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Expansion and Functional Relevance of High-Avidity Myelin-Specific CD4$^+$ T Cells in Multiple Sclerosis

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Multiple sclerosis (MS) is an autoimmune disease in which myelin-specific T cells are believed to play a crucial pathogenic role. Nevertheless, so far it has been extremely difficult to demonstrate differences in T cell reactivity to myelin Ag between MS patients and controls. We believe that by using unphysiologically high Ag concentrations previous studies have missed a highly relevant aspect of autoimmune responses, i.e., T cells recognizing Ag with high functional avidity. Therefore, we focused on the characterization of high-avidity myelin-specific CD4$^+$ T cells in a large cohort of MS patients and controls that was matched demographically and with respect to expression of MHC class II alleles. We demonstrated that their frequency is significantly higher in MS patients while the numbers of control T cells specific for influenza hemagglutinin are virtually identical between the two cohorts; that high-avidity T cells are enriched for previously in vivo-activated cells and are significantly skewed toward a proinflammatory phenotype. Moreover, the immunodominant epitopes that were most discriminatory between MS patients and controls differed from those described previously and were clearly biased toward epitopes with lower predicted binding affinities to HLA-DR molecules, pointing at the importance of thymic selection for the generation of the autoimmune T cell repertoire. Correlations between selected immunological parameters and magnetic resonance imaging markers indicate that the specificity and function of these cells influences phenotypic disease expression. These data have important implications for autoimmunity research and should be considered in the development of Ag-specific therapies in MS. ***The Journal of Immunology*****, 2004, 172: 3893–3904.**

Multiple sclerosis (MS)[2] is the most frequent demyelinating CNS disorder of young adults that leads to significant disability (1). Available therapies are only partially effective and are associated with considerable side effects upon long-term administration (2).

In analogy to experimental autoimmune encephalomyelitis (EAE), an animal model of MS, it is believed that myelin-specific T cells play a crucial role in the pathogenesis of MS. Nevertheless, extensive research efforts failed to demonstrate marked differences in the myelin reactivity between MS patients and healthy donor (HD) controls, and in fact many of these studies concluded that such differences do not exist (3–8). Other studies, focusing on functional characteristics of myelin-specific T cells were generally more successful in this regard (9–12), but even the differences emerging from these studies were viewed as rather unimpressive when compared with available animal data.

There are several reasons that can explain the discrepancy between the strength of evidence for the involvement of myelin-specific T cells in the pathogenesis of MS vs its animal model EAE. For ethical reasons, MS-related immunology is generally restricted to analysis of the peripheral T cell repertoire instead of the target organ, where pathogenic T cells would be preferentially located. In relapsing-remitting disease timing of the assay in relation to inflammatory attacks is virtually unpredictable. In contrast to EAE, the relevant autoantigens in MS are not known for certain. Most likely the immune response is directed to several autoantigens at the same time and these may differ from patient to patient. Consequently, it is not surprising that it has been much more difficult to establish the relevance of myelin-specific T cells in MS when compared with elegant animal models.

Animal experiments including those in EAE demonstrated that T cells recognizing peptide/MHC ligands with high affinity are deleted by thymic-negative selection (13, 14). However, in contrast to the physicochemical affinity between the TCR and its MHC/peptide ligand, the avidity of the interaction between T cell and APC is the sum of multiple factors including coreceptors, costimulatory and adhesion molecules, as well as signaling machinery and others. The outcome of these interactions results in partial or full activation of the responding T cell, which is read out by, for example, proliferation and cytokine secretion. Importantly, some of these components of T cell-APC interactions are highly variable (in contrast to MHC-peptide TCR affinities, which are constant) and are up-regulated during the inflammatory process associated with tissue destruction, like the one present in autoimmune diseases. Therefore, it is more likely that the self-reactive T cells that are relevant in autoimmunity will preferentially recognize those Ags that bind to MHC with lower biochemical affinity, but will compensate by functional changes for this suboptimal MHC-ligand binding. Animal data support this hypothesis: Inbred animal

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[2] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; HD, healthy donor; MBP, myelin basic protein; PLP, proteolipid protein; MOG, myelin oligodendrocyte glycoprotein; CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; TCL, T cell line; Flu-Ha, influenza hemagglutinin; MRI, magnetic resonance imaging; WMLL, white matter lesion load; BFV, brain fractional volume; Gd, gadopentate; BHV, black hole volume; BHFr, black hole fraction; APL, altered peptide ligand.

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strains that develop spontaneous autoimmunity express unique MHC class II alleles that bind peptides with unusually weak affinity, like I-A^{g7} in nonobese diabetic mice (15), and in analogy, in animals with induced autoimmunity, pathogenic T cells are specific for a peptide that binds poorly to MHC class II, e.g., the encephalitogenic myelin basic protein (MBP) peptide Ac_{1-11} to I-A^u in B10.PL mice (13, 16). In addition, human data indicate that autoreactive peripheral blood $CD4^+$ T cells from patients suffering from organ-specific autoimmunity express TCR that recognize peptide-MHC complexes with low affinity, as measured by dissociation of MHC class II tetramers (17).

Based on these considerations, we decided to study avidities of autoreactive $CD4^+$ T cells by using a functional (proliferation) readout. In in vitro assays, the most readily modifiable component contributing to T cell Ag avidity is the concentration of Ag. Therefore, keeping all other components of T cell-APC interaction unaltered, we define the functional avidity of a T cell as the expression of T cell effector functions at a particular concentration of Ag.

In contrast to foreign Ags, comparably higher doses of autoantigens are needed to augment weak proliferative responses to these Ags, and such unphysiologically high Ag concentrations were used in almost all human studies. However, recent data demonstrated that different doses of Ag not only expanded different numbers of T cells, but also modified the repertoire and Ag avidities of the responding population: higher Ag doses recruited higher numbers of low-avidity T cells, whereas lower Ag doses expanded lower numbers of high-avidity cells (18–20). Most importantly in this regard, a previous study of MBP-specific T cells in humans demonstrated that the repertoire of T cells expanded in vitro with low vs high Ag concentrations is generally nonoverlapping, i.e., the high-avidity T cell lines (TCL) were evidently absent from the cultures selected by high concentrations of MBP (20). Consequently, we conclude that the traditional use of high Ag concentrations led to a focus on low-avidity autoreactive T cells in autoimmunity research. It remains questionable whether these T cells can be activated with the autoantigen in vivo or whether they rather represent cross-reactive cells specific for different nominal Ags and are mostly unrelated to the autoimmune processes. The emerging animal studies suggest that the latter is true (13, 21), and our clinical experience with an altered peptide ligand of MBP_{83-99} demonstrated that disease-mediating T cells recognized the native MBP peptide with unexpectedly high functional avidities (22, 23).

Based on these considerations and as a major difference to all previous studies (reviewed in Ref. 24), we decided to use low concentrations of myelin peptides to expand high-avidity autoreactive T cells and compare their frequency and phenotype between MS patients and HD. We demonstrate that high-avidity myelin-specific $CD4^+$ T cells can be readily expanded from the blood of MS patients and that their precursor frequencies are significantly higher than in HD. In addition, these cells have a strong proinflammatory bias and originate more likely from the in vivo-activated T cells, which supports their relevance to the disease process. In agreement with predictions from animal models, the immunodominant epitopes from the high-avidity repertoire are different from those previously reported and are generally characterized by weak predicted MHC class II binding or by lack of thymic expression.

Materials and Methods

Patients and HD

Fifty-five untreated patients (50 with clinically definite MS, 42 relapsing-remitting MS, 8 secondary progressive-MS, and 5 with clinically isolated demyelinating syndrome fulfilling magnetic resonance imaging (MRI) criteria for MS (25) were included. Fifteen age-, sex-, and MHC class II-matched HD were recruited from the National Institutes of Health/Blood Bank database (Table I). The study was approved by the National Institute of Neurological Disorders and Stroke (NINDS) Institutional Review Board.

Antigens

Fifteen peptides from four major myelin proteins were selected based on their immunodominance in humans and/or encephalitogenicity in animals (Table II). Peptides ranged from 13 to 33 aa to include all reported epitopes. Five peptides were derived from MBP, five peptides from proteolipid protein (PLP), three from myelin oligodendrocyte glycoprotein (MOG), and two peptides from 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP). Influenza hemagglutinin (Flu-$HA_{306-318}$) served as positive control. Peptides were synthesized by standard methods (26), purified to >90% by reversed-phase HPLC, and their identities tested by ion spray mass spectrometry.

