Human Autoreactive CD4⁺ T Cells from Naive CD45RA⁺ and Memory CD45RO⁺ Subsets Differ with Respect to Epitope Specificity and Functional Antigen Avidity

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T cells with specificity for self-Ags are normally present in the peripheral blood, and, upon activation, may target tissue Ags and become involved in the pathogenesis of autoimmune processes. In multiple sclerosis, a demyelinating disease of the CNS, it is postulated that inflammatory damage is initiated by CD4⁺ T cells reactive to myelin Ags. To investigate the potential naive vs memory origin of circulating myelin-reactive cells, we have generated myelin basic protein (MBP)- and tetanus toxoid-specific T cell clones from CD45RA⁺/RO⁻ and CD45RO⁺/RO⁻ CD4⁺ T cell subsets from the peripheral blood of multiple sclerosis patients and controls. Our results show that 1) the response to MBP, different from that to TT, predominantly emerges from the CD45RA⁺ subset; 2) the reactivity to immunodominant MBP epitopes mostly resides in the CD45RA⁺ subset; 3) in each individual, the recognition of single MBP epitopes is skewed to either subset, with no overlap in the Ag fine specificity; and 4) in spite of a lower expression of costimulatory and adhesion molecules, CD45RA⁺ subset-derived clones recognize epitopes with higher functional Ag avidity. These findings point to a central role of the naive CD45RA⁺ T cell subset as the source for immunodominant, potentially pathogenic effector CD4⁺ T cell responses in humans. The Journal of Immunology, 2000, 164: 5474–5481.

Immunochemical research on organ-specific autoimmune diseases has focused to a large extent on the identification of candidate self-Ags. In this context, myelin proteins have been studied as potential targets of autoimmune reactivity in the pathogenesis of multiple sclerosis (MS), the most common demyelinating disease of the CNS. Ags such as myelin basic protein (MBP) have been employed as immunogens in vivo to learn from experimental disease in animals (experimental allergic encephalomyelitis, EAE), and in vitro to study the human immune response in MS patients (reviewed in Ref. 1). EAE studies have demonstrated that inflammatory demyelinating CNS disorders can be mediated by CD4⁺ myelin-specific T cells. Extensive data in humans, on the other hand, indicate that myelin-reactive cells are part of the normal T cell repertoire both in MS patients and healthy subjects (2–5). However, these studies on the frequency of self-reactive T lymphocytes did not address whether these cells had been Ag primed in the body, i.e., whether they derived from a memory or naive pool. This is a relevant question, since naive and memory T cells play different roles in the immune response (6–8) and differ with respect to their Ag presentation and activation requirements (9–11), their TCR signaling (12), and their tissue-specific homing receptor-ligand interactions (13–16).

Since human T cells upon antigenic priming down-regulate the high-m.w. CD45RA isoform and reciprocally up-regulate the low-m.w. form CD45RO, these molecules have been proposed as markers for naive (CD45RA⁺/RO⁻) and memory (CD45RA⁻/RO⁺) T cells (17, 18). The following well-established observations corroborate this notion: 1) CD45RO⁺ cells are reduced under conditions of limited antigenic exposure, such as in human cord blood or in neonatal mice; 2) CD45RO⁺ cells are almost absent in the spleens of mice bred in a germfree environment; 3) increased proportions of CD45RO⁺ T cells are found in the peripheral blood of aging or immunized humans and animals; and 4) CD4⁺ CD45RO⁺ T cells predominantly give rise to the human response to recall Ags such as tetanus toxoid (TT) in immunized subjects (19). More recent data, however, demonstrated that changes in CD45RO isoforms are reversible in the rat (20, 21). Since evidence of such reversion in humans is so far only indirect (22), CD45RA vs CD45RO expression has been widely accepted and used to identify and select human naive and memory CD4⁺ T cells, respectively. A concomitant expression of both CD45RA and CD45RO has been associated with the phenotype of effector T cells, but the relationship of this cell population with that of naive and memory cells is not fully understood (8, 23). Thus, we studied human myelin Ag-specific responses from CD4⁺ T cell populations identified by clear expression of either the CD45RA or CD45RO isoform postulating they represent resting naive and memory T cells, and excluded double-positive cells which may either represent a transitional or an effector state.

To better understand the origin, the functional characteristics, and the specificity of CD4⁺ MBP-specific T cells derived from the peripheral blood of MS patients and controls, we asked 1) whether autoreactive T cells can originate both from the putatively naive (CD45RA⁺/RO⁻) and memory (CD45RA⁻/RO⁺) CD4⁺ subsets; 2) whether effector autoreactive cells raised from these subsets...
express different T helper phenotypes; and 3) whether the MBP-reactive T cell repertoires originating from the two subsets differ with respect to frequency, epitope recognition, and antigenic affinity.

