcript ratios in the patient groups and HI (each dot represents the mean Vβ/HPRT transcript ratio for each patient and HI). A Kruskal–Wallis test and a Dunn's multiple comparison test were performed to compare values. A bar indicates the mean value for each group. $^{**}P < 0.01$. (C) Distribution of the Vβ/HPRT transcript ratios between the groups according to a grading scale of 0–2, 2–5 and >5. A χ^2 test was performed to compare the distribution between the groups.

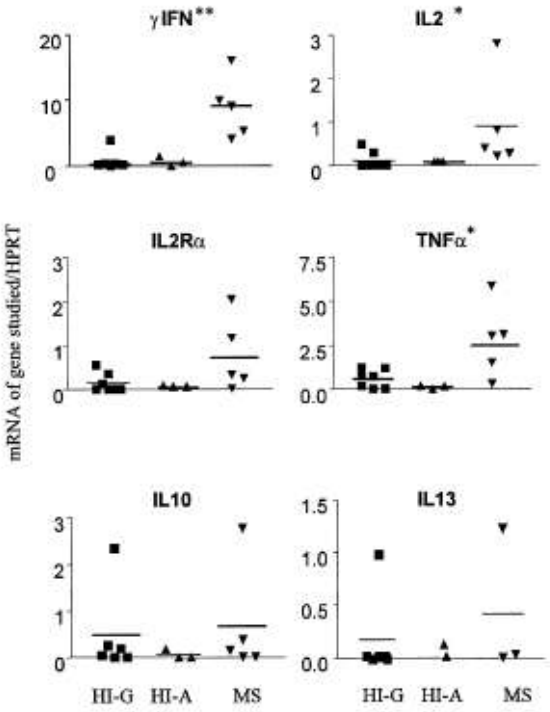


Fig. 7 Cytokine/HPRT transcript ratios from sorted T cells. Cytokine mRNA was studied for T cells sorted from families with CDR3 length alteration >25% in multiple sclerosis patients compared with T cells sorted from Vβ families with a Gaussian-like pattern (HI-G) or an altered CDR3 length (HI-A) from healthy individuals. The median range is indicated for each group. Kruskal–Wallis test, $*P < 0.05$, $**P < 0.01$.

transcripts. The data are summarized in Fig. 7. The mean IFN-γ and IL-2 mRNA levels in sorted Vβ families with altered CDR3-LD from multiple sclerosis patients was significantly higher than for families with a Gaussian-like pattern or altered CDR3-LD in the HI group ($P < 0.01$ and $P < 0.05$, respectively). TNF-α mRNA was also significantly accumulated in the multiple sclerosis group, compared with the HI group ($P < 0.05$). IL-10, IL-13 and IL-2-Rα chain mRNA transcripts were not significantly different between the three groups. Despite the fact that the number of patients analysed was low, these data suggest the presence of T cells with highly altered CDR3-LD able to produce proinflammatory cytokines in the blood of multiple sclerosis patients.

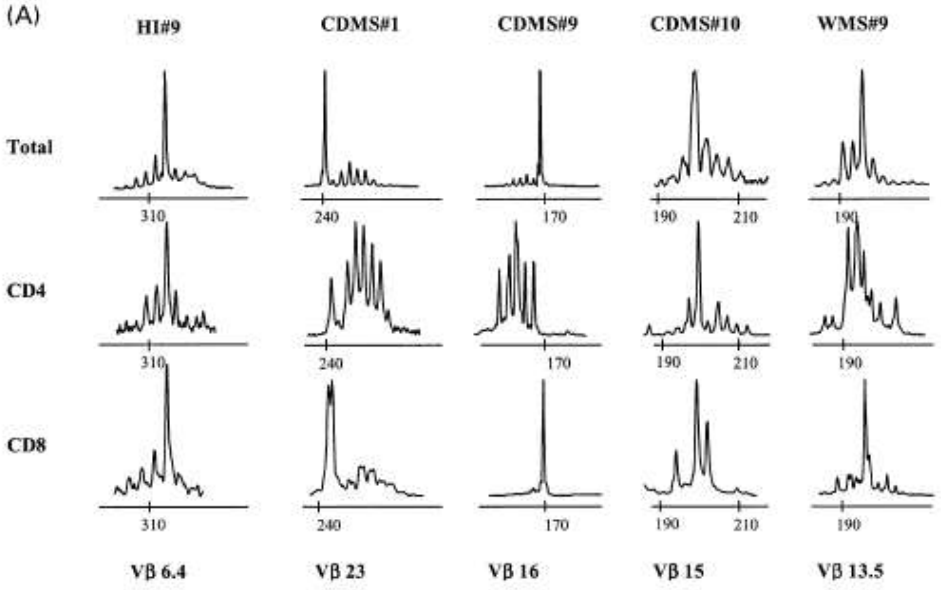
The alterations of CDR3-LD are more prominent in CD8⁺ T cells