Functional assays

The immunological assays are depicted in Table III.

Seeding of PBMC and IL-7-modified primary proliferation assay

PBMC were isolated from leukophereses by density gradients. Leukophereses were collected between 8 and 11 am to exclude diurnal variations and processed within 2 h. PBMC (1×10^5 PBMC/well, 60 wells per Ag) were seeded fresh in 96-well U-bottom plates in serum-free X-vivo 15 medium (BioWhittaker, Walkersville, MD) enriched with IL-7 (10 ng/ml; PeproTech, Rocky Hill, NJ). All peptides were used at 1 μM. Sixty wells without Ag served as negative control. After 7 days of incubation at 37°C

Table I. *Demographic and MRI data for the MS patients and HD controls*

	MS (n = 55)	HD (n = 15)
Demographic data[a]		
Age (median; range)	39.0 (21–56)	35.0 (27–66)
Gender (male:female)	1:1.6	1:1.5
HLA-DR2 (%)	45.5	53.3
HLA-DR4 (%)	29.1	20.0
Clinical and MRI characteristics of MS cohort[b] (median; range)		
Disease duration in years from first clinical symptom	5.8 (0.2–37.0)	N/A
Expanded disability Status Scale (scale from 0 = best to 10 = worst)	2.0 (0–7.0)	N/A
Exacerbation rate (no. of exacerbations per year)	0.6 (0.08–6.25)	N/A
Average no. of contrast-enhancing lesions on brain MRI	1.0 (0–17.38)	N/A

[a] There are no statistically significant differences in demographic data between MS patients and HD.
[b] Clinical and MRI characteristics of MS cohort are given to facilitate the comparison with other published MS cohorts.

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Table II. *Selected peptides used*

Description	Sequence	aa	M_r
$MBP_{13–32}$	KYLATASTMDHARHGFLPRH	20	2351.1
$MBP_{83–99}$	ENPVVHFFKNIVTPRTP	17	2036.3
$MBP_{111–129}$	LSRFSWGAEGQRPGFGYGG	19	2070.6
$MBP_{131–155}$	ASDYKSAHKGLKGVDAQGTLSKIFK	25	2691.1
$MBP_{146–170}$	AQGTLSKIFKLGGRDSRSGSPMARR	25	2718.2
$PLP_{40–60}$	TGTEKLIETYFSKNYQDYEYL	21	2647.0
$PLP_{89–106}$	GFYTTGAVRQIFGDYKTT	18	2066.5
$PLP_{139–154}$	HCLGKWLGHPDKFVGI	16	1848.6
$PLP_{178–197}$	NTWTTCQSIAFPSKTSASIG	20	2141.6
$PLP_{190–208}$	SKTSASIGSLCADARMYGVLP	20	2071.8
$MOG_{1–20}$	GQFRVIGPRHPIRALVGDEV	20	2215.8
$MOG_{11–30}$	PIRALVGDEVELPCRISPGK	20	2190.8
$MOG_{35–55}$	MEVGWYRPPFSRVVHLYRNGK	21	2633.2
$CNP_{343–373}$	EVGELSRGKLYSLGNGRWMLTLAKNMEVRAI	31	3534.2
$CNP_{356–388}$	GNGRWMLTLAKNMEVRAIFTGYYGKGKPVPTQG	33	3683.4
Flu-$HA_{306–318}$	PKYVKQNTLKLAT	13	1508.8

and in 5% CO_2, 50% of the individual cultures were transferred into new 96-well plates ("daughter plates"). While 100 μl of IL-2 (20 IU/ml; National Institutes of Health/National Cancer Institute, Frederick, MD)-enriched T cell medium (IMDM; Life Technologies, Grand Island, NY) containing 2 mM L-glutamine, 50 μg/ml gentamicin, and 100 U/ml penicillin/streptomycin (all Whittaker Bioproducts, Gaithersburg, MD) and 5% pooled human plasma) was added to all "mother plates," the daughter plates were pulsed for 6 h with 1 μCi/well [^{3}H]thymidine (Amersham, Arlington Heights, IL). The incorporated radioactivity (cpm) was measured by scintillation counting (1450 Microbeta; Wallac/PerkinElmer Life Sciences, Boston, MA). Individual wells were considered positive if their cpm were at least 3 SD above the average cpm of 60 control wells.

Restimulation of positive cultures from IL-7PP and confirmation assay in Ag-avidity format

Positive cultures were restimulated on days 10–12 (Table III) in 48 well-plates with 2×10^6 autologous irradiated Ag-pulsed (1 μM) PBMC. Eight to 10 days later, the Ag specificity of each culture was confirmed in a 48-h Ag-avidity assay (27) (Table III, days 18–20). Briefly, T cells were plated in 96-well plates at 2×10^4 T cells/well and irradiated autologous PBMC (1×10^5 cells/well) and Ag (0.001–10 μM) were added in duplicates. Thirty-six hours later, supernatants were collected and stored at −70°C until analysis. For the last 8 h, cells were pulsed with tritiated [^{3}H]thymidine, and proliferation was measured as above. The EC_{50}, i.e., the half-maximal stimulatory concentration, was derived from Ag-avidity curves.

Flow cytometry-based classification of naive vs memory origin of TCL

Confirmed Ag-specific TCL were analyzed by flow cytometry (FACScan; BD Biosciences, Mountain View, CA) using CellQuest software (Table III, days 20–24) for surface expression of CD4, CD45RA, and CD45RO to determine their origin from naive vs memory T cells as described previously (27). This method is based on data that memory-originating $CD4^+$ T cells express the CD45RO, but not CD45RA. Upon Ag-induced clonal expansion their expression pattern of CD45 isoforms does not change (i.e., they remain $CD45RA^-/RO^+$) (27–29). In contrast naive-originating $CD4^+$ T cells express CD45RA, but not CD45RO. Upon Ag-driven expansion, they gradually down-modulate CD45RA and start up-regulating CD45RO ($CD45RA^+/RO^+$ stage); however, several restimulation cycles are necessary to achieve the memory ($CD45RA^-/RO^+$) phenotype (27–29). Therefore, up until the second and third in vitro restimulation cycle, the naive vs memory origin of $CD4^+$ TCL can be assessed by surface CD45RA/RO expression. Based on the time kinetics of down-modulation of CD45RA allele from the cell surface of naive T cells as analyzed in detail in our previous studies (27, 28), when analyzed at the end of the second in vitro restimulation cycle (i.e., days 20–24 ex vivo) memory-originating TCL have consistently >90% $CD45RA^-/RO^+$ T cells (contamination from persistent APC accounts for some CD45RA-expressing cells), whereas naive-originating TCL have <70% $CD45RA^-/RO^+$ T cells and >30% of $CD45RA^+/RO^+$ T cells. By this time there are only negligible amounts of $CD45RA^+/RO^-$ T cells in any in vitro-expanded cultures. TCL with $CD45RA^-/RO^+$

Table III. *Immunological assays used*

Day 0	Seeding	Lymphocytapheresis collected between 8 and 11 am and processed within 2 h ex vivo; Ficoll separation of PBMC PBMC seeded fresh in IL-7 enriched X-Vivo media, 6×10^6 PBMC/Ag, 1 μM concentration of Ag
Day 7	Results of IL-7 primary proliferation (IL-7PP)	Splitting of the cultures into new "daughter plates," which were pulsed with [^{8}H] thymidine for 6 h, then harvested and counted (cpm); IL-2 medium added to mother plates
Day 10–12	Restimulation	Restimulation of "positive cultures" from IL-7PP (cpm > average cpm of negative control + 3 SD) with Ag + APC in 48-well plates
Day 18–20	A Avidity assay (confirmation assay)	Standard 48-h proliferation assay in avidity format: testing Ag-specific proliferation to five doses of Ag ranging from 0.001 to 10 μM concentration Set in 96-well plates, in duplicates, 1×10^5 APC + 2×10^4 T cells/well Supernatants collected in 36 h, at which time cultures were pulsed with [^{3}H] thymidine for last 8 h of incubation
Days 20–24	Flow cytometry analysis	Analysis of the surface expression of CD4, CD45RO, and CD45RA markers and classification of TCL as originating from naive ($CD45RA^+/RO^-$), effector (indeterminate; $CD45RA^+/RO^+$), or memory ($CD45RA^-/RO^+$) T cell pools
Delayed	Cytokine detection assay	Sandwich ELISA from the frozen supernatants from avidity assay Detection of IFN-γ and IL-4 and classification of TCL (Th1, Th0, and Th2) based on IFN-γ: IL-4 ratio

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proportions between 70 and 90% are classified as "indeterminate" because their origin cannot be determined with certainty. From the studied time kinetics of CD45RA and -RO expression, we can logically infer that this group contains cells originating from an in vivo-activated $CD45RA^+/RO^+$ "double-positive" population of "effector" T cells, but this cannot be confirmed with certainty with experimental design (27). Abs (CD45RO-FITC, CD45RA-PE, CD4-Cy) were purchased from BD PharMingen (San Diego, CA) and applied at saturating concentrations. CD45 isotype expression was analyzed on double-gated populations of $CD4^+$ T lymphocytes.