Materials and Methods

Subjects

Donors were typed for HLA class II using standardized molecular typing methods by the Department of Transfusion Medicine, National Institutes of Health. To include well-characterized backgrounds in terms of HLA-restricted T cell response to MBP, we selected patients with haplotypes including DRB1*1501, *1502, or *0401. Age, gender, and HLA-DR type of the subjects were as follows: MS patients: MS1, 31-year-old female, DRB1*0401,07*01, DRB1*1501,04*, MS2, 24-year-old female, DRB1*0403,04, MS4, 24-year-old female, DRB1*0101,1501, and MS5, 39-year-old female, DRB1*1501,04*; MS6, 32-year-old female, DRB1*1502, 0701; and MS7, 38-year-old male, DRB1*0401,1501 and healthy donors: HD1, 33-year-old female, DRB1*0401,07*; HD2, 56-year-old female, DRB1*0101,1301, and HD3, 30-year-old male, DRB1*0301,1101. The study was approved by the Institute Clinical Research Subpanel, and informed consent was obtained from each patient.

Ags

Human MBP was prepared according to the method of Deibler et al. (24). A single batch of MBP of known purity and in vitro immunogenicity was used throughout the entire study. MBP peptides were synthesized on a Beckman 990 peptide synthesizer (Beckman Coulter, Fullerton, CA) using Merrifield’s solid-phase synthesis methodology according to published sequence (5). The superantigens (SAgs) staphylococcal enteroxin D, staphylococcal enteroxin E, and toxic shock syndrome toxin-1 were obtained from Toxin Technologies (Sarasota, FL). TT was purchased from Pasteur Merieux Connaught (North York, Ontario, Canada).

Purification of CD4+ T cell subsets

PBMC were obtained from leukopheresis buffy coats by density gradient centrifugation (Organon Teknika, Durham, NC). T cell subsets were isolated from 1–2 × 10^6 freshly obtained PBMC via high-affinity negative selection using human CD4^+45RO or CD4^+45RA subset columns (R&D Systems, Minneapolis, MN), essentially according to the manufacturer’s protocol. Our improvements included thorough resuspension of the cell pellet by gently passing the cells six times through a 16-gauge needle before loading and keeping a steady cell elution flow by carefully maintaining a 2-ml wash buffer volume in the column reservoir. Cryopreserved cells proved unsuitable for the separation procedure due to reduced viability and an increased tendency for aggregation. Therefore, only data obtained from subset preparations of freshly collected PBMC were included in this study. Composition of the PBMC and purity of the separated T cell subsets were routinely checked by flow cytometry using a FACSscan and CellQuest software (Becton Dickinson, Mountain View, CA) after staining with fluorochrome-labeled anti-CD3, CD34, CD8, CD45RA, and CD45RO mAb as described below. The mean purity of all separations as determined by FACS staining with mAb specific for CD45RA and CD45RO was ≥95% for each subset. Double-positive (CD45RA^+/RO^+) “effectector” cells, variably present in the PBMC (9 and our observation), were selected out. Cell viability was always ≥98% by trypan blue staining exclusion. The yield of purified cells recovered from all cells loaded into the column ranged from 25 to 45%, in accordance to the manufacturer’s specifications. To assess whether cell loss biased the phenotypic characteristics of T cell subsets, we performed FACS analysis of cell surface markers including, in addition to CD45 isoforms, costimulation receptors (CD28), cell adhesion molecules (CD11a, CD49d, and CD62L) and activation markers (CD25 and CD69). The expression of these molecules on the isolated CD45RA^−/RO^− subset reflected the expected phenotypes of naive and memory T cells and matched the profiles found on the corresponding subset analyzed by electronic gating in whole PBMC (data not shown). These experiments also confirmed that the negative selection strategy avoided functional alterations consequent to engagement of CD45 molecules, yielding unaltered cells.

Generation and characterization of Ag-specific T cell clones

T cell clones (TCC) specific for MBP were established from purified T cell subsets using a modification of the split-well method (2). From each donor, purified CD45RA^−/RO^− and CD45 RA^+/RO^− CD4^+ T cells (2.5 × 10^5/well) were seeded into 96-well plates (Nunc, Roskilde, Denmark) along with autologous irradiated PBMC (3000 rad, 1.5 × 10^6/well) as APC and stimulated with 30 μg/ml MBP or 10 μg/ml MBP peptide. Wells containing specific proliferating T cells (stimulation index >2 with a d<sub>0</sub>-en<sub>1000</sub>) were positively (10–15 days) restimulated with Ag and APC and expanded in medium containing 5 ng/ml of human rIL-2 (generously provided by Prof. Sebald, Biozentrum, Wurzburg, Germany). Clonality of T cell cultures was assessed by PCR-assisted TCR Vβ gene usage analysis as described elsewhere (5).

