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Progression of Relapsing-Remitting Demyelinating Disease Does Not Require Increased TCR Affinity or Epitope Spread

Anna E. Kersh,* Lindsay J. Edwards,† and Brian D. Evavold*

In this study, we investigate the basis of T cell recognition of myelin that governs the progression from acute symptoms into disease remission, relapse, and chronic progression in a secondary progressive model of demyelinating disease. Until now, the frequency and affinity of myelin-reactive CD4 T cells that elicit relapsing-remitting disease have not been quantified. The micropipette adhesion frequency assay was used to obtain a sensitive and physiologically relevant two-dimensional measurement of frequency and TCR affinity for myelin, as the inherent low affinity does not allow the use of specific peptide:MHC-II tetramers for this purpose. We found the highest affinity and frequency of polyclonal myelin oligodendrocyte glycoprotein–reactive cells infiltrate the CNS during acute disease, whereas affinities during remission, relapse, and chronic disease are not significantly different from each other. Frequency analysis revealed that the vast majority of CNS-infiltrating CD4 T cells are myelin oligodendrocyte glycoprotein reactive at all time points, demonstrating epitope spread is not a predominant factor for disease progression. Furthermore, time points at which mice were symptomatic were characterized by an infiltration of Th17 cells in the CNS, whereas symptom remission showed an enrichment of cells producing IFN-γ. Also, the ratio of regulatory T cells to Foxp3+ CD4 T cells was significantly higher in the CNS at remission than during acute disease. The results of this study indicate that a high frequency of T cells specific for a single myelin Ag, rather than increased TCR affinity or epitope spread, governs the transition from acute symptoms through remission, relapse, and chronic disease states.

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Multiple sclerosis (MS) is an autoimmune disease characterized by demyelination, inflammation, and gliosis of the CNS. The greatest genetic risk factor for developing MS is in HLA-class II genes, and the disease is predominantly mediated by a CD4 T cell response. Experimental autoimmune encephalomyelitis (EAE) in the NOD mouse is a clinically relevant model of MS due to the fact that these mice develop relapsing-remitting symptoms that become chronic, similar to the disease course experienced by ~80% of MS patients (1). Additionally, the NOD model is ideal to study these mice have a MHC class II–associated proclivity to autoimmune disease and are well studied for their tendencies to develop autoimmune diabetes (2, 3) as well as inducible autoimmune thyroiditis (4) and systemic lupus erythematosus (5).

Although relapsing-remitting disease is the most common disease course experienced by MS patients, the factors that govern the progression from symptomatic disease through remission and relapse remain unclear. One explanation for symptom relapse is epitope spread, which is the concept that the damage that occurs during acute disease releases CNS Ags that prime a second T cell response to a distinct epitope that elicits symptom relapse (6, 7). Proliferation and cytokine data from various mouse models suggest that responses to a secondary myelin Ag can occur, yet it is unclear whether these responses are strong enough to independently elicit symptomatic disease (8–12). Others have attributed the resolution of symptoms observed in relapsing-remitting disease to the activity of regulatory T cells (Tregs) (13). Using the micropipette adhesion frequency assay, we quantify the frequency of myelin oligodendrocyte glycoprotein (MOG)–reactive T cells throughout disease course and the affinity of TCR for myelin: MHC-II for the first time, to our knowledge, in a secondary progressive model of MS (14–16).

Our laboratory has demonstrated that the micropipette assay more sensitively measures the frequency of myelin-reactive cells and defines the binding kinetics as compared with MHC class-II tetramer staining of CD4 T cells isolated from B6 mice and human patients (14, 15). This is in large part because it interrogates the affinity of single TCR:peptide-MHC (pMHC) receptor ligand interactions and does not rely on tetramer-driven avidity for the detection of MOG-reactive cells (17). MHC class-II tetramers are limited in their ability to detect Ag-reactive cells that are present at a low frequency in a population and also those CD4 T cells with moderate to lower affinities for Ag (14, 18). The micropipette-based technology also outperforms surface plasmon resonance (SPR) or three-dimensional (3D) technologies for analysis of polyclonal T cell populations, especially in correlation with pathogenicity or functional output of T cells (19–21). From a practical point of view, the micropipette assay allows us to interrogate TCR affinities in a polyclonal response immediately ex vivo, whereas SPR and other methods may require the generation and analysis of individual T cell clones. In addition, the micropipette assay has been shown to more sensitively measure affinities below the affinity threshold for SPR measurement and that these affinities can be more finely and definitively resolved (19). Thus, the micropipette adhesion frequency assay uniquely allows one to better assess the entire T cell response ex vivo in autoimmune diseases.
this study, we found that the CNS-infiltrating, polyclonal MOG-specific CD4 T cells with the highest affinity occurred during acute disease at onset of symptoms. No significant differences in T cell affinity exist between remission, relapse, and chronic time points. Moreover, the high frequency of MOG-reactive T cells limits the impact of epitope spread as a means for disease progression as the majority of CNS-infiltrating cells were MOG specific at every time point examined, demonstrating that relapsing-remitting disease can be elicited by T cells of a single myelin specificity.