Functional characterization of TCL based on their cytokine production

The secretion of prototypic Th1 (IFN-γ) and Th2 (IL-4) cytokines from the avidity assays was determined by ELISA (Cyto-Sets; BioSource International, Camarillo, CA) in duplicates at 1/5 dilutions for IFN-γ and 1/2 for IL-4 (detection range, 15- 4000 pg/ml). The IFN-γ:IL-4 ratios used for the classification of TCL were: Th1 (ratio $>$100 and IFN-$\gamma > 100$ pg/ml), Th0 ($100 >$ ratio $>$0.1 or IFN-$\gamma < 100$ pg/ml) or Th2 phenotype (ratio $<$0.1). TCL producing $<$100 pg/ml IFN-γ were classified as Th0, even if they did not produce detectable amounts of IL-4.

Phenotypic characterization of TCL was not complete for several subjects: one patient had a genetic defect in down-modulating CD45RA (30); hence, determining TCL origin was not possible. As a result of technical problems, flow cytometry data on four MS patients and one HD and cytokine data on three MS patients and one HD were lost.

Mathematical analysis of MHC binding affinity

Predicted MHC binding affinity was computed by the peptide property models extracted from a database of MHC binders (MHCPEP). The algorithm constructs a statistical model, using biophysical parameters of the amino acids, from a training dataset of binders and nonbinders (31). In this application, the training dataset for DR2 consisted of 493 binders and 102 nonbinders and the DR4 set had 921 binders and 312 nonbinders. Each dataset included data from multiple DR2 [DR4] alleles to increase the sample sizes. The MHC peptide property model consists of the centroid and the covariances of the iteratively discovered binding 9-mers, all in the multivariate space of biophysical properties. The smaller the statistical distance of a 9-mer from this centroid is, the more similar its biophysical properties are to the average values of the binders; therefore, the 9-mer is more likely to bind. The cutoff distance is also estimated from the training data. For each myelin protein, all overlapping 9-mers were converted into multivariate vectors in the space of biophysical properties to compute the distance of every 9-mer from the centroid for each DR. We normalized the distances by the cutoff threshold so that the value of 1 corresponds to the threshold.

MRI analyses

MRIs were performed at 1.5 T (General Electric, Milwaukee, WI) with axial 3-mm slices fast spin echo and T1-weighted SE images before and after injection of gadopentate (Gd) dimeglumine (Magnevist; Berlex, Cedar Knolls, NJ). The number of contrast-enhancing lesions (Gd number) was counted by the consensus of two radiologists. Because of the fluctuating nature of brain inflammatory activity in MS, we averaged the Gd number over 3 consecutive monthly MRIs (average Gd number). All images were analyzed on a Sun workstation (Sun Microsystems, Palo Alto, CA) with a semiautomated software (PV-Wave CL Version 6.2) (32): White matter lesion load (WMLL) and the ratio of cerebral brain matter to total intracranial volume (brain fractional volume (BFV)) were calculated from fast spin echo images; T1-hypointensities ("black hole" volume (BHV)) were defined as hypointense lesions on postcontrast Gd-T1WI; black hole fraction (BHFr) was defined as a proportion of WMLL that was hypointense on Gd-T1WI (BHFr = BHV*100/WMLL).

Statistical analysis

Statistical analysis was performed using commercial software (Sigma-Stat 2.03; Jandel Scientific, San Rafael, CA). The pre-set limit of statistical significance was $p < 0.05$. For continuous data, due to high interindividual variability, comparisons between MS patients and HD were based on the nonparametric Mann-Whitney *U* rank sum test; only when permitted by large datasets with normal distribution (e.g., IFN-γ production) the *t* test was used. For categorical data, χ^2 analysis (cytokine categories) or Fisher's exact test (comparison of demographic data) was used. For correlation analyses between immunological data and MRI variables, we used the nonparametric Spearman correlation.

Results

Reactivity to a set of myelin Ags in an IL-7-modified primary proliferation assay and precursor frequency of myelin-specific TCL

We studied in a cohort of 55 untreated MS patients the T cell reactivity to 15 peptides derived from 4 major myelin proteins, MBP, PLP, MOG, and CNPase (Table II) that were selected based on careful literature review as immunodominant in humans and/or encephalitogenic in animals. For comparison, 15 HD were matched with the MS cohort for demographic parameters and the expression of MHC class II alleles to exclude influences of the MHC binding on the $CD4^+$ T cell repertoire and changes related to immune senescence. As a control Ag for memory responses, we used Flu-HA peptide (306–318) due to its promiscuous MHC class II binding, its immunodominance in humans, and high population prevalence of flu vaccination and/or exposure (33).

The methodological details are depicted in Table III. We tested 6×10^6 PBMC per Ag to detect low precursor frequencies of autoreactive T cells. Addition of IL-7 into the primary proliferation allows the effective expansion of Ag-specific T cells due to its antiapoptotic and growth-promoting effects, but we showed previously that it does not alter cytokine phenotype or avidity of selected TCL (27). All peptides were used at low and equimolar concentrations (1 μM) to detect preferentially high-avidity T cell responses and to allow for comparison of immunodominance between different epitopes. We selected a dose of 1 μM (based on our baseline experiments), which is 10- to 100-fold lower than the doses used in previous studies, but still stimulates discernible T cell responses.

Under these conditions, the T cell reactivity to Flu-$HA_{306-318}$ in primary proliferation assays, expressed as the number of positive wells out of 60 seeded wells (Fig. 1*A*) or as stimulation indices (data not shown), was comparable between MS patients and HD. However, the T cell reactivity to autoantigens was different. Pooling all autoantigens together, MS patients had significantly higher numbers of positive TCL/Ag (Fig. 1*A*, Autoantigens: Mean all; Median 1.4 in MS vs 0.95 in HD; $p = 0.042$) and significantly higher mean stimulation indices (data not shown). Two (3.64%) of 55 MS patients and 1 (6.67%) of 15 HD had no positive culture out of 900 wells seeded per individual (Fig. 1*A*). Since the immunodominant epitopes may be different for different subjects, we also analyzed differences in the numbers of positive wells and mean stimulation index for the immunodominant epitope in each individual (Fig. 1*A*, ImunoDom) and these were also significantly higher in MS patients (Median 6 in MS vs 3 in HD; $p = 0.012$). The differences observed in primary proliferation assays were further enhanced in confirmation assays (Fig. 1*B*, *second panel*). However, only several myelin epitopes contributed to the increased reactivity observed in MS patients: MBP epitopes (MBP_{13-32}, $MBP_{111-129}$, and $MBP_{146-170}$), $PLP_{139-154}$, and MOG (MOG_{1-20} and MOG_{35-55}) (Fig. 1*B*). The reactivity for the other peptides was comparable between MS patients and HD. When we pooled these six discriminatory autoantigens together and compared the reactivity of MS vs HD cohorts (Fig. 1*A*, Mean 6-select), the difference was clearly more pronounced than when pooling all 15 autoantigens (Median 2.3 in MS vs 1.0 in HD; $p = 0.010$). There was only one myelin Ag for which HD had overall higher reactivity, both in primary proliferation and confirmation assays: MBP_{83-99}. However, the differences between MS and HD did not reach statistical significance for any single myelin epitope. In terms of reactivity to multiple myelin epitopes, MS patients recognized on average 8.4 $\pm$ 3.947 peptides (Median 9) in primary