Concentrations of the soluble factors IFN-γ, TNF-α, IL-4, IL-10, and GM-CSF were measured by ELISA using CytoSets (BioSource, Camarillo, CA) according to an optimized standard protocol. Aliquots of cell culture supernatant (50 μl) were harvested 48 h after Ag stimulation and stored at −80°C until analysis. Supernatants were diluted 1:5 with assay buffer and used in a total assay volume of 75 μl/well. For each TCC tested, we measured cytokine secretion upon the following conditions: no Ag, 10 and 100 μg/ml MBP, 10 and 100 μg/ml specific MBP peptide. All measurements were performed in duplicate wells, and the specificity of the assay was ascertained in every instance.

Antigenic peptide specificities of the MBP-reactive TCC were defined by using a panel of 16 overlapping 19-mers in standard proliferation assays. Epitopes within the immunodominant region of MBP(111–129) were further mapped using a set of 16 peptides truncated at the amino (112 to 119–129) and carboxyl (111–121 to 128) termini at equimolar concentrations (5). The overall functional Ag affinity was estimated by determining the Ag concentration required to induce 50% of the maximum proliferation (EC<sub>50</sub>). This was accomplished in dose-response proliferation assays covering Ag concentrations between 10^-6 and 10^-5 μg/ml.

The expression of cell surface markers of MBP-specific TCC was assessed by three-color flow cytometry staining. Taking into account that the expression of several lymphocyte surface molecules, including CD45 isoforms varies during cell cycle progression (25), we performed FACS staining always on resting TCC at the end of each restimulation cycle (day 12), thus avoiding to introduce a bias. To monitor a possible variation of cell surface molecules after repeated antigenic contact, analysis included TCC which underwent a number of restimulations ranging from three to nine cycles. For FACS staining, 1 × 10^6 T cells/well were plated in 96-well plates and washed twice with 200 μl cold staining buffer (PBS 1% FCS-0.1% (w/v) sodium azide). The following mAb combinations were added at saturating concentrations: 1) IgG1 FITC, IgG1 PE, IgG1 Cy-Chrome; 2) CD45RA FITC (clone HI100), CD45RO PE (clone UCHL1), and CD4 Cy-Chrome; 3) CD28 FITC, CD62L PE, and CD4 Cy-Chrome; 4) CD29 FITC, CD54 PE, and CD4 Cy-Chrome; 5) CD25 FITC, CD40 ligand (CD40L), PE, and CD4 Cy-Chrome; 6) HLA-DR FITC, CD2 PE, and CD4 Cy-Chrome; 7) CD11a FITC, CD95 PE, and CD4 Cy-Chrome; 8) CD49d FITC, CD27 PE, and CD4 Cy-Chrome; 9) CD80 FITC, CD86 PE, and CD4 Cy-Chrome; 10) CD30 FITC, CD58 PE, and CD4 Cy-Chrome; 11) CD26 FITC, CD44 PE, and CD4 Cy-Chrome; 12) CD134 FITC, CD152(CTLA-4) PE, and CD4 Cy-Chrome; and 13) unstained cells. Abs were purchased from Becton Dickinson (CD2 PE, CD69 PE, and HLA-DR PE), Exalpha (Boston, MA), IgG1 FITC, IgG1 PE, Immunotech (Westbrook, ME; CD11a FITC, CD25 FITC, and CD49d FITC), and PharMingen (San Diego, CA). After 30 min incubation on ice in the dark, cells were washed twice, resuspended in 200 μl washing buffer, and transferred to 5-ml tubes for FACS scanning. Of each stained sample, 5000 events were acquired in a forward scatter/sideward scatter lymphocyte gate. Cell surface marker expression was analyzed on the CD4+ lymphocyte population.

Statistical methods

Given the difficulty in obtaining large numbers of purified T cells to set up full limiting dilution experiments, we chose culture conditions aimed to obtain MBP-specific T cell lines arising from a single precursor cell per well. Such expectation was confirmed by the clonal profiles of TCR usage of the T cell lines (data not shown). This allowed us to estimate T cell precursor frequency of MBP-responding cells by dividing the number of responding wells by the number of wells seeded, multiplied by the number of cells per well.