Sorted CD4⁺ and CD8⁺ T cells from nine patients (16 Vβ families) and two HI (six Vβ families) were studied and their immunoscope profile compared with that of unselected PBLs (Fig. 8A and B). In some cases, the CD4 or CD8 nature of a selected CDR3 length was obvious (Fig. 8A, examples CDMS1 and CDMS9). In other cases (Fig. 8B: HRMS12, Vβ 14; CDMS4, Vβ 15, Vβ 17 and Vβ 21; WMS7, Vβ 7; WMS9, WMS10 and HI9, Vβ 6.4), the correspondence of the immunoscope profiles of total PBLs with that of the fraction studied (CD4 or CD8) suggested that the selected CDR3 length in question belonged to this T-cell subpopulation, which was corroborated by the comparison of the frequency of the prominent CDR3 length in the three cellular fractions. Finally, in other Vβ families (Fig 8B: CDMS4, Vβ 7; CDMS7; CDMS10; WMS7, Vβ 4 and Vβ 15; HI9, Vβ 21, Vβ 22 and Vβ 24; HI7, Vβ8 and Vβ 24), no clear assignment to CD4⁺ or CD8⁺ cells was possible. Using this approach, 11 of the 16 Vβ families from multiple sclerosis patients and one of the six Vβ families from HI with an altered CDR3 length were found to have a more prominent skewing in the CD8⁺ T cells, suggesting a more pronounced bias in the CD8⁺ repertoire in multiple sclerosis than in HI.

Sorted T cells from Vβ families with an altered CDR3-LD from multiple sclerosis patients produce IFN-γ in the presence of human MBP

IFN-γ ELISPOT assays were performed with T cells from Vβ families with altered CDR3–LD from six multiple sclerosis patients (three from the HRMS group, one from the CDMS group and two from the WMS group) and two HI. In addition, because the analysis of Vβ families with Gaussian-like CDR3-LD from patients already studied for their skewed Vβ family was technically impossible (due to an insufficient quantity of cells), three Vβ families with Gaussian-like CDR3-LD were studied from three other patients. Only background ELISPOT reactivity (<10 spots) was detected when irradiated PBMCs (used as APCs) were tested (data not shown). Figure 9A shows the ELISPOT score obtained when human MBP was added to a culture of purified sorted T cells and irradiated PBMCs used as APCs (black boxes). For comparison, the same cultures were tested with human albumin (white boxes). In addition, the irradiated PBMCs alone were also stimulated by human MBP (grey boxes). The figure shows that purified T cells from multiple sclerosis patients were highly reactive in response to human MBP ($P < 0.01$ versus human albumin). Some reactivity was also observed in the irradiated PBMC fraction when human MBP was present in the culture. However, the sorted T-cell response was much higher than that of the PBMCs alone in four out of seven cases. Importantly, Fig. 9B shows that sorted T cells from the HI counterparts cultured in the same conditions with syngenic PBMCs were not reactive to human MBP compared with altered Vβ families from multiple sclerosis patients ($P < 0.05$). Furthermore, the number of IFN-γ spots obtained with the Gaussian Vβ families from the three additional patients was as low as for the altered Vβ families from HI (mean number of spots: 2.3 ± 1), suggesting a specific response of the skewed Vβ families from multiple sclerosis patients.