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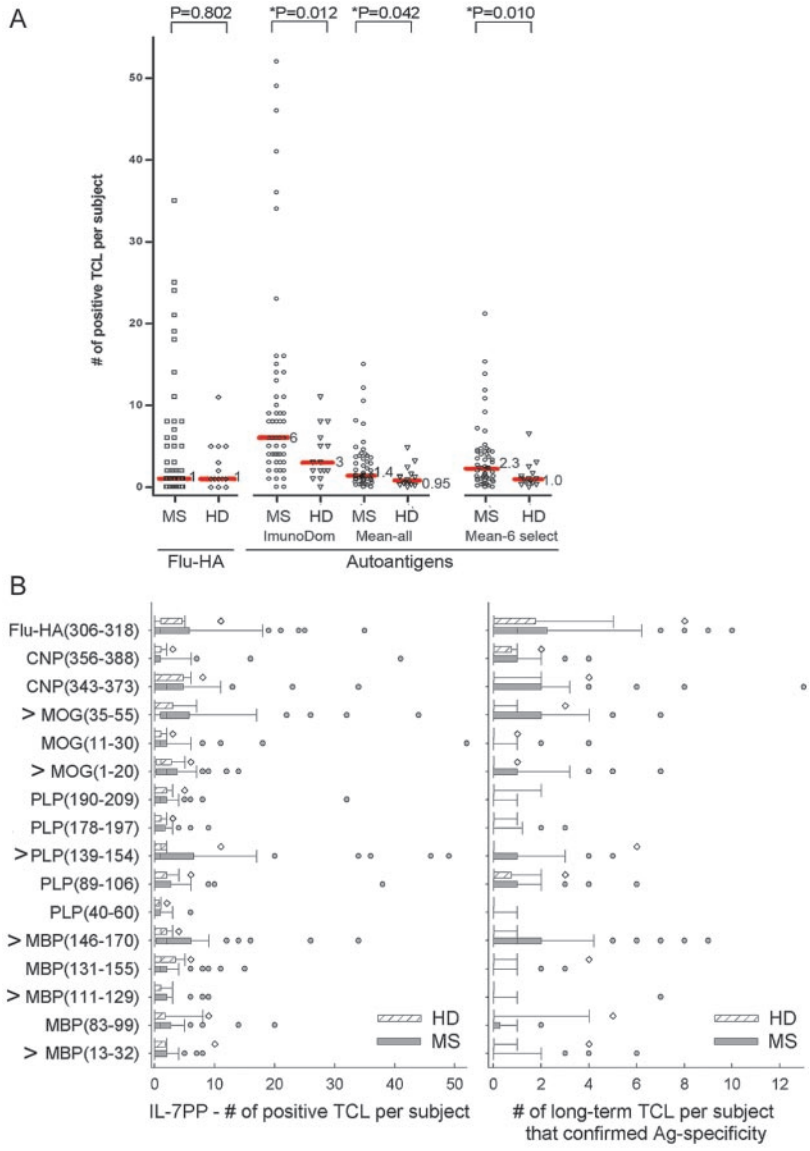

FIGURE 1. Results of IL-7 primary proliferation assay: differences between MS patients and HD. Because of the difference in numbers of tested subjects between MS and HD, we highlighted group medians in all scatter plots and specified them by number. As a result of high interindividual variability in response between subjects, all statistics are nonparametric by the Mann-Whitney *U* rank sum test; statistically significant differences are highlighted by an asterisk (∗). *A*, Number of positive TCL per subject: Comparison of T cell reactivity to Flu-HA and to autoantigens: ImunoDom, average number of positive cultures from 60 for single myelin peptide that was most dominant for individual subject; Mean-all, average number of positive cultures from 900 wells seeded per total of 15 myelin peptides; Mean-6 select, average number of positive cultures for six most discriminatory autoantigens: MBP ($MBP_{13–32}$, $MBP_{111–129}$, and $MBP_{146–170}$), $PLP_{139–154}$, and MOG ($MOG_{1–20}$ and $MOG_{35–55}$). *B*, Box plots demonstrating results of IL-7 primary proliferation assay (average number of positive cultures from 60 wells seeded per Ag per subject) and results of confirmation assay (number of confirmed Ag-specific TCL per subject) − comparison between MS patients and HD. Six most discriminatory autoantigens are highlighted. There are no statistically significant differences between MS patients and HD for any single Ag.

proliferation assay, whereas HD recognized 7.467 ± 4.257 peptides (Median 7; $p = 0.427$).

Next, we asked whether our protocol indeed expanded high-avidity myelin-specific T cells. For the purpose of the study, we arbitrarily defined as high-avidity TCL those with an EC_{50} (i.e., dose of Ag that leads to 50% maximal stimulation) <1 μM. In our experience and based on the literature review, the majority of myelin-specific $CD4^+$ TCL have EC_{50} values between 1 and 50 μM (20, 28). We expanded all positive cultures from the primary proliferations (293 HD-derived TCL and 1901 MS-derived TCL) and assessed their Ag avidity by dose titration experiments with Ag doses ranging from 0.001 to 10 μM (Table III). The confirmatory rate for autoreactive TCL was on average 37.15% for MS and 15.63% for HD ($p = 0.005$). For Flu-HA, the confirmatory rate was 51.46% for MS and 31.82% for HD subjects ($p = 0.117$). In the MS cohort, on average, 58.57% of all confirmed autoreactive TCL fulfilled the definition of high-avidity TCL, whereas in the HD cohort this proportion was 31.82% ($p = 0.003$). For Flu-HA-specific TCL, 88.25% were of high avidity in the MS group and 88.0% in the HD cohort ($p = 0.927$). As demonstrated in Fig. 2*A*, the number of high-avidity myelin-specific T cells per subject was more than four times higher in MS patients as compared with HD (mean 5.67 in MS vs 1.27 in HD; $p < 0.001$). Only 7 (12.73%) of 55 MS patients vs 8 (57.14%) of 14 HD had no high-avidity autoreactive T cells ($p = 0.00068$, χ^2). In contrast, the numbers of high-avidity TCL specific for Flu-HA were virtually identical between MS and HD (Fig. 2*A*). In addition, within the high-avidity TCL, the Ag avidities of MS-derived autoreactive TCL were still significantly higher then in HD (Fig. 2*B*). Again, the difference in Ag avidities of Flu-HA-specific TCL was not significant between the groups. The same myelin epitopes that were most discriminatory between MS and HD in primary proliferation also differed the most in the avidity assays (data not shown).

Phenotypic differences between TCL derived from MS vs HD cohorts

Cytokine phenotype. Proinflammatory (Th1) T cells are considered pathogenic in EAE and MS, and therefore we measured the prototype Th1 (IFN-γ) and Th2 (IL-4) cytokines to classify TCL into Th1, Th0, or Th2 phenotype. We applied a conservative classification of IFN-γ:IL-4 ratios (Th1: IFN-γ:IL-4 ratio $\geq$100; Th2: $\leq$ 0.1; Th0: ratios in between). Fig. 3*A* depicts the relative distribution of TCL from MS patients and HD that are categorized as

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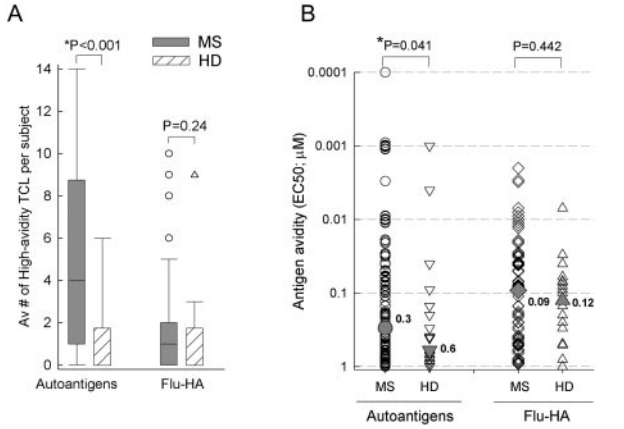

FIGURE 2. High-avidity TCL: comparison of precursor frequencies and Ag avidities between MS patients and HD. *A,* Box blots depicting number (group medians, 25th and 75th percentile and outliers) of high-avidity TCL specific for all autoantigens pooled together and for Flu-HA. Comparison between MS patients and HD. Statistics are based on nonparametric Mann-Whitney *U* rank sum test. *B,* Scatter plots delineating Ag avidities of individual high-avidity autoreactive- vs Flu-HA-specific TCL derived from MS patients or HD. Median values are highlighted. MS patients have significantly higher precursor frequency of high-avidity autoreactive TCL and also on average higher mean Ag avidity (EC_{50}; specified in scatter plots) as compared with HD.