Variance was measured by calculating SD. Differences between groups were evaluated by the unpaired t test. For data not normally distributed, the Mann-Whitney U rank sum test was applied. Statistical calculations were performed using SigmaStat software (Jandel Scientific, San Rafael, CA).

Results

The human T cell response to MBP originates predominantly from the CD45RA^−/CD4^+ T cell subset

To estimate the frequency of autoantigen-specific T cells in the naive and memory resting CD4^+ T cell population, we obtained
highly purified CD45RA⁺/RO⁻ and CD45RA⁻/RO⁺ cell subsets by negative selection. To assess the maximum proliferative capacity of the isolated subsets in response to potent stimuli, we compared the primary response to SAg (staphylococcal enteroxin A, staphylococcal enterotoxin B, SEC1–SEC3, staphylococcal enterotoxin D, staphylococcal enterotoxin E, and toxic shock syndrome toxin-1) at concentrations ranging from 10⁻² to 10² ng/ml in dose-response proliferation assays. Cells from both subsets proliferated comparably well, indicating that universal T cell stimuli elicit similar responses both in naive and memory T lymphocytes. In contrast, the short-term reaction to TT in primary proliferation assays clearly resided in the CD45RO⁺ subset, as expected from a response to a recall Ag in immunized subjects. However, although a vigorous primary proliferation to TT (assayed after 3 days) could be detected only in the CD45RO⁺ subset, short-term TT-specific cell lines could be raised after rechallenge with the Ag from the CD45RA⁺ subset as well (data not shown).

We next dissected the T cell response of the subsets to MBP using purified T cell subsets and APCs. Interestingly, it was the CD45RA⁺ T cell population that originated predominant responses to MBP, both in MS patients and in healthy controls (Table I). The estimated precursor frequency of MBP-reactive T cells in the CD45RA⁺ subset was significantly higher than in the CD45RO⁺ subset of MS patients, (6.10 ± 3.41 × 10⁻⁶ vs 2.59 ± 2.13 × 10⁻⁶; p = 0.040). Although this was true also for healthy donors (7.24 ± 1.79 × 10⁻⁶ vs 2.67 ± 1.33 × 10⁻⁶), the small number of subjects available for analysis does not allow us to compare their precursor frequency of MBP-reactive cells with that of MS patients.

Table I. Generation of MBP/peptide-specific TCC from the naive (CD45RA⁺/RO⁻) and resting memory (CD45RA⁻/RO⁺) T cell subsets

| Subjects | MBP-Reactive Wells from Naive T Cell Subsets | MBP-Reactive Wells from Memory T Cell Subsets |
|----------|---------------------------------------------|---------------------------------------------|
|          | No. positive | % positive | No. positive | % positive |
| MS patients |               |            |               |            |
| MS1      | 30/120       | 25         | 7/60          | 12         |
| MS2      | 24/120       | 20         | 0/60          | 0          |
| MS3      | 6/30         | 20         | 6/42          | 14         |
| MS4      | 11/110       | 10         | 12/220        | 5.4        |
| MS5      | 12/60        | 20         | 1/30          | 3.3        |
| MS6      | 21/180       | 11.7       | 8/90          | 8.9        |
| MS7      | 2/30         | 2.1        | 2/120         | 2.4        |
| Total MS | 104/650      | 16.0       | 36/622        | 5.8        |
| HD*      |               |            |               |            |
| HD1      | 20/90        | 32         | 9/90          | 10         |
| HD2      | 3/16         | 3           | 4/60          | 6.7        |
| HD3      | 16/120       | 14         | 4/120         | 3          |
| Total HD | 39/226       | 17.2       | 17/270        | 6.3        |
| Total all subjects | 143/876 | 16.3       | 53/892        | 5.9        |

* HD, healthy donors.

MBP-specific TCC originating from the CD45RA⁺ and CD45RO⁺ subsets recognize different antigenic epitopes within individual subjects

To assess the Ag-fine specificities of TCC originating from either subset, we tested MBP-reactive TCC in proliferation assays using a panel of 16 overlapping MBP peptides. As summarized in Fig. 1, the overall specificities corresponded well to the pattern of immunodominant MBP peptides previously identified by others and by us (3–5, 26–28). However, a clear skewing in the specificities of TCC derived from either subset emerged. Most important, 13 of 14 TCC recognizing the immunodominant epitope MBP(81–99) originated from the CD45RA⁺ subset. T cells responding to other immunodominant regions of MBP, such as MBP(61–79) in the central region of the protein and MBP(141–159) toward the carboxyl terminus, resided as well in the naive pool (nine naive vs 1 memory TCC and seven vs none, respectively). Strikingly, when considering individual patients, the MBP epitopes recognized by TCC from either subset excluded each other almost completely (Table II). Even in the case of naive- and memory-derived TCC from a given subject responding to the same peptide MBP (111–129) (patient MS4), further epitope mapping with truncated peptides delineated different nested epitopes, probably recognized by