Materials and Methods

Mice

NOD mice were purchased from The Jackson Laboratory and Taconic facilities. Mice were housed and bred in the Emory University Department of Animal Resources facility and used in accordance with the Institutional Animal Care and Use Committee–approved protocols. Female mice were used in all EAE experiments, whereas male mice were used for lymph node priming. Mice were used for experiments at 8–11 wk of age.

EAE induction

EAE was induced with a single (day 0) s.c. injection in the hind flank of 300 μg MOG35–55 (MEVGWYRSPFSRVVHLYRNGK) emulsified in CFA containing 5 mg/ml heat-inactivated Mycobacterium tuberculosis (Difco). MOG35–55 peptide and truncated peptides within MOG35–55 (Fig. 2A, 2B) were synthesized on a Prelude Peptide Synthesizer (Protein Technologies). Mice also received 250–300 ng pertussis toxin (List Biological Laboratories) i.p. on days 0 and 2. Disease severity was assessed using the following scoring rubric: 0, no disease; 0.5 weak tail; 1, flaccid tail; 2, hind limb weakness/poor grip; 3, hind limb paralysis; 4, hind limb paralysis and forelimb weakness; 5, moribund/death.

CNS mononuclear cell isolation

Mice were euthanized with CO2 and perfused through the left ventricle with PBS. The brain and spinal cord were harvested from each animal and homogenized through a 100-μm filter. Mononuclear cells were isolated using a Percoll (Sigma-Aldrich) gradient, washed, and counted using a hemocytometer. Cells were then prepared for flow cytometric analysis or stained with tetramer immediately post-Percoll isolation. Mononuclear cells were isolated from the CNS and spleen of immunized animals were stained extracellularly with anti-CD4 FITC, anti-CD8a V450, anti-CD11b PerCP, anti-CD11c PerCP, and anti-CD19 PerCP for 30 min on ice, fixed, and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) for EAE experiments and the Fix&Perm Cell Permeabilization Kit (Life Technologies) for lymph node cultures. Both kits were used, according to manufacturer’s protocol. Cells were then stained for intracellular cytokines using anti-GM-CSF PE (MP1-XT22; BioLegend), anti–IL-17A allyloxyaceticin (eBio17B7; eBioscience), anti–IFN-γ allyloxyaceticin-Cy7 (XMG1.2; BD Pharmingen), and anti–TNF-α PE-Cy7 (MP6-XT22; BioLegend) for 30 min on ice.

Foxp3 stain

Cells isolated from the CNS and spleen of immunized animals were stained extracellularly with anti-CD4 FITC, anti-CD8 V450, anti-CD11b PerCP, anti-CD11c PerCP, and anti-CD19 PerCP for 30 min on ice. Cells were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer set (eBioscience), per manufacturer’s instructions, and then stained intracellularly using anti-Foxp3 PE (FJK-16s; eBioscience) for 30 min on ice.

Micropipette adhesion frequency assay

A detailed description of the micropipette adhesion frequency assay has been published elsewhere (14, 19, 22). Briefly, human RBCs were bio- tinylated using Biotin–X-NHS (EMD Biosciences). Cells were again coated with streptavidin (Thermo Scientific) and biotinylated peptide: MHC-II monomers. Mononuclear cells were isolated from brains and spinal cord of MOG-induced mice, and CD4 T cells were isolated from the pooled samples by CD4-positive magnetic separation using MACS L7T4 CD4-positive selecting beads (Miltenyi Biotec). A single pHMHC-coated RBC and T cell were aspirated onto opposing micropipettes in a cell chamber mounted on the stage of an inverted light microscope. Using micromanipulators, cells were aligned against each other and a computer-controlled piezo-electric actuator brought the T cell into contact with a stationary pHMHC-coated RBC. Cells remained in contact for 2 s, and, upon retraction, a PHMHC-TCR-biding event was visualized as an elongation of the ultrasoft RBC membrane. In the absence of a binding event, the RBC membrane instantaneously reverts to its spherical shape. Cells were brought into contact with 50 times with the same contact time and area (A,), and an adhesion frequency (F) was calculated. Quantification of surface pHMHC and TCR-β densities was determined by flow cytometry and BD QuantITRITE PE Beads for standardization (BD Biosciences). Surface densities as well as adhesion frequency were then calculated to two-dimensional affinity using the following equation: A=A,=ln(1−P,(o)/m,m), where m and m represent TCR and pHMHC surface densities, respectively. Geographic mean affinity values are reported ± SEM.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software (Software for Science). Significance was calculated using one-way ANOVA and Student t tests.