(A)



(B)

Patient	Altered Vβ studied	Percentage of the selected CDR3 in CD4+CD8	CD4	CD8	CD4/CD8
HI#7	Vβ 8	52	**50**	**49**	**Both**
	Vβ 24	41	**36**	**38**	**Both**
HI#9	Vβ 6.4	56	41	**57**	**CD8**>CD4
	Vβ 21	31	**30**	**32**	**Both**
	Vβ 22	42	**43**	**40**	**Both**
	Vβ 24	36	**33**	**35**	**Both**
HRMS#12	Vβ 8	100	45	**100**	**CD8**>CD4
	Vβ 14	57	17	**60**	**CD8**>CD4
CDMS#1	Vβ 23	53	12	**54**	**CD8**>CD4
CDMS#4	Vβ 7	61	**42**	**38**	**Both**
	Vβ 15	41	28	**43**	**CD8**>CD4
	Vβ 17	54	37	**55**	**CD8**>CD4
	Vβ 21	50	32	**45**	**CD8**>CD4
CDMS#7	Vβ 15	49	**50**	**48**	**Both**
CDMS#9	Vβ 16	75	15	**91**	**CD8**
	Vβ 23	85	0	**60**	**CD8**
CDMS#10	Vβ 15	41	**29**	**32**	**Both**
WMS#7	Vβ 4	44	**41**	**45**	**Both**
	Vβ 7	41	16	**33**	**CD8**>CD4
	Vβ 15	40	**25**	**27**	**Both**
WMS#9	Vβ 13.5	46	**34**	**57**	**CD8**>CD4
WMS#10	Vβ 23	68	0	**100**	**CD8**

Fig. 8 CD4/CD8 phenotype of blood T cells from Vβ families with an altered CDR3 length. (A) Examples from Vβ families with an altered CDR3 length. The phenotype was obtained by comparison of the immunoscope profile of each population (CD4, CD8 and unselected PBLs). (B) Summary of the CD4/CD8 pattern of the Vβ families studied. CD8>CD4 means that the $CD8^+$ fraction of T cells is more represented in the selected CDR3 length than the $CD4^+$ fraction (see Material and methods). The percentage indicated represents the frequency of the more prominent CDR3 length in the Vβ family studied. The comparison of the percentages for the same CDR3 length in the different cell fractions (PBL, CD4 and CD8) indicates the fraction that is responsible for the skewing.

Discussion

In this study, we analysed the TCR Vβ chain transcriptome both qualitatively (alteration of CDR3-LD) and quantitatively (amount of transcripts concerned) in three groups of patients with relapsing–remitting multiple sclerosis at various stages of their disease compared with age-matched HI. Despite the fact that our study only concerned Vβ biases (analysis of Vα chain patterns may provide further information), significant T-cell repertoire alterations, which were more prominent in $CD8^+$ T cells and mostly concerned Vβ families reactive to

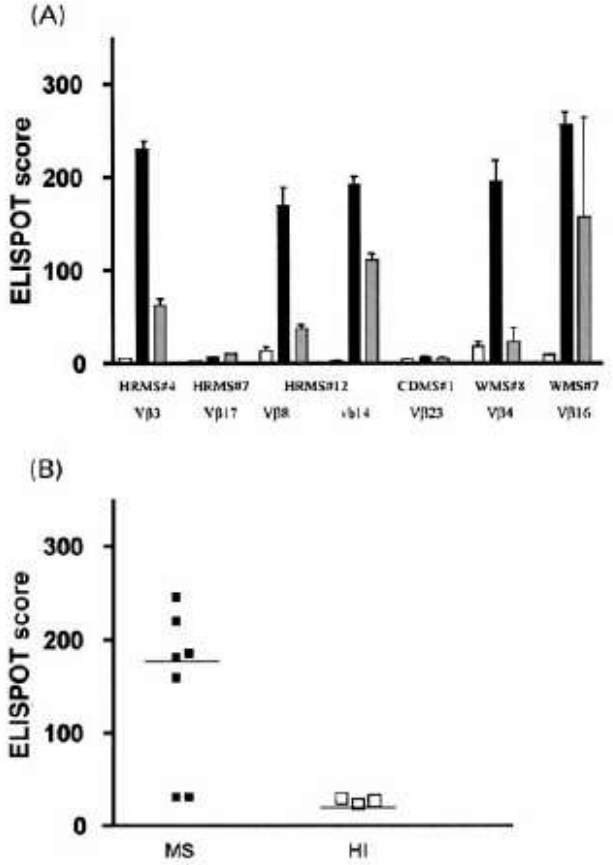


Fig. 9 IFN-γ ELISPOT in T cells sorted from Vβ families with an altered CDR3 length. (A) ELISPOT score of irradiated PBMCs (APCs) in multiple sclerosis patients (grey boxes) and irradiated PBMCs with T cells from sorted CDR3-LD-altered Vβ families in the same multiple sclerosis patients (black boxes) in the presence of human MBP. White boxes represent the control score obtained with PBMCs and sorted T cells from the same multiple sclerosis patients in the presence of human albumin. (B) Comparison of reactivity for human MBP in irradiated PBMCs with T cells sorted from CDR3-LD-altered Vβ families (black squares) or HI (white squares). Mann–Whitney test, $P < 0.01$.