![FIGURE 1. Summary of MBP peptide specificities of CD45RA⁺ vs CD45RO⁺ subset-originating TCC. The fine Ag specificity of 85 TCC was tested against a set of overlapping peptides spanning the MBP sequence. Included are data from both MS patients and controls. The number of TCC specific for each MBP peptide is plotted on the x-axis, distinct in CD45RA⁺ subset-derived (hatched bars) and CD45RO⁺ subset-derived (filled bars). Remarkably, T cell lines specific for the immunodominant epitopes MBP(61–79, 81–99, and 141–159) predominantly emerged from the CD45RA⁺CD4⁺ subset.](image-url)
distinct TCC. Consistent with this finding, in this patient naive subset-derived MBP(116–124)-specific TCC MD4-RA2 and -RA11 and memory-derived MBP(115–125)-specific TCC MD4-RO1, -RO3 and -RO7 expressed different TCR β genes, i.e., TCRBV3 and TCRBV2, respectively.

Cytokine secretion profiles of CD45RA + and CD45RO + subset-originating TCC

To define the T helper subtype of MBP-specific T cells emerging from the CD45RA + and CD45RO + subsets of MS patients, we measured the secretion of pro (IFN-γ, TNF-α) and anti-inflammatory cytokines (IL-4, IL-10) in culture supernatants of 41 TCC raised from 4 subjects, as well as the APC-activating factor GM-CSF. The conventional types of T helper cells (Th1, Th2, and Th0) emerged from both the naive and the memory subsets (Table III). By using the IFN-γ to IL-4 quotient as a marker of Th1- vs Th2-type cells, we failed to detect clear-cut systematic differences in the cytokine secretion of TCC derived from the naive and memory subsets (Fig. 2). Although the small number of memory subset-derived TCC available for analysis precludes a definitive statement on their phenotype, naive subset-originating TCC were predominantly Th1 or Th0, similar to observations on PBMC-derived TCC specific for immunodominant MBP epitopes (5, 29).

Higher functional avidity of epitope recognition by CD45RA + subset-derived TCC does not depend on higher expression of costimulatory/accessory molecules

To estimate the functional avidity for the Ag of naive vs memory subset-derived MBP-specific T cells, we determined the EC50 of subset-derived MBP(116 –124) for TCC emerging from both the naive and memory subsets (Table III). To define the T helper subtype of MBP-specific T cells emerging from the naive and memory subset, respectively. These clones expressed different TCR (see Results).

Table II. Skewed peptide specificity of naive and memory subset-derived MBP-reactive TCC from individual MS patients

| Patient MS1 (DRB1*1501, 04) | Patient MS3 (DRB1*0403, 1501) | Patient MS4 (DRB1*0401, 1301) | Patient MS6 (DRB1*1502, 0701) |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| TCC                         | Peptide                     | TCC                         | Peptide                     | TCC                         | Peptide                     |
| Naive subset-derived TCC    | RA2B(8)3                    | 1–19                        | RA7                         | 81–99                       | RA1                         | 1–19                        |
| RA1A(5)                     | 81–99                       | RA B2                       | 61–79                       | RA2                         | 111–129 a                    | RA7                         | 21–39                       |
| RA1B(6)4                    | 81–99                       | RA B9                       | 61–79                       | RA11                        | 111–129 a                    | RA4                         | 41–59                       |
| RA1B(6)6                    | 81–99                       | RA D2                       | 61–99                       | RA6                         | 131–149                      | RA10                        | 61–79                       |
| RA2B(8)3                    | 141–159                     | RA D4                       | 81–99                       | RA4                         | 131–149                      | RA11                        | 61–79, 81–99                |
| RA2B(8)6                    | 151–170                     | RA B6                       | 141–159                     | RA1                         | 141–159                      | RA2, RA16,                 |
| Memory subset-derived TCC   | RA(5)7                      | 11–29                       | RO B9                       | MBP b                       | RO2                         | MBP b                       | RO2                         | 11–29                       |
| RO2A(7)3                    | 11–29                       | RO E4                       | MBP b                       | RO4                         | 71–89                       | RO7                         | 11–29                       |
| RO1B(10)4                   | MBP b                      | RO B10                      | 151–170                     | RO5                         | 31–49                       | RO8                         | 11–29                       |
| RO2B(8)2                    | 71–89                       | RO C5                       | 151–170                     | RO1 (11–29),                 | 111–129, c                   | RO6(2)                      | 31–49                       |
| RO1B(10)3                   | MBP b                      | RO D4                       | 151–170                     | RO3                         | 111–129, c                   | (121–139)                   | RO6                          | 101–119                     |
|                             |                             | RO F1                       | 151–170                     |                             |                             |                             |                             |