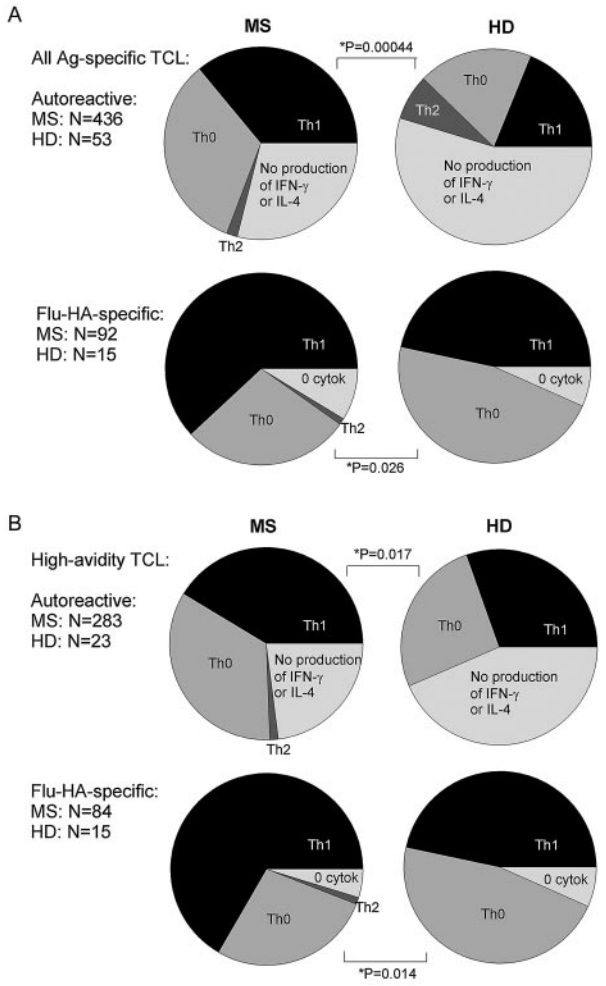

FIGURE 3. Cytokine phenotype of confirmed Ag-specific TCL: differences between MS patients and HD. Pie graphs depicting proportions of autoreactive vs Flu-HA-specific TCL derived from MS patients vs HD that are classified into the Th1, Th0, or Th2 phenotype based on production of IFN-γ and IL-4. Values of *p* are based on χ^2 2 × 4 contingency table. The number of tested TCL for MS patients and HD for each condition is specified. *A,* For all generated TCL; *B,* for high-avidity TCL only. TCL of MS patients are significantly skewed toward proinflammatory Th1 phenotype. In HD, >50% of all autoreactive TCL do not produce detectable levels of either IFN-γ or IL-4.

Th1, Th0, Th2 or those that did not produce detectable levels of either IFN-γ or IL-4. Most strikingly, >50% of autoreactive TCL selected with this low concentration of autoantigens in the HD cohort did not produce detectable levels of either cytokine, despite the fact that cytokine production was measured from wells with effective Ag-specific proliferation (Fig. 3*A*). This lack of cytokine secretion was not due to an intrinsic defect, because the percentage of flu-specific TCL not producing IFN-γ or IL-4 in HD was 6.7%, which was in fact lower than the proportion detected in the MS cohort (8.7%). Less than 20% of the HD-derived autoreactive TCL had pure Th1 phenotype, whereas this proportion was twice as high in MS patients. Overall, the differences in cytokine phenotype of autoreactive TCL between MS patients and HD were statistically significant ($p = 0.000044$; χ^2 4 × 2 contingency table). Furthermore, MS-derived autoreactive TCL produced significantly higher amounts of IFN-γ (average, 560.28 pg/ml vs 165.15 pg/ml; $p < 0.001$).

Next, we analyzed the cytokine phenotypes of only high-avidity TCL (Fig. 3*B*). Although the data did not change for Flu-HA-specific TCL, because the majority of these were of high avidity, it became clear that high-avidity autoreactive TCL were enriched for cytokine-producing TCL (especially of Th1 phenotype) both in MS patients and HD. Even if less striking, the cytokine phenotype of high-avidity autoreactive TCL remained significantly different between MS patients and HD ($p = 0.017$). With respect to the fine Ag specificity, the differences between MS patients and HD were again driven by reactivity to the above-mentioned six myelin peptides (data not shown).

The importance of high-avidity autoreactive TCL in MS was further supported by their higher average IFN-γ production (731.13 pg/ml) compared with low-avidity TCL (324.46 pg/ml; $p < 0.001$; data not shown).

Naive vs memory origin. The determination of naive vs memory origin of $CD4^+$ TCL is based on the analysis of their surface expression of CD45RA and -RO isoforms at the end of the second restimulation cycle (i.e., days 20–24 after ex vivo isolation; Table III; see *Materials and Methods*). We analyzed the expression of CD45RA and RO isoforms in TCL that confirmed their Ag specificity in a proliferation assay and compared naive- vs memory-originating TCL between MS patients and HD. All of these TCL were $CD4^+$. Considering all Ag-specific TCL (Fig. 4*A*), the proportions of naive-, indeterminate-, and memory Flu-HA-specific TCL were virtually identical between the MS and HD cohorts. As expected for recall Ag, the majority of Flu-HA-specific TCL originated from the memory T cell pool. Similarly, the precursor frequencies of autoreactive TCL originating from the naive T cell pool were identical between MS and HD. In sharp contrast, the precursor frequencies of in vivo-activated autoreactive TCL (either memory-originating or indeterminate/effector) were significantly increased in MS (Fig. 4*A*).

Similar differences between MS patients and HD were found for high-avidity TCL (Fig. 4*B*). The importance of high-avidity autoreactive TCL is stressed by the observation that they were enriched

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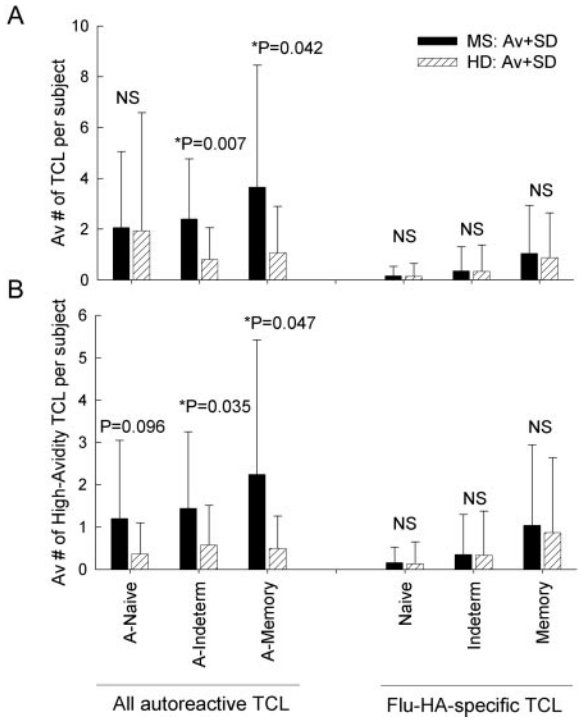

FIGURE 4. Naive vs memory origin of confirmed Ag-specific $CD4^+$ TCL: differences between MS patients and HD. All confirmed Ag-specific $CD4^+$ TCL were analyzed by flow cytometry at days 20–24 ex vivo for the surface expression of CD45RA and CD45RO isoforms to detect whether the TCL originated from naive vs memory T cell pools in vivo. TCL that could not be classified unequivocally into naive vs memory origin were classified as indeterminate. The proportions of autoreactive vs Flu-HA-specific TCL originating from naive vs memory T cell pools are depicted. *A*, For all generated TCL; *B*, for high-avidity TCL only. MS patients have a significantly higher precursor frequency of autoreactive TCL originating from memory and indeterminate pools as compared with HD. No such differences were observed for autoreactive TCL originating from a naive T cell pool and no differences were observed for Flu-HA TCL. Differences between MS patients and HD are based on the nonparametric Mann-Whitney *U* rank sum test.

for potentially in vivo-activated cells (i.e., memory or indeterminate phenotype) in both MS ($p < 0.001$) and HD ($p = 0.031$) cohorts.

We also analyzed the differences in Ag avidities among naive-, indeterminate-, and memory-originating TCL. For Flu-HA-specific TCL those originating from the naive pool were of relatively lower avidity (i.e., had significantly higher EC_{50}; median 0.55 μM) as compared with in vivo-activated TCL (indeterminate/effector TCL, 0.04 μM; memory, 0.1 μM; $p = 0.044$). In contrast, for autoreactive TCL the Ag avidities were not significantly different between the categories (naive TCL, median of 0.8 μM; indeterminate/effector, 0.6 μM; and memory, 0.75 μM; data not shown).

Again, the most striking differences between MS patients and HD in terms of memory vs naïve origin of autoreactive TCL were observed for TCL specific for the six myelin epitopes mentioned previously (data not shown).

