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Does the Frequency and Avidity Spectrum of the Neuroantigen-Specific T Cells in the Blood Mirror the Autoimmune Process in the Central Nervous System of Mice Undergoing Experimental Allergic Encephalomyelitis?1


In humans, studies of reactive T cells that mediate multiple sclerosis have been largely confined to testing peripheral blood lymphocytes. Little is known how such measurements reflect the disease-mediating autoreactive T cells in the CNS. This information is also not available for murine experimental allergic encephalomyelitis (EAE); the low number of T cells that can be obtained from the blood or the brain of mice prevented such comparisons. We used single-cell resolution IFN-γ ELISPOT assays to measure the frequencies and functional avidities of myelin basic protein (MBP:87–99)-specific CD4 cells in SJL mice immunized with this peptide. Functional MBP:87–99-specific IFN-γ-producing cells were present in the CNS during clinical signs of EAE, but not during phases of recovery. In contrast, MBP:87–99-specific T cells persisted in the blood during all stages of the disease, and were also present in mice that did not develop EAE. Therefore, the increased frequency of MBP:87–99-reactive T cells in the blood reliably reflected the primed state, but not the inflammatory activity of these cells in the brain. The functional avidity of the MBP:87–99-reactive T cells was identical in the brain and blood and did not change over 2 mo as the mice progressed from acute to chronic EAE. Therefore, high-affinity T cells did not become selectively enriched in the target organ, and avidity maturation of the MBP:87–99-specific T cell repertoire did not occur in the observation period. The data may help the interpretation of measurements made with peripheral blood lymphocytes of multiple sclerosis patients. The Journal of Immunology, 2005, 174: 4598–4605.

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1 Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; LN, lymph node; dLN, draining LN; MRP, myelin basic protein; PLP, proteolipid protein; MOG, myelin oligodendrocyte glycoprotein; PTX, pertussis toxin.

Received for publication September 27, 2004. Accepted for publication January 1, 2005.

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1 This work was supported by grants to P.V.L. from the National Institutes of Health (NS-39434) and to M.T.-L. (AI-47756). T.G.F. was supported by Grants NS-42809 from the National Institutes of Health and RG3322 from the National Multiple Sclerosis Society. H.H.H. was supported by a fellowship of the Studienstiftung des Deutschen Volkes.

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CNS Ags (myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG)) are present in the blood of MS patients; the responses detected are mostly weak and frequently present in healthy individuals as well. Does this outcome result from the sequestration and eventual deletion of the disease-related high-affinity T cell clones in the CNS of MS patients? Therefore, are MBP-reactive cells detectable in the blood of MBP-immunized mice during all phases of EAE?

Studies of the fate of the autoreactive T cell were initially done using T cell lines and T cell clones using the passive EAE model. More recently, TCR-transgenic cells have started to emerge as a powerful new tool for studying the behavior of neuroantigen-specific T cells in EAE (5, 6). However, both T cell clone- and TCR-transgenic-cell-based approaches have the limitation of not being able to account for affinity-based repertoire selections. Affinity maturation was observed in NOD diabetes (7), and has been postulated to play a central role in the progression of T cell-mediated autoimmunity. It is conceivable that high-affinity clones within the repertoire engaged by immunization are further expanded by the endogenous autoantigen, and that among these clones those with the highest affinity are expanded most. Unlike for B cells, affinity maturation via the mutation of Ag receptors is not common for T cells. However there are a number of mechanisms that can lead to a change of affinity for Ags in a T cell. RAG recombinase can be reactivated in peripheral T cells (8, 9). The Vα gene of the TCR has been shown to be capable of multiple rearrangements (10, 11). There are reports of changes in Vα chain expression leading to T cell affinity maturation (12). Furthermore, the activation state of the T cell can contribute to its affinity (better defined as functional avidity, or Ag Responsiveness) (13). To the contrary, scenarios can be proposed that lead to a decrease in the affinity of the neuroantigen-specific T cell repertoire. If autoantigen-recognition in the CNS predisposes T cells to apoptosis, then high-affinity neuroantigen-specific T cells (that recognize Ag in the brain) will preferentially die, and eventually the neuroantigen-specific repertoire will consist of “irrelevant” clones that were activated by excess peptide during the peripheral immunization but are not of high enough avidity to recognize the endogenous autoantigen and to mediate EAE. Affinity maturation being central to the understanding of the immune pathology of EAE can only be addressed by studying the full T cell repertoire of wild-type mice. We asked whether affinity maturation occurs in EAE by measuring longitudinally the functional avidity of the neuroantigen-specific T cell repertoire during various phases of the disease process.