human MBP, can be detected in the blood of these patients from the onset of the disease [occurrence of first clinical symptoms (HRMS)]. Furthermore, we show that sorted, circulating T cells from families with altered CDR3-LD are characterized by a transcriptional pattern with significant accumulations of IFN-γ, IL-2 and TNF-α mRNA, without any further *in vitro* stimulation. Our data provide new information that supports the concept of early peripheral T-cell activation in multiple sclerosis. Our results also suggest that multiple sclerosis patients could benefit from immunoregulatory treatment early in the course of their disease.

TCR biases in patients with multiple sclerosis have been reported by several groups in the past (Kotzin *et al.*, 1991; Oksenberg *et al.*, 1993; Musette *et al.*, 1996; Gran *et al.*, 1998; Lozeron *et al.*, 1998; Muraro *et al.*, 2002; Matsumoto *et al.*, 2003). However, this study is the first to analyse additionally a cohort of patients identified as HRMS patients. Although healthy age-matched individuals also exhibited some level of CDR3-LD alterations (~10%), HRMS patients displayed highly significant global CDR3-LD alterations (~16%). However, the Vβ/HPRT transcript ratios did not suggest blood accumulation of the T-cell populations using the altered CDR3 Vβ transcript species. This apparent lack of peripheral accumulation may be related to a continuous flux of these selected T cells toward other compartments including the CNS. Such a flux has been suggested recently by the effect of natalizumab, a monoclonal antibody inhibiting T-cell homing into the CNS compartment through endothelial cells (Miller *et al.*, 2003).

Significant global CDR3-LD alteration at a stage where the disease is in fact only suspected suggests that strategies aimed at inhibiting activated T-cell transfer to the CNS or at regulating activation of selected T cells could be useful in the very early stage of the disease. One study has analysed patients at the onset of a confirmed disease (CDMS) (Musette *et al.*, 1996). However, only a partial exploration of the T-cell repertoire was performed, with only a few Vβ families analysed. This latter study of patients with confirmed disease (i.e. at a later stage than HRMS) was performed on Vβ 5 and Vβ 17 and also reported CDR3-LD alterations. Our data confirm and extend these initial observations by showing that most of the patients at the CDMS stage have both significantly more families with altered CDR3-LD and a more altered global transcriptome (allowing a statistical comparison) than patients at the HRMS stage (see Figs 5 and 6A). The fact that 11 of the 26 Vβ families exhibited selected CDR3-LD at this stage of the disease suggests early epitope spreading. Spreading of T-cell responses against different epitopes has been well characterized in experimental models of multiple sclerosis (Vanderlugt and Miller, 2002). In humans, Tuohy *et al.* (1999) have also described a serial analysis showing the decreasing frequency of clones directed against a single epitope during the progression of the disease from a monosymptomatic demyelinating syndrome (a group of patients similar to our HRMS group) to the stage of CDMS and the appearance of new clones directed against other epitopes of the same molecule or of other myelin proteins.

It is known that the CD8 T-cell repertoire in healthy subjects is more altered than the CD4 repertoire (Gorochov *et al.*, 1998). In this study, only six altered Vβ families from two HI were observed and only one alteration clearly belonged to the $CD8^+$ cell fraction. To our knowledge, the CD4/CD8 distribution of selected T cells in the blood of multiple sclerosis patients has rarely been investigated (Monteiro *et al.*, 1996). The reason for a prominent bias in $CD8^+$ T cells in multiple sclerosis may be due to a response against viruses since it has been shown that the relapses of the disease are favoured by infections (Edwards *et al.*, 1998; Buljevac *et al.*, 2002). However, this explanation is not likely because patients with a recent or ongoing clinical infection were not enrolled in the study. Furthermore, the patients from the WMS group were not included at the moment of a relapse. Five patients of the CDMS group were able to be analysed