Comparison of predicted MHC class II binding of myelin peptides to HLA-DR2 and -DR4 alleles

The myelin epitopes that were identified as immunodominant in our study differed substantially from those identified previously. Most strikingly, $MBP_{83–99}$ was not immunodominant in our MS cohort despite the fact that 45.5% of our patients expressed HLA-DR2 (DRB1*1501, DRB5*0101). We believe that this different immunodominance pattern is related to the low Ag concentration used in our study and explained by our hypothesis that T cells specific for self-Ags that bind with high affinity to expressed MHC class II alleles will be deleted during thymic-negative selection or anergized in the periphery.

To explore this hypothesis, we related the observed immunodominance pattern to the binding affinity of tested peptides to the MHC class II molecules. Previously published data demonstrated that the vast majority (>90%) of myelin-specific $CD4^+$ TCL derived from MS patients are restricted by HLA-DR alleles, in contrast to extremely rare DQ- or DP-restricted TCL (5, 8, 34). As evident from Table I, almost 70% of all tested subjects in our study expressed either HLA-DR2 or DR4 alleles. The majority of the $DR2^+$ subjects expressed HLA-DRB1*1501/DRB5*0101. In contrast, $DR4^+$ patients had greater diversity in molecular phenotypes: DRB1*0401, *0402, *0404, *0405, and *0411. Because of this diversity in HLA-DR expression and our interest in comparing the relative strength of peptide-MHC binding across several related DR alleles rather than in exact binding affinity for distinct HLA-DR, we used an approach that is more generally applicable and uses mathematical modeling for comparative prediction of MHC class II binding (31). This method was originally developed for tumor and vaccine immunology to identify peptides that are capable of binding to multiple MHC molecules and is based on peptide property models constructed using biophysical parameters of the constituent amino acids and a training set of known binders and nonbinders.

We applied this biomathematical strategy, calculating predicted binding values for overlapping 9-mer peptides scanning the whole sequences of MBP, PLP, MOG, CNP, and Flu-HA. Numerical binding values were shade-coded for display with darkest shades indicating highest predicted binding affinity. Only data for MBP, PLP, and MOG are displayed (Fig. 5*A*). These analyses led to several interesting observations: the areas of the myelin proteins containing peptides with high-affinity binding are quite similar for DR2 and DR4 alleles, although the differences in fine-epitope recognition are obvious. Furthermore, previously identified immunodominant epitopes are not necessarily located within these high-affinity binding areas of the proteins. We next analyzed the peptides used in our study and selected the best-binding 9-mers among all contained within individual epitopes and displayed this best-predicted binding index for DR2 and DR4 alleles (Fig. 5*B*). The most discriminatory epitopes between MS patients and HD identified in our study (Fig. 5*B*, highlighted) are epitopes with the weakest predicted binding affinity to HLA-DR2 and -DR4 alleles.

Because only ~70% of MS patients in our cohort expressed DR2 or DR4 alleles, we reanalyzed the results of IL-7PP and confirmation assays and demonstrated that the DR2/DR4 subcohort is fully representative of the whole cohort (data not shown).

Correlations between immunological data and clinical and MRI disease characteristics in MS cohort

To investigate the potential pathogenic role of the autoreactive TCL that were expanded in MS patients as compared with HD, we compared the demographic, clinical and MRI data and the number and phenotype of autoreactive TCL in the MS cohort. We found no significant correlations between any of the demographic data (i.e., age, sex, disease duration, family history of MS, or other autoimmune diseases) and any immunological parameters. We found no

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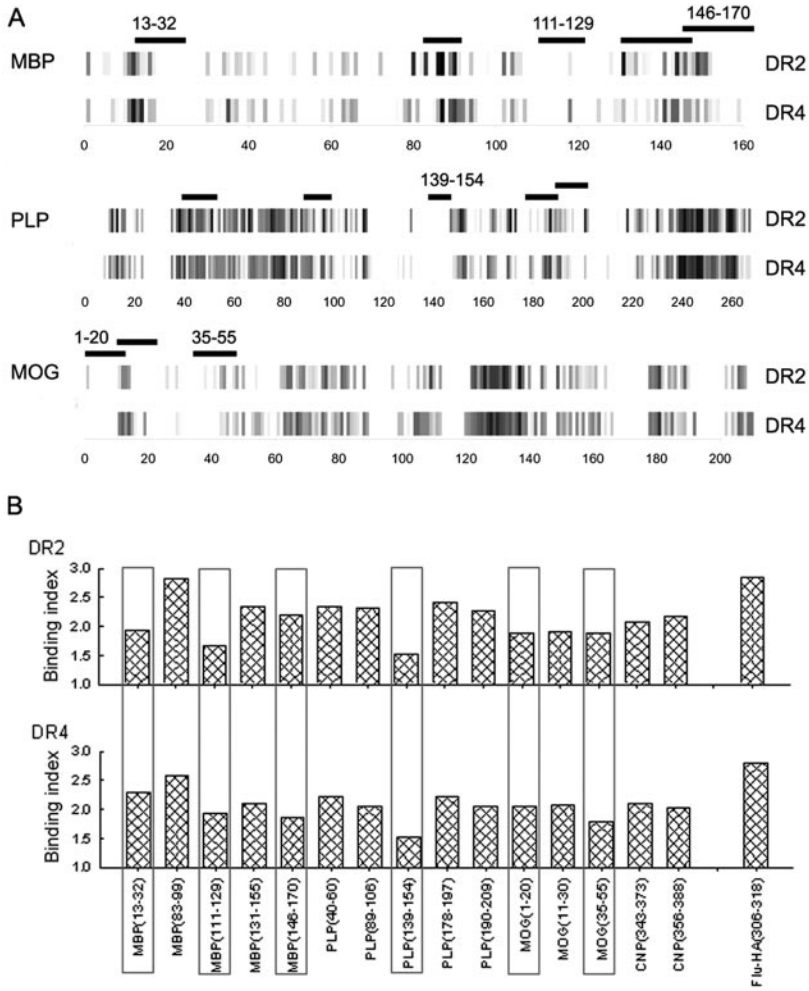

FIGURE 5. Mathematically predicted binding of selected myelin epitopes using DR2- and DR4-specific peptide models. *A*, The image plots of 9-mer profiles for MBP, PLP, and MOG displaying the theoretical binding indices of individual 9-mers. A 9-mer is represented on the horizontal axis by the position of the first amino acid within the protein. Predicted high-affinity 9-mers are dark, predicted low-affinity light, and nonbinders are white in the gray scale. Every overlapping 9-mer was assigned a distance from the model centroid using DR2- and DR4-specific peptide property models separately (see *Materials and Methods*). The distances were normalized to facilitate comparison across the two DR models. The bars above the image plots correspond to the epitopes tested in our study. *B*, The comparison of binding indices of the individual epitopes. The binding index for each epitope corresponds to the highest among the 9-mers that lie within the peptide. The binding index was heuristically defined as 3 (normalized distance from peptide property model centroid), since the distance from the model centroid is inversely correlated to the binding affinity for that MHC. The boxed bars correspond to the immunodominant epitopes labeled in the image plots.

significant correlations between total numbers of all autoreactive TCL per patient and any of the clinical (i.e., sustained disability scores or rate of progression of disability) or brain MRI parameters (i.e., brain inflammatory activity reflected by average number of contrast-enhancing lesions from 3 monthly MRIs (average Gd number); volume of all MS plaques: WMLL; complement to BFV (1-BFV) as a measure of diffuse brain destruction or brain atrophy and BHFr (the amount of WMLL that is hypointense on postcontrast T1WI) as a measure of focal brain tissue destruction.