a Epitope MBP(116–124).
b Epitope MBP(115–125). In patient MS4, TCC reacting to MBP(111–129) were observed from both the naive and memory subset. However, performing a more detailed analysis of these TCC with a set of truncated peptides revealed the recognition of two distinct minimal epitopes, MBP(116–124) and MBP(115–125), by clones derived from the naive and memory subset, respectively. These clones expressed different TCR (see Results).

c Denotes lack of recognition of the overlapping MBP peptide set despite confirmed specificity to the whole protein.

This comprehensive analysis demonstrated that the lower Ag concentration required by CD45RA subset-derived TCC was not secondary to a more favorable expression of molecules for accessory interactions, in particular ligands for costimulation or adhesion molecules. Although it should be pointed out that recognition
of epitopes with higher binding affinity for the MHC complex such as MBP(81–99) by naive-derived cells might contribute to their lower EC50 values, our data show that T cells responding to MBP with overall greater efficiency are selected from the CD45RA+ repertoire.

Discussion

In this study, we have dissected the response of highly purified, putative naive and memory-resting CD45RA+/RO− and CD45RA+/RO+ CD4+ T cell subsets against the human self-Ag MBP. Our findings suggest a major role of the naive T cell subset in the origin and the maintenance of the self-reactive effector CD4+ peripheral T cell repertoire.

This notion is first supported by the ~2-fold higher frequency of MBP-reactive T cells in the naive CD45RA+/RO− subset that did not depend on a biased viability, proliferative potential, or coreceptor/adhesion molecule density of the progenitor T cell population. In fact, viability and response to potent stimuli like SAg did not differ in the naive and memory cell subsets. Levels of expression of IL-2R, adhesion molecules, and ligands for costimulation were lower in the purified CD45RA+ subset, thus penalizing rather than favoring its proliferative potential (11). Our results are in contrast to recent data reporting a higher frequency of MBP-specific T cells in memory CD45RO− subsets from MS patients (30). The different experimental setting might account for this discrepancy. Burns et al. (30) generated MBP-specific T cell lines from seeding PBMC populations depleted of cells expressing CD45RA or CD45RO. Since not only lymphocytes, but also dendritic cells and monocytes express CD45R isoforms (Refs. 3 and 32 and our observation), isomorph depletion may affect Ag presentation by accessory cells, which is crucial for the activation of naive T lymphocytes (6, 11, 33). Different from that study, we compared the precursor frequency in the two subsets using exact numbers of isolated CD45RA+ and CD45RO+ T cells and delivering antigenic presentation by means of unmanipulated PBMC.

The second major finding of our study is that the human T cell response to immunodominant MBP epitopes mainly resides in the CD45RA+/RO− naive subset. This observation is in line with results obtained in experimental models of autoimmunity. Encephalitogenic T cells with specificity for the immunodominant regions of MBP could be selected in vitro from PBL and spleen cells of nonimmunized healthy Lewis rats (34). Similarly, the T cell repertoire of naive healthy rats harbored diabetogenic T cells (35). In addition, CD45RBh+ cells have autoggressive potential toward several other target organs (36, 37). Unlike the case in inbred animal strains, however, MBP-reactive cells from either the naive or memory CD4+ T subsets of MS patients did not show the strong bias toward a Th1 phenotype which is characteristic for disease-mediating effector cells in experimental models of autoimmune diseases, but were phenotypically more diverse.

Although a prevalent T cell reactivity to MBP from the naive subset in MS patients could also be seen as a lack of involvement of this autoantigen in the pathogenesis of the disease, the detection of proinflammatory and activated MBP-specific T cells in MS patients by different functional assays argues against this possibility (38–40). Moreover, the increased frequency of MBP-specific T cells which can be raised in the absence of CD28/B7-mediated costimulation in MS patients (41, 42) has suggested the presence