A major obstacle to obtaining unambiguous answers to the questions mentioned above has been the technical limitations of methods available to study the neuroantigen-specific T cells in vivo (14). First, except for the brief accumulation of these T cells in the dRLN shortly after immunization, their frequency is low in freshly isolated cell material (at, or below the detection limits of standard T cell assays). Second, such T cell assays require a rather high number of T cells (several millions), a number that cannot be readily obtained from murine blood or the CNS. We used a new generation of computer-assisted ELISPOT assay to overcome these limitations (15). Measuring the Ag-induced cytokine production by individual T cells, this test system excels in detecting the few Ag-specific T cells in the test cell populations, even when the specific cells occur in the frequency range of 1:100,000 to 1:1,000,000, which is below the detection limit of flow cytometry-based alternative approaches, including intracytoplasmic cytokine or tetramer staining. Because the ELISPOT approach measures cytokine production by individual cells (16), it permits measurement of the accurate frequencies of the Ag-specific T cells. Requiring only few cells, the ELISPOT approach also permits multiple measurements on the limited blood and CNS-derived lymphocytes titrating the recall peptide concentration. In this way, the ELISPOT approach permits the measurement of the functional avidity of the T cells as defined by the concentration of the nominal peptide at which 50% of the peptide-specific T cells become activated (17). Taking advantage of the unique features of this ELISPOT approach, we set out to study in MBP:87–99 induced active EAE the frequency and functional avidity of the MBP:87–99-specific T cells in the dRLN, spleen, blood, and CNS of mice.

Materials and Methods

Animals, Ags, and treatments

Male and female SJL (H-2d) mice, age 6–8 wk, were purchased from The Jackson Laboratory and maintained in specific pathogen-free animal facilities of Case Western Reserve University. MBP peptide 87–99 was the kind gift of Dr. A. Gaur at Neurocrine Biosciences (San Diego, CA). IFA was purchased from Invitrogen Life Technologies and CFA was prepared by mixing Mycobacterium tuberculosis H37RA (Difco Laboratories) at 2.5 mg/ml into IFA. For inducing autoimmune disease, MBP:87–99 was mixed with CFA to yield an emulsion whose maximal pathogenic dose has been previously established, being 2.5 mg/ml for MBP:87–99. Of this emulsion 100 μl (250 μg/mouse) was injected once, s.c., at two sites of the flank. Pertussis toxin (PTX, 200 ng; List Biological) was injected i.p. in 500 μl of saline at the time of the CFA immunization and a second time, 24 h later. The mice were assessed daily for the development of paralytic symptoms and the severity of disease was recorded according to the standard scale: grade 1, floppy tail; grade 2, hind leg weakness; grade 3, full hind leg paralysis; grade 4, quadriplegia; grade 5, death. Mice that were in between the clear cut gradations of symptoms were scored intermediate in increments of 0.5. In particular, for scoring a mouse as ‘‘diseased’’ in this study, we required signs of floppy tail with some indication of hind leg weakness, that is, a minimal score of 1.5.

Cell preparation from the various organs

Mouse blood was obtained using heparin as an anticoagulant. The blood was diluted with sterile PBS. PBMCs were obtained by density gradient centrifugation over Ficoll, washed three times with DMEM, and counted afterward. Finally, the cells were plated at various cell numbers (ranging from 5 x 104 to 5 x 105 per well) in HL-1 medium (BioWhittaker; supplemented with 1% glutamine) together with Ag and 5 x 108 irradiated naive spleen cells functioning as additional APC. Cells from the spinal cord were purified as described (the number of cells washed twice twice the standard: grade 1, floppy tail; grade 2, hind leg weakness; grade 3, full hind leg paralysis; grade 4, quadriplegia; grade 5, death. Mice that were in between the clear cut gradations of symptoms were scored intermediate in increments of 0.5. In particular, for scoring a mouse as “diseased” in this study, we required signs of floppy tail with some indication of hind leg weakness, that is, a minimal score of 1.5.