more closely for the phenotype of the T cells of Vβ families with altered CDR3-LD, and six out of the nine Vβ families analysed turned out to correspond in the majority to $CD8^+$ cells (see Fig. 8A and B). The fact that the altered CDR3 length species represent a large majority of the T cells of these families (as assessed from the area under the curve of the immunoscope pattern of the amplified CDR3 segments) strongly suggests that these selected clones belong more to the CD8 than the CD4 phenotype. These observations are in agreement with the findings of Battistini *et al.* (2003) who have reported that circulating $CD8^+$ T cells from multiple sclerosis patients express adhesion molecules allowing them to cross the blood–brain barrier and may explain the significant $CD8^+$ T-cell infiltrate seen in CNS lesions of multiple sclerosis (Booss *et al.*, 1983; Gay *et al.*, 1997). Moreover, these data suggest that, even if HLA class II-restricted $CD4^+$ cells play a role in disease susceptibility (Haines *et al.*, 1998), spreading involving different class I restricted effectors occurs rapidly throughout the course of the disease, possibly by a phenomenon of cross-presentation (for a review see Carbone *et al.*, 1998). In this respect, these data also suggest that besides class II tetramers (Reddy *et al.*, 2003), class I tetramers may be a pertinent tool to test the blood T cells of multiple sclerosis patients. Interestingly, the only patient analysed at the HRMS stage also displayed alterations of $CD8^+$ T cells (Fig. 8A and B). The concept that $CD8^+$ T cells may be important in multiple sclerosis has been suggested by the possibility of inducing autoreactive $CD8^+$ T cells for myelin peptides (Tsuchida *et al.*, 1994). Furthermore, the role of CD8 T cells has been highlighted recently by the findings that the majority of cells with clonal expansions and memory phenotype in the CSF and brain lesions of patients with multiple sclerosis were also $CD8^+$ T cells (Babbe *et al.*, 2000; Jacobsen *et al.*, 2002). These data and our own showing circulating $CD8^+$ CDR3-LD selected T cells in the blood of multiple sclerosis patients support the possibility that $CD8^+$ cytotoxic T lymphocytes could damage class I-expressing brain cells including oligodendrocytes and neurons (Medana *et al.*, 2001; Liblau *et al.*, 2002; Neumann *et al.*, 2002). Our observations also suggest a potential use of selected anti-Vβ antibodies in patients exhibiting expansions of circulating T cells with altered CDR3-LD as an alternative therapy.

This trend of $CD8^+$ selection was also found in two out of five Vβ families with altered CDR3-LD in the WMS group. However, these patients, who had been defined as presenting an exacerbated disease (see Material and methods; all of them were included in a mitoxantrone regimen and complied with the usual criteria for worsening disease), exhibited fewer alterations of their Vβ transcriptome than CDMS patients, despite still being significantly different from age-matched HI. Furthermore, these patients did not have more Vβ/HPRT transcript ratios with altered CDR3-LD in their blood than normal individuals. Whether this lack of more severe global Vβ transcriptome alterations in this group of patients with active disease is related to the fact that the blood sampling was not concomitant with disease exacerbation, that most of these patients (as opposed to those of other groups) had previously received immunologically oriented treatments or that an exacerbated flux of activated circulating T cells into the CNS occurred is unknown.

We were able to analyse three patients further (one from each of the clinical groups) for their transcriptional profile (real-time PCR) of the major Th1/Th2-related cytokines as well as TNF-α. This study was performed immediately following cell sorting using anti-Vβ antibodies, without *in vitro* stimulation to avoid artefactual selection. Interestingly, despite the low number of Vβ families studied, the transcriptional pattern of T cells from altered families from multiple sclerosis patients differed significantly from those observed in families with either biased or Gaussian CDR3-LD from normal individuals, reinforcing the idea that these expanding peripheral T cells may be involved in the disease process. Furthermore, IFN-γ ELISPOT assays were performed to test T cells from Vβ families with altered CDR3 lengths in each group of patients and HI. There was a striking difference in response to human MBP between T cells from Vβ families with altered CDR3-LD in multiple sclerosis patients and those from the controls. The T cells from more than half of such Vβ families from multiple sclerosis patients produced IFN-γ when stimulated with MBP, whereas none of the Vβ families tested from HI did so. The reactivity of these cells to human MBP is probably due to the CD4 fraction of these cells, since it is known that peripheral APCs may not be optimal for cross-presentation. The lack of response of three Vβ families from multiple sclerosis patients could be explained by a reactivity to other myelin or non-myelin antigen(s) or a production of cytokines other than the one tested.