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Table III. Cytokine secretion of naive and memory subset-derived TCC

| TCC      | IFN-γ | TNF-α | IL-4 | GM-CSF | IL-10 |
|----------|-------|-------|------|--------|-------|
| MS3-RAB2 | 165.6 | 307.2 | 222.8| 710.7  | 20.7  |
| MS3-RAB9 | 38.5  | 315.7 | 96.7 | 212.6  | 0     |
| MS3-RAC6 | 56.5  | 131.1 | 260.2| 390.5  | 0     |
| MS3-RAD4 | 0     | 0     | 14.9 | 40.9   | 0     |
| MS3-ROC5 | 0.8   | 125   | 80.2 | 76.2   | 3.1   |
| MS3-ROD4 | 0     | 0     | 985.3| 649.5  | 42.7  |
| MS4-RAL  | 571.1 | 2264.9| 23.9 | 2231.8 | 130.6 |
| MS4-RAL1 | 83.2  | 683.8 | 88.2 | 757.4  | 44.1  |
| MS4-RAL2 | 32.1  | 356.7 | 46.6 | 43.6   | 1.2   |
| MS4-RAL4 | 63.9  | 416.9 | 27.9 | 59     | 11.1  |
| MS4-RAL7 | 30.9  | 0     | 1553.3| 4.7   |
| MS4-RAL8 | 129.9 | 807.9 | 0    | 253.6  | 0     |
| MS4-RAL2 | 74.8  | 179.2 | 216.7| 120.3  | 29.6  |
| MS4-RAL3 | 21.8  | 0     | 37   | 27.8   | 26.7  |
| MS4-RAL4 | 436.5 | 738.7 | 0    | 467.3  | 0     |
| MS4-RAL5 | 175.5 | 376.4 | 100.9| 388.7  | 77.7  |
| MS5-RAL  | 6221.5 | 1913  | 36.9 | 2596.5 | 17.8  |
| MS5-RAL2 | 4619.5| 1328.4| 18.5 | 3037.4 | 0     |
| MS5-RAL4 | 5984.5| 2281.4| 17.5 | 3475.5 | 0     |
| MS5-RAL5 | 6286.6| 2658  | 30.7 | 2724.1 | 35.4  |
| MS5-RAL6 | 2079.8| 2339.3| 20.5 | 2527.9 | 56.4  |
| MS5-RAL7 | 6406.1| 2339.3| 20.5 | 2527.9 | 56.4  |
| MS5-RAL8 | 3680  | 836.1 | 33.8 | 1628   | 22.2  |
| MS6-RAL  | 1293.4| 1039.6| 0    | 625.6  | 0     |
| MS6-RAL2 | 1441.4| 685.7 | 20.7 | 742.9  | 0     |
| MS6-RAL4 | 2985.4| 1833.8| 25.8 | 716.0  | 23.4  |
| MS6-RAL5 | 678   | 1441.4| 79.5 | 1411.8 | 60.4  |
| MS6-RAL6 | 1401.1| 1983.1| 29   | 2453.7 | 2.5   |
| MS6-RAL9 | 1130.5| 2677.6| 43.3 | 2275.9 | 6.4   |
| MS6-RAL10| 42.5  | 185.5 | 19.1 | 59     | 0     |
| MS6-RAL5 | 17.5  | 177.2 | 19.2 | 94.4   | 0     |
| MS6-RAL7 | 702   | 2364.3| 23.6 | 1553.3 | 29.4  |
| MS6-RAL9 | 28.5  | 1274.5| 79.5 | 757.4  | 78.8  |
| MS6-RAL20| 30.1  | 1045.9| 163.3| 291.4  | 0     |
| MS6-RAL21| 338.6 | 2888.9| 54.3 | 1368.9 | 0     |
| MS6-RAL7 | 389.8 | 952.8 | 19.1 | 781.9  | 117.3 |
| MS6-RAL8 | 1979.8| 2545  | 51.8 | 1380   | 10    |
| MS6-RAL9 | 5094.3| 2929.9| 31.1 | 2084.4 | 56.8  |
| MS6-RAL7 | 625.9 | 1672.1| 22.5 | 792    | 0     |
| MS6-RAL8 | 4262.3| 3813.6| 22.1 | 4047.2 | 131.1 |
| MS6-RAL6 | 658.4 | 2889.9| 0    | 1512.7 | 117.2 |
| MS6-RAL7 | 207.3 | 947.6 | 0    | 1021.1 | 0     |

* For all cytokines, values refer to 48-h supernatants obtained after stimulation with 10 μg/ml MBP peptide. Background values were subtracted from those of stimulated cultures.
of myelin-reactive activated/memory cells. However, MBP-specific responses in those studies were not examined with respect to their origin in the CD45RA<sup>+</sup> vs CD45RO<sup>+</sup> compartments. The CD45RA<sup>+</sup> T cell response to recall Ag increases 3- to 5-fold in the presence of anti-CD28 Ab (43), but it remains to be determined to which extent this population contributes to the overall response of PBMC in the absence of costimulation.