Results

NOD mice develop a relapsing-remitting EAE disease course

To characterize relapsing-remitting demyelinating disease, NOD mice were assessed for weight and clinical score daily after induction to track disease progression. With the described induction protocol, mice become acutely sick with an incidence of 78.4% (Table I) and experience symptom onset as early as 11 d post-induction (Fig. 1A). Acute disease was defined as a score of 1 or greater for at least 2 d. In experiments with mice originating from The Jackson Laboratory, acute disease occurred within the first 20 d postinduction. Interestingly, acute disease occurs later post-induction in mice procured from Taconic facilities. To ensure these differing timelines did not affect results, clinical scores were plotted against time, and areas under the curve (AUCs) were calculated. AUCs were calculated to standardize and quantify cumulative disease experience by each mouse used in experiments. The day at which mice experienced acute symptoms, relapse and chronic progression varied from mouse to mouse and variation was more pronounced when comparing mice sourced from different vendors. To confirm the accuracy of our comparisons of mice from different experiments isolated at the same point in disease, AUCs were calculated to ensure the similarity of both duration of disease.
and symptom severity. Mice from both facilities experience acute disease followed by entrance into remission, symptom relapse, and chronic progression. Expectedly, AUCs for acute and remission time points did not vary significantly (6.59 ± 0.55 and 9.63 ± 0.79, respectively), whereas mean AUC increased for both relapse (22.32 ± 2.99) and chronic (39.03 ± 3.80) time points (of note, relapse incidence of disease as well as mean day of onset of acute, relapsing, and chronic symptoms in EAE experiments is summarized. Mice from Taconic and The Jackson Laboratory as well as Emory-bred mice originating from both facilities are included. Maximum score for each time point within each experiment is reported. Mice that developed diabetes (blood glucose >250 mg/dl) were excluded from experiments and all incidence calculations. Mice that did not become acutely sick were sacrificed and not used in experiments (or incidence calculations for relapse and chronic disease) but were included in calculation of acute incidence. Incidence of chronicity was calculated from all mice that developed acute symptoms regardless of whether a relapse in symptoms was observed. After mice were sacrificed for use in experiments, they were not included in further incidence calculations. For example, experiments 1–10 involved the characterization of mice experiencing acute disease or symptom remission, and thus, no incidence of relapse or chronicity was calculated on these mice (with the exception of one surviving mouse in experiment 7).

**Table I. Incidence and disease course of secondary progressive MOG-induced EAE in NOD mice**

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<th>Maximum Acute Score</th>
<th>Mean Day of Relapse</th>
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Incidence of disease as well as mean day of onset of acute, relapsing, and chronic symptoms in EAE experiments is summarized. Mice from Taconic and The Jackson Laboratory as well as Emory-bred mice originating from both facilities are included. Maximum score for each time point within each experiment is reported. Mice that developed diabetes (blood glucose >250 mg/dl) were excluded from experiments and all incidence calculations. Mice that did not become acutely sick were sacrificed and not used in experiments (or incidence calculations for relapse and chronic disease) but were included in calculation of acute incidence. Incidence of chronicity was calculated from all mice that developed acute symptoms regardless of whether a relapse in symptoms was observed. After mice were sacrificed for use in experiments, they were not included in further incidence calculations. For example, experiments 1–10 involved the characterization of mice experiencing acute disease or symptom remission, and thus, no incidence of relapse or chronicity was calculated on these mice (with the exception of one surviving mouse in experiment 7).

Mice that did not develop acute symptoms were sacrificed and not used in experiments (but are included in acute incidence calculations). Of note is that NOD mice are prone to develop autoimmune diabetes with an incidence of 85% in females by 30 wk for The Jackson Laboratory mice and 60% in 6-mo-old females from Taconic facilities. Blood glucose levels were monitored throughout experiments, and mice that developed diabetes (non-fasting blood glucose of 250 mg/dl or greater) were sacrificed and not included in analysis. We found that 12.0% of mice used in these experiments became diabetic postinduction and were thus excluded from analysis, as it is unknown what effects autoimmune diabetes has on the immune response in demyelinating disease.