We next performed analyses focusing only on high-avidity autoreactive TCL: Again, there was no correlation between the total number of these TCL and any clinical or MRI parameters. However, when we focused on specific Ag specificities of high-avidity TCL, we found mild to modest correlations to several MRI measures. We first analyzed in detail the cytokine phenotypes of TCL specific for each autoantigen (Fig. 6*A*): this figure demonstrates that TCL specific for MBP epitopes 13–32 and 111–129 were highly skewed toward the Th1 phenotype by both the proportion of Th1 TCL and by high IFN-γ production. When analyzing high-avidity Th1 TCL specific for each one of the three discriminatory MBP epitopes ($MBP_{13–32}$, $MBP_{111–129}$, and $MBP_{154–170}$), we noticed trends toward correlation between the number of these TCL/patient and the MRI measures of brain tissue destruction. Therefore, we pooled all high-avidity Th1 TCL specific for these three epitopes per patient and repeated the Spearman correlation analyses between this combined Th1 MBP reactivity and MRI measures (Fig. 6*B*, *last two panels*). We found statistically significant correlations between this compound Th1 MBP reactivity and both measures of brain tissue destruction: focal brain destruction: BHFr ($R = 0.294$; $p = 0.0296$) and diffuse brain destruction: 1-BFV ($R = 0.296$; $p = 0.0282$). Similarly, as evident from Fig. 6*A*, TCL specific for both immunodominant MOG epitopes ($MOG_{1–20}$ and $MOG_{35–55}$) show less pronounced IFN-γ production and relatively higher proportions of Th0/2 or noncytokine-producing T cells. We therefore pooled these specificities together and found significant negative correlation between this compound Th0/2 MOG reactivities and brain inflammatory activity (Fig. 6*B*, *first panel*; $R = -0.32$; $p = 0.0185$) as well as a negative correlation between the number of these TCL/patient and disability progression index based on the Scripps neurological rating scale ($R = -0.297$; $p = 0.040$; data not shown). We found no other significant correlations between any immunological parameters and any clinical or MRI parameters.

Hence, our data appear to indicate a deleterious effect of Th1 high-avidity TCL specific for MBP epitopes on brain tissue destruction and beneficial/protective effect of Th0/2 immunodominant MOG reactivities on brain inflammatory activity. We therefore asked whether the combination of these two immunological parameters would strengthen the relationship to MRI outcome measures and consequently devised a parameter in which we subtracted the number of high-avidity Th0/2 MOG specificities from Th1 MBP specificities (no. of Th1MBP − no. of Th0/2 MOG) per

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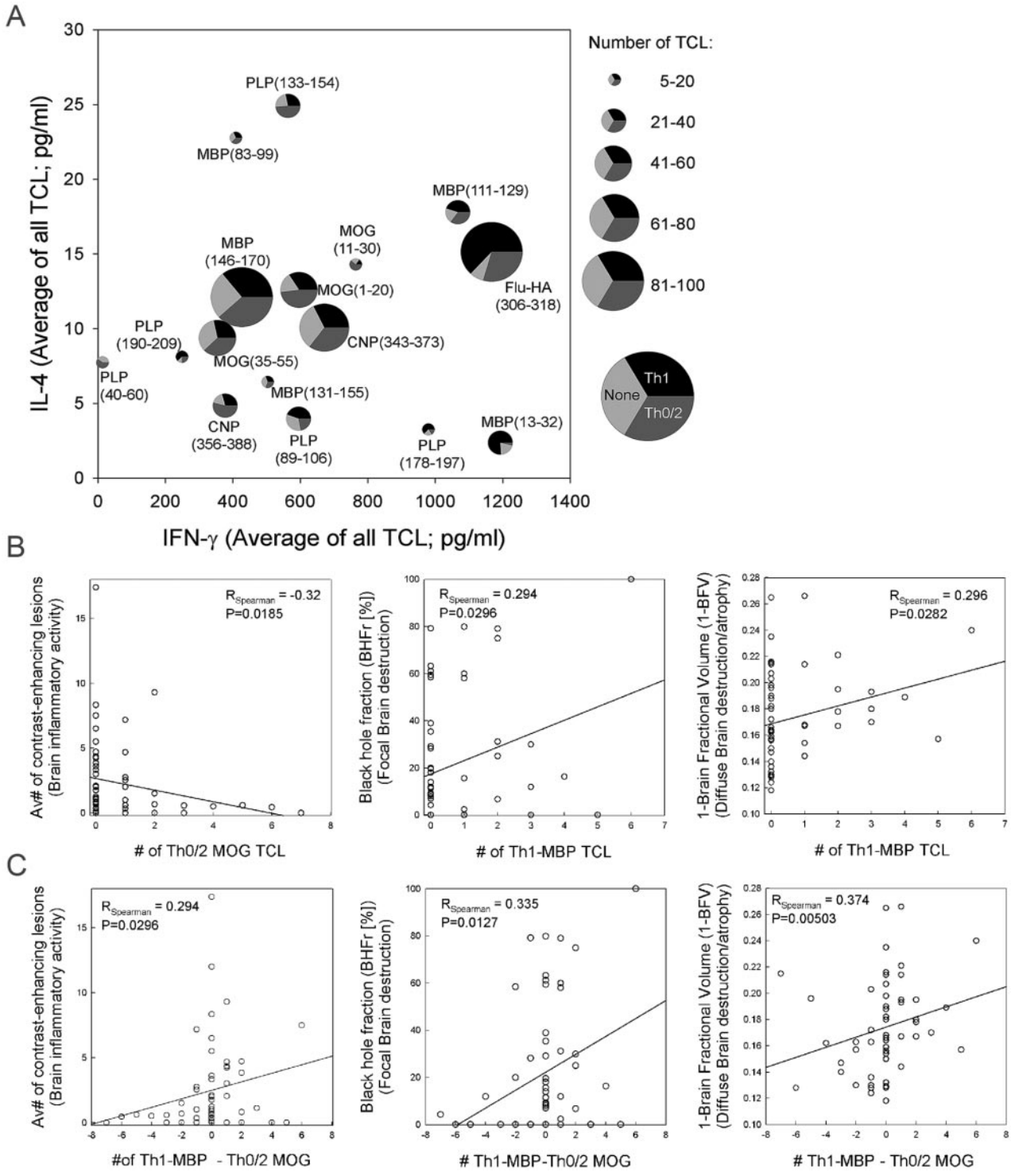

FIGURE 6. Cytokine phenotype of TCL specific for each autoantigen and correlations between MRI disease characteristics and number of high-avidity TCL of specific phenotype per MS patient. *A,* Cytokine phenotype of all MS-derived TCL specific for each Ag: the number of TCL from which the information is derived is coded by size of each pie graph. The position of each pie graph in the *x*/*y* (IFN-γ/IL-4) axes is determined by average production of these two cytokines from all TCL specific for that particular Ag. The proportion of Th1, Th0/2, and no-cytokine-producing TCL in each Ag specificities is coded by gray shading. *B,* Spearman correlations between combined high-avidity TCL of Th0/2 phenotype plus noncytokine-producing TCL specific for $MOG_{1–20}$ and $MOG_{35–55}$ and brain inflammatory activity and between high-avidity TCL of the Th1 phenotype specific for MBP ($MBP_{13–32}$, $MBP_{111–129}$, and $MBP_{146–170}$) and MRI measures of focal and diffuse brain destruction. *C,* Spearman correlation between compound immunological measure subtracting above-mentioned Th0/2 MOG reactivities from Th1 MBP reactivities and MRI measures.

each patient (Fig. 6*C*). This compound index further strengthened the Spearman correlations with the above-mentioned MRI measures (Fig. 6*C*) which is not intuitive or expected, because there is no significant correlation between the average number of contrast-enhancing lesions and measures of brain tissue destruction in our cohort (data not shown).

These exploratory immunological/MRI correlations are very interesting, but we have to caution that the above *p* values are

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unadjusted for multiple comparisons and therefore potentially are associated with higher than 5% false discovery rate.

Discussion

T cell reactivity to myelin Ags has been studied extensively in MS (5, 7, 9, 34–42) and while these studies led to many important findings, they failed to demonstrate profound differences between MS patients and controls that would be analogous to the data from EAE. In the Introduction, we formulated several reasons that explain why the data from humans and animals are not directly comparable. As one of the most important explanations, the nonphysiologically high concentrations of Ags that were used in previous studies (including our own) resulted in missing potentially the most relevant component of autoimmune responses, i.e., high-avidity autoreactive T cells.

By using between 10- and 100-fold lower Ag concentrations than those described previously, we compared the reactivity patterns of PBMC to the 15 immunodominant peptides from 4 myelin proteins between 55 untreated MS patients and 15 matched HD. This strategy documented profound quantitative and qualitative differences in the repertoire of myelin-specific $CD4^+$ T cells between these two cohorts: the precursor frequency of high-avidity myelin-specific T cells in MS was on average more than four times higher than in HD; MS-derived autoreactive T cells had significantly higher Ag avidity, were notably skewed toward the proinflammatory phenotype, and originated more often from in vivo-activated T cells. In contrast, low-avidity T cells contained increased proportions of cells with undetectable cytokine production; they predominated in HD, and were enriched for the cells originating from naive T cells. All of these data strongly argue against the notion that these low-avidity cells had been expanded by autoantigen in vivo. They rather represent marginally cross-reactive cells with "primary" specificity for unrelated Ag, e.g., foreign Ag. The observation that such a high proportion of low-avidity T cells did not produce detectable levels of IFN-γ or IL-4 has several potential explanations: the TCR signal by the autoantigen was either insufficient to trigger full effector functions of these cells, i.e., the self-peptide served as an altered peptide ligand (APL) (22), or, alternatively, these cells produced other cytokines, which were not assayed in our study. Especially interesting would be information about their production of regulatory cytokines like IL-10 and TGF-β, but unfortunately at the time this work was initiated, the multiplex cytokine assays that would enable measurement of several cytokines from limited amounts of supernatants were not yet available.