The differences we found in the human CD45RA<sup>+</sup> and CD45RO<sup>+</sup> MBP-specific T cell repertoires offer important clues to speculate on the possible mechanisms of regulation and maintenance of the autoreactive effector T cell pool. In this context, particularly significant are the more efficient recognition of antigenic epitopes by naive-originating T cells. Given the lower density of coreceptors like CD28 and CD29 observed on naive subset-derived cells, their Ag-specific responses at 10-fold lower concentrations appear to be significant and indicate that a greater functional avidity of TCR-MHC-peptide interactions outweighed poorer costimulation. The absence within each individual of memory T cells responding to

**FIGURE 3.** Functional antigenic avidity of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> subset-derived TCC. A, Summarizes the analysis of the EC<sub>50</sub> of naive (n = 25) and memory (n = 10) subset-derived TCC, which revealed a significantly more efficient recognition of antigenic epitopes by naive-originating T cells. Central tendency measure is the median. Boxes frame the 5th to the 95th percentiles, and error bars represent SD. Representative examples of dose-response proliferation curves of a naive-derived TCC (B, TCC MS6-RA17) and a memory-derived TCC (C, TCC MS6-RO6) are given.

**FIGURE 4.** Differential expression of cell surface molecules on naive vs memory subset-derived TCC. An extensive mAb panel was used to compare cell surface marker expression on 20 naive and 15 memory subset-derived TCC (see Materials and Methods). Only statistically significant differences are shown. The upper plots show CD45 isoform-expressing cell percentages (left panel) and mean fluorescence intensity (MFI, center panel) for naive-derived (open circles) and memory-derived (cross-hatched circles) TCC. A relatively decreased CD27 expression was detectable in memory subset-derived T cells as compared with the naive derived (upper right panel). The lower plots show the significantly different proportions (% positive) and intensity of expression (MFI) of CD28 (left panel), CD29 (center panel), and CD134 (right panel). Visualization of all TCC may be hampered in some cases by the overlapping of circles corresponding to very close values. Asterisks at p values indicate the use of the Mann-Whitney U rank sum test.
epitopes recognized by the naive repertoire is difficult to reconcile with the hypothesis that primed MBP-specific cells might revert to a canonical naive phenotype, as shown in other systems (44). Rather, the lack of MBP epitopes shared by the naive and memory subsets in MS patients suggests that naive T cells specific for the immunodominant regions of MBP do not evolve beyond effector stage into resting long-term memory cells with CD45RA+/RO− phenotype after priming and activation in vivo.

We propose that in MS, upon effective Ag priming probably due to a potent cross-reacting stimulus (45), sufficient TCR affinity, and adequate costimulation, naive CD45RA+ T cells become activated and proliferate in secondary lymphoid organs, acquiring a double-positive (CD45RA+activated and proliferate in secondary lymphoid organs, acquiring a double-positive (CD45RA+/RO−) effector cell phenotype. These activated cells can reach the brain vascular endothelium via the peripheral blood and cross the blood-brain barrier. Their recognition of immunodominant epitopes of myelin Ag may elicit proinflammatory T cell functions and initiate a demyelinating attack. To limit the autoimmune process and restore self-tolerance, high-affinity MBP-primed T cells need to be neutralized by peripheral mechanisms, including clonal deletion and high-dose energy (46, 47). This concept is supported by the recent observation of a strong T cell response to an immunodominant epitope in MBP-deficient (shiverer) mice, and the inactivation of the high-avidity MBP-specific repertoire in congenic MBP-expressing (C3H) mice (48). According to a recently proposed model (23), memory CD45RO+ cells, which we found to recognize subdominant MBP epitopes with low affinity, may have differentiated directly from the naive pool as a result of partial activation, bypassing the proliferative/effector stage. These cells could survive in the peripheral blood as a result of cross-reactive stimulation, facilitated by their high density of adhesion molecules and receptors for costimulation (46, 49).

Our interpretation implies that effector CD4+ cells mediating demyelination in MS are generated de novo from the CD45RA+/RO− T cell pool and differentiate either into short-lived effector or long-term memory cells. Defining how naive cells can be recruited into an inflammatory focus leading to organ-specific immune attack warrants further studies. The detection of prevalent, immunodominant autoantigen-specific responses from the CD45RA+/RO− CD4+ T cell compartment points to this subset as the main reservoir of self-reactive cells, continuously available to trigger human autoimmune processes upon the appropriate stimuli.

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