ELISPOT assays and ELISPOT image analysis

ELISPOT assays were performed as described previously (19). Briefly, ImmunoSpot M200 plates (Cellular Technology) were coated overnight with the capture Abs in sterile PBS. R46A4 at 4 μg/ml (isolated and purified from hybridoma) was used for IFN-γ, JES6-1A12 at 3 μg/ml (BD Pharmingen, San Diego CA) was used for IL-2, 1B11 at 2 μg/ml (isolated and purified from hybridoma) was used for IL-4, and TRFK5 at 5 μg/ml (isolated and purified from hybridoma) was used for IL-5. The plates were blocked for 1 h with sterile PBS containing 1% BSA and washed 3 times with PBS. The cells (in the numbers specified) were plated in HL-1 medium (enriched with 1% glutamine) and Ag. In selected experiments, cells and Ags were titrated. The plates were incubated at 37°C, 5% CO2 for 24 h (IFN-γ and IL-2 assays) or for 48 h (IL-4 and IL-5 assays). After washing first with PBS then with PBS 0.025% Tween 20 (PBST), detection Abs were added overnight. XMG1.2-biotin (isolated and purified from hybridoma in our laboratory) was used for IFN-γ. Rat anti-mouse IL-4-biotin BVD6-24G2, BD Pharmingen) was used for IL-4, and rat anti-mouse IL-2-biotin (JES6-5H4, BD Pharmingen) was used for IL-2. Biotinylated TRFK4 (BD Pharmingen) was used for IL-5. The plates were then washed 3 times in PBST. Streptavidin-AP (DakoCytomation) was added at 1:1000 dilution in PBST as a third reagent for IL-2, IL-4, and IL-5 for 2 h, followed by three washes with PBS. The plates were developed using NBT/4599The Journal of Immunology

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Results


We immunized SJL mice with MBP:87–99 peptide in CFA, including PTX injections as required for EAE induction. Of the 140 mice immunized in six experiments, 113 (81%) developed signs of clinical EAE with a score of at least 1.5; the mean disease onset was between day 9 and 15. At various time points after immunization, and at various stages of the disease, mice were sacrificed. Mononuclear cells were isolated from their drLN, blood, spleen, and CNS and the cell isolates were tested separately for each mouse. MBP:87–99 peptide-induced IFN-γ ELISPOT formation was measured in the freshly isolated cells in assays of 24 h duration. Although MBP:87–99 peptide did not induce IFN-γ spots in any of the organs of unimmunized or OVA:CFA/PTX control immunized mice (data not shown), this peptide induced responses in all of the MBP:87–99 immunized mice (Fig. 1). In all the organs, very clear Ag-induced signals were detected. Dedicated ELISPOT image analysis software permitted clear definition of the individual spots over various levels of background: an example of the image analysis is provided in Fig. 2. With the exception of the CNS isolates during the first days of EAE onset, the medium control wells contained essentially no or only a few spots (<3/million cells). The MBP:87–99 peptide-induced production of IFN-γ in the CNS could be entirely blocked by adding anti-CD4 Ab (data not shown). When the cell isolates from either of these organs of MBP:87–99 immunized mice were serially diluted in the presence of a constant number of APC, the number of MBP:87–99-induced spots detected and the number of cells plated gave a linear function that passed through the origin (data not shown and Refs. 15, 18, and 19). Thus, in addition to drLN and the spleen, clear-cut MBP:87–99-specific recall responses could be detected in the blood and in the CNS of MBP:87–99 immunized mice. Based on spot size and linear titration, these IFN-γ spots signify the cytokine production by single cells, i.e., the measurements reflect actual frequencies of IFN-γ-producing MBP:87–99-specific CD4 cells.

Table I summarizes the frequency of MBP:87–99 peptide-induced IFN-γ spot forming cells per million mononuclear cells isolated from the organs, at various phases of EAE. The results for three to four representative individual mice are shown for each of the following time points: 3 days and 7 days after immunization (preonset EAE), acute EAE (within 2 days of the first manifestation of the disease), chronic EAE (mice with EAE persisting for at least 14 days), and mice that were tested in the recovery phase. The data are described in detail in the following section.

MBP:87–99-specific IFN-γ-producing cells appear first in the drLN and become detectable in the blood by day 7

In MBP:87–99 immunized mice, peptide-induced IFN-γ-inducible T cells were first detected in the drLN, on day 3 after immunization; these cells occurred in the frequency range of 10–30 per million (Table I). By accounting for the absolute number of cells recovered from the drLN (between 10 and 30 million), we have established that in average 433 (between 250 and 780) MBP:87–99-specific, IFN-γ-producing CD4 Ag-experienced memory/effector cells are present in the drLN on day 3 (Fig. 3). Because T cell proliferation and differentiation into cytokine-expressing Ag-experienced memory/effector cells occur in the drLN after such s.c. immunization (20), and because Ag-experienced memory/effector cells at this time point were not yet detectable in other tissues (Table I and Fig. 3), it appears that these cells have not disseminated yet, and that their entire clonal size in the mouse is reflected by their numbers in the drLN.