These observations fit with other studies which also reported IFN-γ and/or TNF-α mobilization in this disease (Huang *et al.*, 1999; Pelfrey *et al.*, 2000; Tejada-Simon *et al.*, 2001) despite the fact that in two of these three studies, the patterns were analysed following *in vitro* stimulation with a putative multiple sclerosis antigen and that TNF-α/IFN-γ-producing $CD8^+$ T-cell clones directed against myelin peptides were found (Tsuchida *et al.*, 1994).

Taken together, our data support the concept of circulating TCR-selected T cells in multiple sclerosis and that $CD8^+$ T cells may be important in the pathophysiological processes of the disease. Our data also suggest that surveying the blood T-cell abnormalities in multiple sclerosis may be helpful for drug response studies, particularly when global Vβ transcriptome analysis is used.

Acknowledgements

We wish to thank Nelly Robillard and Yannick Oudinet for excellent technical help (cell sorting), Joanna Ashton for manuscript editing, and Mrs M. J. Mérienne, Dr T. Ronzière and Dr E. Lepage for clinical data harvesting (Rennes, France). This work was supported in part by grants from the

ARSEP (Association pour la Recherche sur la Sclerose En Plaques), the Collège des Enseignants de Neurologie, the Académie Nationale de Médecine and the Direction of Clinical Research at Nantes University Hospital.

References

Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, et al. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J Exp Med 2000; 192: 393–404.

Barkhof F, Filippi M, Miller DH, Scheltens P, Campi A, Polman CH, et al. Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. Brain 1997; 120: 2059–69.

Battistini L, Piccio L, Rossi B, Bach S, Galgani S, Gasperini C, et al. CD8+ T cells from patients with acute multiple sclerosis display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1. Blood 2003; 101: 4775–82.

Booss J, Esiri MM, Tourtellotte WW, Mason DY. Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. J Neurol Sci 1983; 62: 219–32.

Brouard S, Vanhove B, Gagne K, Neumann A, Douillard P, Moreau A, et al. T cell repertoire alterations of vascularized xenografts. J Immunol 1999; 162: 3367–77.

Buljevac D, Flach HZ, Hop WC, Hijdra D, Laman JD, Savelkoul HF, et al. Prospective study on the relationship between infections and multiple sclerosis exacerbations. Brain 2002; 125: 952–60.

Carbone FR, Kurts C, Bennett SR, Miller JF, Heath WR. Cross-presentation: a general mechanism for CTL immunity and tolerance. Immunol Today 1998; 19: 368–73.

CHAMPS Study Group. MRI predictors of early conversion to clinically definite MS in the CHAMPS placebo group. Neurology 2002; 59: 998–1005.

Douillard P, Pannetier C, Josien R, Menoret S, Kourilsky P, Soulillou JP, et al. Donor-specific blood transfusion-induced tolerance in adult rats with a dominant TCR-Vbeta rearrangement in heart allografts. J Immunol 1996; 157: 1250–60.

Edan G, Miller D, Clanet M, Confavreux C, Lyon-Caen O, Lubetzki C, et al. Therapeutic effect of mitoxantrone combined with methylprednisolone in multiple sclerosis: a randomised multicentre study of active disease using MRI and clinical criteria. J Neurol Neurosurg Psychiatry 1997; 62: 112–8.

Edwards S, Zvartau M, Clarke H, Irving W, Blumhardt LD. Clinical relapses and disease activity on magnetic resonance imaging associated with viral upper respiratory tract infections in multiple sclerosis. J Neurol Neurosurg Psychiatry 1998; 64: 736–41.

Gagne K, Brouard S, Giral M, Sebille F, Moreau A, Guillet M, et al. Highly altered V beta repertoire of T cells infiltrating long-term rejected kidney allografts. J Immunol 2000; 164: 1553–63.

Gay FW, Drye TJ, Dick GW, Esiri MM. The application of multifactorial cluster analysis in the staging of plaques in early multiple sclerosis. Identification and characterization of the primary demyelinating lesion. Brain 1997; 120: 1461–83.

Gorochov G, Neumann AU, Kereveur A, Parizot C, Li T, Katlama C, et al. Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy. Nat Med 1998; 4: 215–21.