At first, it appears surprising that using an Ag concentration that is "only" 10- to 100-fold lower than those used previously would show strong functional differences. However, evidence from animal studies indicates that a 10-fold difference in concentration of Ag or in Ag affinities may be functionally highly relevant. For example, in an informative EAE study (43), a 10-fold difference in peptide binding to the MHC class II molecule (achieved by a single amino acid substitution of MBP (Ac_{1-9}) in a I-A^u-binding position) was sufficient to change the encephalitogenic properties of the peptide, i.e., the weaker binder was encephalitogenic, while the stronger binding peptide was not. Likewise, experimental systems suggest that the 10- to 100-fold difference is adequate to provide a "chemical margin of safety" on which the thymic tolerance is based: the number of specific peptide-MHC complexes required to eliminate immature $CD4^+CD8^+$ thymocytes by negative selection is only 10-fold lower than the amount of peptide-MHC complexes necessary to trigger mature memory T cells in the periphery, and 100-fold lower than the amount necessary to prime naive T cells (44, 45). All of these data support the notion that using Ag concentrations that are 10- to 100-fold lower than those used traditionally will make a profound difference in the phenotype and potential disease relevance of expanded autoreactive T cells.

In agreement with the experimental data mentioned above, our study confirmed that the repertoire of T cells expanded with low Ag concentration differs from the one selected by higher Ag doses. As one striking example, MBP_{83-99}, which many studies (including our own) identified as immunodominant in MS, was subdominant in the present study. The precursor frequencies of MBP_{83-99}-specific T cells were not higher in MS patients compared with HD (Fig. 1*B*), but also high-avidity MBP_{83-99}-specific T cells were virtually undetectable in $DR2^+$ individuals. We believe that the unusually high binding affinity of this peptide to HLA-DRB1*1501/B5*0101 alleles (Fig. 5 and Ref. 34) leads to deletion of high-avidity MBP_{83-99}-specific T cells from the peripheral repertoires of DR2-expressing individuals. This does not rule out that the T cell responses to this epitope are relevant in some MS patients, and in fact we could show that MBP_{83-99}-specific T cells mediated disease exacerbation of patients treated with an APL based on the $MBP_{83-99)}$ sequence (22, 23). However, none of the patients in whom APL immunization led to the expansion of high-avidity MBP_{83-99}-specific T cell-expressed DR2 alleles.

The following myelin epitopes were immunodominant and most discriminatory between patients and controls in the present study: MBP_{13-32}, $MBP_{111-129}$, and $MBP_{154-170}$, $PLP_{139-154}$, and MOG_{1-20} and MOG_{35-55}. Their immunodominance was based on their high precursor frequencies, their high Ag avidities, and functional studies. Nevertheless, the mere presence of high-avidity myelin-specific TCL was not specific for the disease process in MS. We observed high interindividual variability in the numbers and peptide specificity of high-avidity autoreactive TCL. Therefore only "pooling" of all autoreactive epitopes demonstrated strong statistical significance between MS patients and controls. This is not too surprising in view of the heterogeneity in MHC and TCR genotypes in human populations and in view of several potential autoantigens in MS that are likely different from patient to patient. We believe that this "immunological disease heterogeneity" in MS is responsible for many "negative" comparative studies between MS patients and HD if the numbers of studied subjects are limited. This was also the most likely reason for failure to demonstrate significant differences between MS patients and HD in our previous study using very low concentrations of MBP (20). In contrast to this study, we focused in the current one only on those myelin epitopes that were already found to be immunodominant in humans, and, furthermore, the number of subjects studied, the amount of PBMC seeded per Ag, and the complexity of functional assays render the two reports incomparable.

To further explore our hypothesis that the immunodominance profile detected in the current study is related to binding affinities of myelin epitopes to HLA-DR alleles, we used mathematical modeling to predict the binding affinity of the best epitope comprised in peptide sequences used in our study to multiple DR2 and DR4 alleles. Although this method is only an estimation and cannot compare with biochemical methods measuring exact binding affinity of the epitopes to the concrete molecular HLA-DR allele, binding hierarchy derived from this method (Fig. 5*B*) correlated well with the published binding affinities for those myelin peptides for which the binding data are known (8, 34). When we compared the predicted binding affinity of the epitopes identified as immunodominant and discriminatory between MS patients and HD in our study to the two HLA-DR molecules expressed in the majority of our study subjects, we observed a clear negative correlation between immunodominance and strength of HLA-DR binding.

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These data, which concur with animal studies (16, 43), contradict the hypothesis that the disease-associated MHC molecules contribute to the development of autoimmunity by efficient binding of the autoantigens involved in the pathophysiology of the disease. In contrast, they support the concept proposed by Ridgway et al. (46) that these MHC molecules permit escape of potentially deleterious high-avidity autoreactive T cells due to their poor peptide-binding properties. This alternative hypothesis, which stresses the importance of central and peripheral tolerance, explains the apparent paradox that chemical dominance (i.e., binding to MHC) does not necessarily translate into immunodominance (14, 47) or pathogenicity in autoimmune disorders (16).

One further important factor merits mentioning, because it might have influenced the immunodominance pattern that we observed. Most previously published studies examined reactivity to whole myelin proteins and then established the epitope specificity of TCL by proliferation to overlapping peptides (5, 6, 8). Since we based the selection of peptides on these data, we can assume that most of them can be processed from the intact protein. However, peptides loaded onto MHC molecules exogenously may assume a conformation that is different (type B conformation; e.g., after proteolytic cleavage of the protein) from that of peptides presented via endogenous processing (type A conformation) (48). Although type A T cells can be efficiently stimulated by both protein and peptide, type B T cells recognize only peptide-loaded MHC but are not stimulated by the whole protein (45, 49). Epitopes that are presented in the thymus assume type A conformation, and hence only type A T cells undergo negative selection. In contrast, during inflammatory responses in the target organs tissue destruction occurs in a protease-rich environment (50), which may permit or even favor external type B MHC loading and subsequently may lead to activation of type B autoreactive T cells. Our previously published study with CNPase demonstrated that type B autoreactive TCL specific for this protein are actually highly prevalent (>90% of all TCL selected by peptides (51)), and additional unpublished observations with MBP-specific TCL demonstrated that, depending on the epitope and the origin of TCL, up to 50% of peptide-selected TCL may be type B. By using peptides instead of proteins, the present study allowed us to detect both type A and type B T cells, which may have contributed to the observed differences between MS patients and HD. The question of type B vs type A TCL in autoimmunity is probably very important; however, it is difficult to address particularly in the context of myelin proteins, since one would need to test reactivity to the intact protein in an avidity assay format (i.e., with a wide range of concentrations) to definitely exclude its recognition. This poses a major problem with PLP, MOG, and CNP because these proteins are very difficult to isolate in sufficient quantities.

Perhaps the most critical question that remains is whether our study supports a pathogenic role of high-avidity myelin-specific T cells in MS. Direct proof, for example, similar to an adoptive transfer experiment in EAE, is impossible to obtain in MS patients. However, the fact that the numbers, Ag avidities, cytokine phenotype, and naive vs memory origin of Flu-HA-specific TCL were so strikingly similar between MS patients and HD provides compelling evidence that the differences found for myelin-specific T cells were Ag specific and disease related. We are not aware of any evidence that tissue destruction under physiological conditions can prime autoreactive T cells to the proinflammatory phenotype and therefore the striking skewing of these cells toward a Th1 phenotype in MS argues against them being an epiphenomenonon of the disease process. Furthermore, as suggested by correlation analyses, the influence of the presence and numbers of several myelin specificities in the autoreactive high-avidity T cell repertoire on MRI disease phenotype is very encouraging, although we have to view these data as preliminary that need to be followed up and confirmed.

In summary, our data strongly indicate that high-avidity myelin-specific T cells are pathogenetically relevant in MS. The marked differences to previous studies with respect to immunodominance patterns need to be considered in future therapies that aim at Ag-specific immunomodulation.

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