By day 5 after immunization, MBP:87–99-specific, IFN-γ-producing Ag-experienced memory/effector T cells started to become detectable in the blood, and by day 7 they were present in the blood of all mice, occurring in 30–150 per million frequency range (Table I). At this point in time, IFN-γ-producing T cells became also detectable in the spleen, in the frequency range of 50–200 per

![Figure 1](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)
During acute and chronic EAE, MBP:87–99-reactive T cells become detectable in the CNS and continue to be present in the blood.

Unlike in pre-EAE-onset mice, the animals tested shortly after the clinical onset of EAE (1–7 days after onset of the paralytic symptoms) showed high frequencies of MBP:87–99-reactive cells in the CNS isolates. These MBP:87–99-reactive cells were CNS-resident (as opposed to contaminating blood cells) because the CNS isolates yielded up to 28-fold higher frequencies of MBP:87–99 induced spots than measured in the blood of the same animals (Table I). Also the fact that no peptide-induced spots were detected in the CNS before the onset of the disease while such cells were present in high frequencies in the blood (Table I) argues against blood cell contamination affecting these frequency measurements in the CNS.

The numbers of MBP:87–99-reactive cells were rather high in the CNS of mice with acute EAE as compared with the frequencies detected in the immune periphery (Table I), yet the highest number detected among all mice in acute EAE was 2,599:1,000,000 (that is, 1:385). The CNS frequencies were considerably lower in mice with chronic EAE (Table I). These data suggest, that among the cells that infiltrate the CNS in active EAE, only a minor fraction is specific for the disease-inducing peptide, a notion supported by previous reports (18, 21).

When the frequencies of MBP:87–99-specific cells in the CNS were corrected for absolute numbers, they constituted a minor fraction of the MBP:87–99-specific Ag-experienced memory/effector cells that infiltrate the CNS in active EAE, only a minor fraction is specific for the disease-inducing peptide, a notion supported by previous reports (18, 21).
detected in the immune periphery (spleen and blood) of mice with active EAE, but the majority of these cells reside there.

In mice that recover from EAE, MBP:87–99-specific cells can no longer be detected in the CNS, but such mice continue to have high frequencies of peptide-reactive cells in the blood. The last group of mice which was studied consisted of animals that, after passing through a phase of acute EAE, had recovered from the neurological symptoms. These mice continued to have MBP:87–99-specific IFN-γ-producing cells in their blood and spleens occurring at frequencies comparable to mice that undergo active disease (Table I). However, CNS isolates of the mice that stably recovered invariably failed to show strong MBP:87–99-induced IFN-γ production: the frequencies were <50-fold lower compared with the diseased mice, and frequently no MBP:87–99 induced spots were detected at all. In such stably recovered mice the testing of blood and spleen revealed MBP:87–99-reactive T cells in relative high frequency (Table I, Fig. 3). Therefore, in the stably recovered mice, like in the mice that were immunized but never developed disease, immune reactivity in the periphery did not mirror the autoimmune activity in the target organ.

The functional avidity of the MBP:87–99-specific T cell repertoire is comparable in the blood, spleen, and the CNS. T cell avidity is comprehensively defined as the peptide concentration at which the activation threshold of T cell is reached (17). In wild-type mice (and in humans) Ag-specific T cells tend to be polyclonal, encompassing a spectrum of clones with avidities ranging from low to high. The avidity of a T cell population can be defined as the peptide concentration at which 50% of the maximally inducible Ag-specific T cells become activated. Less peptide is needed for activation if the avidity of the T cell population increases, subsequently the dose-response curve will be shifted to the left. A right shifting of the dose-response curve signifies a decrease in avidity of the peptide-specific T cell repertoire. To study the functional avidity of the MBP:87–99-specific cells, we established the MBP:87–99 peptide-induced dose-response curves for the T cells isolated from different organs. In this assay, the peptide can bind directly to the restricting I-A^d molecule on the surface of APC (not requiring processing) and all until saturation is reached, the peptide concentration is directly proportional to the number of I-A^d:MBP:87–99 complexes on the APC available for stimulation of the specific T cells.