Gran B, Gestri D, Sottini A, Quiros Roldan E, Bettinardi A, Signorini S, et al. Detection of skewed T-cell receptor V-beta gene usage in the peripheral blood of patients with multiple sclerosis. J Neuroimmunol 1998; 85: 22–32.

Guillet M, Sebille F, Soulillou J. TCR usage in naive and committed alloreactive cells: implications for the understanding of TCR biases in transplantation. Curr Opin Immunol 2001; 13: 566–71.

Guillet M, Brouard S, Gagne K, Sebille F, Cuturi MC, Delsuc MA, et al. Different qualitative and quantitative regulation of V beta TCR transcripts during early acute allograft rejection and tolerance induction. J Immunol 2002; 168: 5088–95.

Haines JL, Terwedow HA, Burgess K, Pericak-Vance MA, Rimmler JB, Martin ER, et al. Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group. Hum Mol Genet 1998; 7: 1229–34.

Huang WX, Huang P, Link H, Hillert J. Cytokine analysis in multiple sclerosis by competitive RT–PCR: a decreased expression of IL-10 and an increased expression of TNF-alpha in chronic progression. Mult Scler 1999; 5: 342–8.

Jacobsen M, Cepok S, Quak E, Happel M, Gaber R, Ziegler A, et al. Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients. Brain 2002; 125: 538–50.

Kotzin BL, Karuturi S, Chou YK, Lafferty J, Forrester JM, Better M, et al. Preferential T-cell receptor beta-chain variable gene use in myelin basic protein-reactive T-cell clones from patients with multiple sclerosis. Proc Natl Acad Sci USA 1991; 88: 9161–5.

Liblau RS, Wong FS, Mars LT, Santamaria P. Autoreactive CD8 T cells in organ-specific autoimmunity: emerging targets for therapeutic intervention. Immunity 2002; 17: 1–6.

Lozeron P, Chabas D, Duprey B, Lyon-Caen O, Liblau R. T cell receptor V beta 5 and V beta 17 clonal diversity in cerebrospinal fluid and peripheral blood lymphocytes of multiple sclerosis patients. Mult Scler 1998; 4: 154–61.

Matsumoto Y, Yoon WK, Jee Y, Fujihara K, Misu T, Sato S, et al. Complementarity-determining region 3 spectratyping analysis of the TCR repertoire in multiple sclerosis. J Immunol 2003; 170: 4846–53.

Medana I, Martinic MA, Wekerle H, Neumann H. Transection of major histocompatibility complex class I-induced neurites by cytotoxic T lymphocytes. Am J Pathol 2001; 159: 809–15.

Miller DH, Khan OA, Sheremata WA, Blumhardt LD, Rice GP, Libonati MA, et al. A controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med 2003; 348: 15–23.

Monteiro J, Hingorani R, Peroglizzi R, Apatoff B, Gregersen PK. Oligoclonality of CD8+ T cells in multiple sclerosis. Autoimmunity 1996; 23: 127–38.

Muraro PA, Bonanni L, Mazzanti B, Pantalone A, Traggiai E, Massacesi L, et al. Short-term dynamics of circulating T cell receptor V beta repertoire in relapsing–remitting MS. J Neuroimmunol 2002; 127: 149–59.

Musette P, Bequet D, Delarbre C, Gachelin G, Kourilsky P, Dormont D. Expansion of a recurrent V beta 5.3+ T-cell population in newly diagnosed and untreated HLA-DR2 multiple sclerosis patients. Proc Natl Acad Sci USA 1996; 93: 12461–6.

Neumann H, Medana IM, Bauer J, Lassmann H. Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. Trends Neurosci 2002; 25: 313–9.

Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. N Engl J Med 2000; 343: 938–52.

Oksenberg JR, Panzara MA, Begovich AB, Mitchell D, Erlich HA, Murray RS, et al. Selection for T-cell receptor V beta–D beta–J beta gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. Nature 1993; 362: 68–70.

Pannetier C, Even J, Kourilsky P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol Today 1995; 16: 176–81.

Pelfrey CM, Rudick RA, Cotleur AC, Lee JC, Tary-Lehmann M, Lehmann PV. Quantification of self-recognition in multiple sclerosis by single-cell analysis of cytokine production. J Immunol 2000; 165: 1641–51.

Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Ann Neurol 1983; 13: 227–31.