Only spleen cells were available in sufficient numbers to permit establishing the dose-response curves for individual mice over a wide range of peptide concentrations. Fig. 4A shows results testing spleen cells of three individual mice which were all immunized 14 days earlier; all three mice exhibited clinical EAE at the time of testing. The maximal number of cells that could be activated to produce IFN-γ by adding increasing concentrations of the peptide was reached in all mice at ~10 μg/ml. The peptide concentration at which 50% of the maximal cell number became activated (K_{eff}, defining functional avidity) was between 0.5 and 5 μg/ml of the MBP:87–99 peptide. Therefore, while the clonal sizes (the frequencies) of peptide-specific IFN-γ-producing T cells in the individual mice showed up to 2-fold interindividual variation (which corresponds to only a single additional round of cell division), the avidity spectra of the clones engaged in the spleens of the individual mice showed K_{eff} values in a rather narrow range.

![FIGURE 3. Absolute numbers of MBP:87–99-specific T cells in various organs at different stages of EAE. The frequencies of peptide-specific cells in the organs of individual mice (as summarized in Table I) have been recalculated by multiplying with the cell numbers harvested from each organ.](http://www.jimmunol.org/)

![FIGURE 4. Dose-response curves of MBP:87–99-induced IFN-γ spot formation in various organs and at different stages of EAE. A, Spleen cells of three individual mice were isolated on day 14 while the mice were diseased with acute EAE. The spleen cells were activated in the presence of the specified concentration of MBP:87–99 peptide, and the number of ELISPOTs induced were measured. B, Pooled spleen cells, PBMC, and CNS isolates were tested from immunized mice, and the peptide concentrations at which 50% of the peptide-specific cells became activated (K_{eff} values) were calculated from dose-response curves. Cells from three mice for each organ were pooled at three different stages of EAE: day 7, before EAE onset (pre.), during acute active EAE, day 14 (act.), and after 2 mo, when the mice recovered from EAE (recov.). Results of two experiments are shown, E. 1 and E. 2, respectively. NA, No lymphocytes were recovered from CNS. C—E, Dose-response curves were established in the spinal cord (C), spleen (D), and the blood (E) at the time points specified by symbols. •, Day 7, before EAE onset; □, acute EAE (day 14); and △, after 2 mo, when the mice recovered. The cells of three mice were pooled for each organ. The results were reproduced in two independent experiments.](http://www.jimmunol.org/)
Pooling the cells isolated from the CNS and from the blood of the same three mice, we established the dose-response curves for these isolates as well. The pooled spleen cells were tested simultaneously. The $K_{\text{eff}}$ values obtained for the blood and the CNS were essentially identical to those in the spleen (Fig. 4B, E, 1). In a separate experiment, we reproduced these data testing pooled cells from mice with acute EAE (Fig. 4B, E, 2). Therefore, the avidity spectrum of MBP:87–99-specific clones in the blood, spleen, and the CNS was comparable in acute EAE; we found no evidence of retention of high-affinity cells in the CNS of mice with acute disease. The avidity spectrum of the MBP:87–99-specific cells in the blood reflected the avidity spectrum present in the target organ at the same time.

The MBP:87–99-specific repertoire does not undergo avidity maturation in the course of EAE

Deletion of the high-affinity end of the MBP:87–99-specific repertoire appeared to be a possible explanation for the observation that peptide-specific cells are no longer detectable in the CNS of mice that recovered from EAE, but continue to be present in high frequencies in the blood and the spleen. If this was the case, then the dose-response curves of spleen and blood cells should be shifted to the right in the mice that recovered from EAE, as compared with the preonset or acute stage of the disease. Moreover, in this scenario, a higher dose of peptide might be required to activate low avidity cells in the CNS. Therefore, we measured the $K_{\text{eff}}$ values for CNS, blood, and spleen for mice at the preonset, acute EAE, or recovery stage (Fig. 4, C–E). The $K_{\text{eff}}$ values were found to be in the 0.5–5 μg/ml concentration range similar to the $K_{\text{eff}}$ values for individual mice tested during acute EAE (Fig. 4A). Because we observed 1,000- to 10,000-fold $K_{\text{eff}}$ increases as the consequence of repertoire selection during central tolerance development to MBP (22), these data suggest that there is no significant avidity maturation of the MBP:87–99-specific T cell repertoire in the course of MBP:87–99-induced EAE.

Discussion

In this study we followed the natural history of the autoimmune T cell response to MBP:87–99 in EAE induced by immunization with this peptide in SJL mice. One aim was to establish the numbers of Ag-experienced memory/effector T cells in the CNS, blood, and the CNS over the course of the disease. This part of the data is meant to address the ability to generalize observations we have previously made measuring the distribution of the PLP:139–151-specific T cells in SJL mice (18). The parallels seen for these organs in MBP:87–99 and the PLP:139–151 are striking, including the kinetics of cytokine secretion. We also observed very similar organ distributions of the neuroantigen-specific T cells in the course of EAE in the MOG:35–55 model of C57BL/6 mice (H. H. Hofstetter, A. Y. Karulin, T. G. Forsthuber, M. Tary-Lehmann, and P. V. Lehmann, manuscript in preparation). Therefore, the “reference values” provided in this paper for LN, spleen, and CNS might broadly apply to EAE models, and possibly to MS. However, the primary objective of this study was to determine how the numbers of neuroantigen-specific T cells in the blood compare at different stages of EAE to the numbers of these cells in lymphoid tissues and the target organ. Moreover, in this study we tested whether avidity distributions and/or avidity maturation occurs in EAE including whether measurements of the neuroantigen-specific T cell activity in the immune periphery, including the blood, reflect the T cell activity in the CNS itself.

After immunization, the initial proliferation and differentiation of the MBP-specific T cells occurred in the dLN, suggesting that the precursor cells to this response were L-selectin+ naïve T cells. Consistent with this notion we did not detect MBP:87–99-induced production of IFN-γ at early time points in the spleen, nor in any cell material (LN, spleen, blood, CNS) derived from immunized mice. Therefore, the MBP:87–99-specific cells were not preactivated to an Ag-experienced memory/effector state either by the endogenous autoantigen or by cross-reactive environmental priming. Because the detection limit of our assay as performed is 1:1,000,000 cells (16), we can conclude that if preprimed MBP:87–99-specific cells exist in nonimmunized SJL mice, such Ag-experienced memory/effector cells occur in a frequency range <1 in a million.

Between day 3 and day 14, the total number of peptide-specific IFN-γ-producing CD4 cells in dLN and spleen increased ~125-fold, corresponding to six cell divisions. This number is likely to slightly under represent the extent of clonal expansion because, while most Ag-experienced memory/effector T cells reside in the spleen, these cells also disseminate early into extra lymphoid tissues, including the lung, the liver, the gut, and skin (23). Indeed, studying the MBP:87–99-specific recall response in cells obtained from the peritoneal cavity, by lavage, we found high frequencies of peptide-specific IFN-γ-producing cells as early as day 8 after the immunization (data not shown).

The maximal frequencies of MBP:87–99-specific CD4 cells induced by immunization with CFA and PTX in the immune periphery (~1:2000) are one to two orders of magnitude lower than has been measured by tetramers or ELISPOT for CD8 cells during early phases of viral infections (24, 25). We observed similar frequencies (~1:2000) of PLP:139–151-reactive CD4 cells in EAE induced by immunization with that peptide in SJL mice (18), and in the MOG:35–55 peptide-induced EAE of B6 mice (H. H. Hofstetter et al., unpublished data). The adjuvant-driven clonal expansion of CD4 cells in standard EAE models therefore does not seem to induce clonal sizes comparable to viral infections. This difference might be of importance in MS, where the priming of the first wave of effector T cells is potentially thought to be induced by a cross-reactive infection.

The frequency of the MBP:87–99-specific T cells reached its peak in the blood and spleen around day 14 of immunization, the time point when EAE typically developed. During clinical disease, the frequency of IFN-γ-producing MBP:87–99-specific cells reached higher numbers in the CNS isolates compared with lymphoid tissues and the blood showing selective recruitment and retention of the specific cells in the target organ. Although the CNS isolates consisted of 20–50% CD4 lymphocytes, the frequencies of the MBP:87–99-specific IFN-γ-producing cells did not exceed 1:300. Therefore, even at the peak of the first EAE attack, T cells specific for the immunizing peptide represented only a minor fraction of the T cells that accumulated in the CNS.

If in MBP:87–99-induced EAE only 1:300 cells in the CNS are MBP:87–99-specific cytokine-producing functional Ag-experienced memory/effector cells, what is the specificity of these other T cells that constitute the vast majority of the infiltrate? Many MBP:87–99-specific cells could be rendered nonfunctional in the CNS as a consequence of the prevalent apoptosis induction (26, 27). Other T cells could be specific for third party neuroantigens, engaged by determinant spreading (4, 28). Yet other cells could be bystander cells nonspecifically recruited into the CNS. In models of passive EAE, the recruitment of host-derived T cells by the injected neuroantigen-specific T cells is a well established phenomenon (21); it is likely to apply for active EAE as well.