Reddy J, Bettelli E, Nicholson L, Waldner H, Jang MH, Wucherpfennig KW, et al. Detection of autoreactive myelin proteolipid protein 139–151-specific T cells by using MHC II (IAs) tetramers. J Immunol 2003; 170: 870–7.

Sebille F, Gagne K, Guillet M, Degauque N, Pallier A, Brouard S, et al. Direct recognition of foreign MHC determinants by naive T cells

mobilizes specific Vbeta families without skewing of the complementarity-determining region 3 length distribution. J Immunol 2001; 167: 3082–8.

Tejada-Simon MV, Hong J, Rivera VM, Zhang JZ. Reactivity pattern and cytokine profile of T cells primed by myelin peptides in multiple sclerosis and healthy individuals. Eur J Immunol 2001; 31: 907–17.

Tsuchida T, Parker KC, Turner RV, McFarland HF, Coligan JE, Biddison WE. Autoreactive CD8+ T-cell responses to human myelin protein-derived peptides. Proc Natl Acad Sci USA 1994; 91: 10859–63.

Tuohy VK, Yu M, Yin L, Kawczak JA, Kinkel RP. Spontaneous regression of primary autoreactivity during chronic progression of experimental autoimmune encephalomyelitis and multiple sclerosis. J Exp Med 1999; 189: 1033–42.

Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. Nat Rev Immunol 2002; 2: 85–95.

Wucherpfennig KW, Ota K, Endo N, Seidman JG, Rosenzweig A, Weiner HL, et al. Shared human T cell receptor V beta usage to immunodominant regions of myelin basic protein. Science 1990; 248: 1016–9.

Etude du répertoire T et de la spécificité des lymphocytes T CD8+ chez des patients atteints de sclérose en plaques

La sclérose en plaques (SEP) est une maladie chronique inflammatoire du système nerveux central (SNC) associée à la présence de lymphocytes T auto-réactifs. Les patients atteints de SEP présentent plus d'altérations du répertoire T dans le sang que des individus sains, plus marquées dans le compartiment lymphocytaire T CD8+. Dans une première étude, le répertoire Vβ dans le sang de patients atteints de SEP a été analysé longitudinalement et en parallèle les données cliniques ont été récoltées par imagerie par résonance magnétique. La proportion de familles Vβ altérées corrèle avec le volume lésionnel et le nombre de lésions positives au Gadolinium. De plus, l'apparition de nouvelles familles Vβ altérées dans le sang corrèle avec la formation de nouvelles lésions au niveau du SNC. L'étude du répertoire T chez des patients atteints de SEP et traités par l'acétate de glatiramère (GA) n'a cependant pas montré de modifications majeures après traitement. Au niveau du SNC, les cellules infiltrantes présentent un répertoire T extrêmement altéré à la fois au niveau des lésions actives et chroniques mais également au niveau de la substance blanche d'apparence normale, renforçant l'idée d'un état général d'inflammation. Concernant la spécificité des lymphocytes T CD8+ dans la SEP, nous avons décrit 69 nouveaux épitopes de la myéline. Il n'y a pas de différence de fréquences de cellules CD8+ auto-réactives dans le sang des patients atteints de SEP et des individus sains. L'activation ou la régulation des effecteurs cellulaires semblent par conséquent importantes dans la pathologie de la SEP.

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

Study of T cell repertoire and CD8+ T cell specificity in multiple sclerosis patients

Multiple sclerosis (MS) is a chronic inflammatory disease of central nervous system (CNS) associated with auto-reactive T cells. MS patients show increased alterations in T cell repertoire, predominantly in CD8+ T cell compartment. In a first study, Vβ repertoire in MS patient blood was analyzed in a longitudinal study, collecting in parallel clinical data obtained from magnetic resonance imaging. The proportion of altered Vβ families correlates with lesional volume and the number of Gadolinium positive lesions. Moreover, the apparition of new altered Vβ families correlates with the formation of new SNC lesions. In contrast T cell repertoire study in MS patients treated with glatiramer acetate (GA) did not show major modifications after treatment. SNC infiltrated T cells show a highly altered T cell repertoire in active and chronic SNC lesions but also in normal appearing white matter, confirming general inflammation. Concerning CD8+ T cell specificity, we have described 69 new myelin epitopes. There is no difference of auto-reactive CD8+ T cell frequency in blood of MS patients and healthy individuals. Activation or regulation of effectors seem to be important in MS pathology.

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