In all mice we studied, the frequencies of MBP:87–99-specific T cells was considerably lower in chronic EAE than in acute EAE. The most likely explanation for this finding is that the T cells that
reach the CNS die in the target organ. As we have shown previously, the first wave effecter cells exhaust in EAE over time (18). Simultaneously, in several other models the occurrence of determinant spreading was noted (18, 28), and it is likely that also in this MBP:87–99 model second wave effecter cells become engaged and drive the autoimmune pathology in the chronic phase of the disease when the first wave of effecter cells have declined in numbers.

Calculating the total numbers of MBP:87–99-specific functional T cells in the CNS and comparing these with the numbers of such cells in the peripheral lymphoid tissues, we found that even in acute EAE only a minor fraction of the MBP:87–99-specific functional T cells were present in the CNS; up to 60 times higher number of these cells were present in the spleen. The approach that we used is likely to underestimate the total numbers of MBP:87–99-reactive T cells present in the CNS because we isolated cells from the spinal cord only, and because during the cell isolation process from the spinal cord tissue we most likely do not recover all (or even the majority) of the infiltrating T cells. However, our assessment of the peripheral repertoire in the lymphoid tissues is also certainly an under representation of the total number of MBP:87–99-specific cells outside the CNS (23, 29). Because we always detected high frequencies of MBP:87–99-specific cells in the peritoneal cavity (representing a random extra lymphoid compartment from which T cells can be readily sampled), a considerable mass of the Ag-experienced memory/effector cells is likely to reside in other extra lymphoid tissues as well. The lungs, the liver and the gut represent sites to which Ag-experienced memory/effector cells preferentially migrate, but being solid tissues, they cannot be readily studied. Taking this “missing mass” in both the CNS and in the “rest of the body” into account, it might still be safe to conclude that the vast majority of the MBP:87–99-specific Ag-experienced memory/effector cells reside outside of the CNS, even in the acute phase of the disease. The numbers in Fig. 3 indicate that even if our isolation method only yields one-tenth of the T cells that are actually present in the inflamed CNS, they will still be outnumbered >10-fold by the numbers of specific T cells present in the immune periphery. This notion contradicts results obtained in a model of passively induced EAE, in which the majority of the transferred neuroantigen-specific T cells was reported to migrate to the CNS (4). The difference might be explained by the nature of the disease model: in passive EAE the transferred T cells occur in much larger clonal sizes and are fully synchronized by the reactivation in vitro.

No major differences were seen when the functional avidity of the MBP:87–99-specific T cell repertoire was tested in the different anatomic compartments and at different stages of EAE. The frequencies of the Ag-specific T cells was different for the individual mice and the different stages of the disease, however, the $K_{eff}$ values denoting functional avidity were surprisingly similar (between 0.5 and 5 μg/ml Fig. 4). We have observed that avidity changes of T cell repertoires caused by clonal selection result in up to 10,000-fold changes in $K_{eff}$ values (22) and that T cell activation state-dependent avidity changes of a single T cell clone can affect the $K_{eff}$ value up to 50-fold (13). Therefore, the maximally 10-fold interindividual variations and differences that we observed in this study between organs and time points show that if avidity distribution or maturation occurs in EAE, it is on a minor scale. Confirming the notion that neuroantigen-reactive T cells are not retained in the inflamed CNS, no dramatic differences have so far been found between T cell repertoires in PBMC of MS patients and healthy donors.

In apparent contrast to our data generated in an EAE model, avidity maturation was reported to be responsible for disease progression in another classic animal model of autoimmune disease, the diabetic NOD mouse (7). These differences might be explained by the different T effector cells studied, CD8 T cell reactivity was investigated in the study mentioned above. CD4 and CD8 cells might undergo different tuning mechanisms of avidity regulation. Furthermore, in autoimmune diabetes the endogenous autoantigen is not sequestered behind an immunological barrier, while CNS autoantigens are not readily accessible for the autoimmune T cell attack. Therefore, we can envisage two fundamentally different interpretations of our finding of stable functional T cell avidity in EAE. First, there might be a unidirectional flow of the neuroantigen-specific T cells from the periphery to the CNS. From the sizeable peripheral reservoir, MBP:87–99-specific cells (high- and low-affinity cells alike) might migrate at a rather slow rate to the CNS where autoantigen recognition causes them (high- and low-affinity cells alike) to undergo apoptosis locally. In this scenario, Ag encounter in the brain causes T cells to die, not to undergo affinity maturation. The other possibility is that the encounter of the autoantigen in the CNS drives the clonal expansion of these T cells, whereby low- and high-affinity cells expand at the same rate; if the resulting daughter cells recirculate between the CNS and the immune periphery, this would also result in a constant avidity spectrum. Although it is clear that T cells entering the CNS parenchyma are prone to die, this does not apply to the cells residing in the mesenchymal compartments, including the perivascular cuffs, where the majority of the infiltrating lymphocytes reside (2, 21). Indeed, our previous studies have shown that determinant spreading occurs in the brain itself (18). Therefore, the inflamed CNS is capable of supporting clonal expansions of T lymphocytes. Irrespective of which of the two mechanisms apply, our data clearly shows that in the EAE model studied, avidity maturation over time, or the selective retention of high avidity clones in the CNS does not occur. As far as the affinity spectrum of the autoimmune repertoire was concerned, the blood therefore was fully representative of the autoreactive repertoire present in other organs of the mouse, including the target organ itself.

It is striking that in disease recovery, while there are no MBP:87–99-specific cells present in the CNS, MBP:87–99-specific cells continue to be abundant in the spleen and the blood. The cytokine profile of these T cells showed no significant shift toward a possibly nonpathogenic Th2 class, nor was the functional avidity of these MBP:87–99-reactive cells shifted so as to suggest the deletion of the pathogenic high-avidity clones. Although naive autoactive T cells being ignorant of the autoantigen was previously observed (30), those data also suggested that once naive T cells become activated to the Ag-experienced memory/effector state, they become autoaggressive. Our observation suggests that Ag-experienced memory/effector cells can be ignorant of the autoantigen as well, although this might apply for the EAE model only. To penetrate the blood-brain barrier, T cells have to be in a recently activated/blast state (31, 32). By the time of recovery, there might not be sufficient MBP:87–99-specific blasts present in the immune periphery to fuel the autoimmune attack on the CNS although there apparently still are sufficient numbers of (resting) Ag-experienced memory/effector cells left. When reactivated in vitro for adoptive transfers, or reactivated in vivo by superantigens (33), these “ignorant” Ag-experienced memory/effector cells can become pathogenic. Gradual loss of the hyperactivated state of the first generation of effector cells (MBP:87–99-specific T cells in MBP:87–99-induced EAE), therefore, might account for the initial recovery from EAE. Their reactivation, or the engagement of new generations of effector cell blasts by determinant spreading, might account for the relapsing disease. By extrapolation, primed neuroantigen-specific T cells might also be detectable in the blood of
humans without signifying an active autoimmune process in the target organ.

In conclusion, the major scope of these experiments was to establish to what extent measurements of the autoimmune T cell repertoire in blood (as pertinent for human studies) or in lymphoid tissues (as has been done so far in the mouse) reflect the autoimmune T cell repertoire present in the CNS. With the exception of the first 5 days, before T cells start to disseminate after immunization, the presence of primed, MBP:87–99-specific Ag-experienced memory/effector cells was reliably reflected in blood, even during the peak of the disease. During active EAE, blood displayed the same clonal avidity spectrum as the CNS, showing that the same repertoire is present in both organs, that is, high avidity clones do not become selectively enriched in the CNS leaving only the low avidity cells in recirculation. The frequency of the neuropeptid-specific cells in blood reflected neither the number of cells present in the CNS, nor whether the mice were diseased. For MS, these data suggest that measurements done on peripheral blood leukocytes might reliably reflect the primed state of the autoreactive T cell repertoire. However, neither the mere presence of primed T cells, nor their exact frequencies/clonal sizes permits conclusions as to their overall numbers in the body or in the CNS. Perhaps, more importantly, the detection of these cells, even in high frequencies, does not necessarily reflect on the pathological activity in the target organ itself. In addition, it should have therapeutic implications to know that even at the peak of the disease the vast majority of the autoreactive T cells reside outside the target organ.

Acknowledgments
We thank R. Trezza and T. Ansari for excellent technical assistance, Drs. P. Heeger and Z. Fabry for valuable discussions.

Disclosures
The authors have no financial conflict of interest.

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