**MOG42–55 is the encephalitogenic I-A\(^{d}\)–restricted epitope**

The 21-aa-long MOG\(_{35-55}\) peptide could contain several possible T cell epitopes and MHC-binding registers. To determine the I-A\(^{d}\)–restricted MOG epitope, lymph nodes were primed in vivo with MOG\(_{35-55}\) in CFA and enriched for MOG-specific cells by culturing on MOG\(_{35-55}\) peptide. Seven peptides truncated from the C and N termini of MOG\(_{35-55}\) were synthesized and used to stimulate cytokine production to determine the core pathogenic epitope(s) presented by I-A\(^{d}\). Of the fragments tested, MOG\(_{42-55}\) was the only epitope able to elicit a significant production of IFN-\(\gamma\) and IL-2 by MOG-primed T cells (Fig. 2A, 2B). The 42–55 peptide was selected as the epitope for monomer and tetramer synthesis by the National Institutes of Health Tetramer Core Facility.

To verify functionality of the tetramer, mice were primed with MOG\(_{35-55}\) emulsified in CFA, and draining lymph nodes were harvested and stimulated with MOG\(_{35-55}\) peptide in culture. After Ag-specific enrichment, ∼7% of cells were found to be MOG specific by tetramer staining (Fig. 2C, 2D). Interestingly, cells isolated from the CNS of mice acutely sick with MOG-induced EAE did not stain with MOG-I-A\(^{d}\) significantly above back-ground control I-A\(^{d}\) tetramers (Fig. 3C). Importantly, intracellular cytokine stain assays on MOG-primed lymphocytes in culture were able to detect 2-fold more MOG-reactive cells than MOG-I-A\(^{d}\) tetramers, indicating, as we have seen in the B6 model of MOG-induced EAE, that MHC class II tetramers fail to identify most Ag-reactive T cells (14).
CD4 T cell affinity for MOG is greatest during acute phase of disease

To investigate the frequency and affinity of MOG-reactive CD4 T cells infiltrating the CNS during progression from acute disease through symptom remission, relapse, and chronic progression, the micropipette assay was chosen over MHC class II tetramers because tetramer technology uses avidity-based interactions that miss the known contribution of lower-affinity T cells to autoimmunity. Furthermore, in our own preliminary experiments using I-A\(^{\beta}\) tetramers, no MOG-specific CD4 T cells were identified in CNS isolates from acute disease time points (Fig. 3C). For the micropipette assay, CD4 T cells were isolated from the CNS of mice sacrificed at acute, remission, relapse, and chronic time points and loaded into a microscope chamber along with RBCs coated with peptide-loaded MHC molecules of different surface densities. RBCs serve as biosensors in the micropipette assay, allowing for visualization of a single TCR-pMHC-binding event. The micropipette assay is preferable for assessing the frequency and affinity of CD4 T cells for Ag in that both TCR and pMHC in this assay are cell anchored, thus providing a biologically relevant assessment of binding events. In the assay, an adhesion frequency is calculated after 50 contacts between cells, and, from this adhesion frequency as well as quantified surface densities of both TCR and pMHC, the affinity can be calculated (19). In these experiments, MOG\(_{35-55}\) I-A\(^{\beta}\)-coated RBCs were used as the experimental Ag, and monomers presenting irrelevant peptide epitopes of hen egg lysozyme or hCLIP were used as negative controls. At the acute disease time point, none of the CD4 T cells bound to RBCs loaded with control Ags, indicating the affinity for these Ags was below the level of detection of the micropipette assay (<1 \times 10^{-7} \ \mu m^2) (data not shown).

CD4 T cells in the CNS during acute disease have a significantly higher mean affinity for MOG than at remission or chronic time points (2.24 \times 10^{-5} \pm 1.04 \times 10^{-5} for acute, 8.65 \times 10^{-6} \pm 1.01 \times 10^{-5} for remission, 1.30 \times 10^{-5} \pm 2.95 \times 10^{-6} for relapse, and 1.11 \times 10^{-5} \pm 4.40 \times 10^{-6} for chronic; Fig. 3). Furthermore, the affinities for all time points adhere to a Gaussian distribution (Fig. 3A, 3C) with \(r^2\) values >0.830 for remission, relapse, and chronic time points. The lowest \(r^2\) value of 0.673 was observed for the curve fitted to affinities from acute disease, which is expected, as the greatest range of affinities was observed at this time point. The breadth of affinities is best highlighted on inspection of the distributions and shows 10-fold greater spread during acute disease compared with disease remission, 5-fold greater than during relapse, and twice as great as during chronic disease. Of note, no significant difference exists between the affinities measured during remission, relapse, and chronic time points. MOG-reactive T cells predominate in CNS at all time points

The frequency of myelin-reactive T cells in the CNS is a driving force for disease (14, 23–25). In addition to the two-dimensional (2D) affinity, micropipette analysis gives the most sensitive measure currently available as to how frequent a given CD4 T cell specificity occurs in the immune repertoire. Our laboratory has previously shown that, in the B6 model of EAE, nearly 10% of CNS-infiltrating CD4 T cells are MOG specific by I-A\(^{\beta}\) tetramer staining, whereas >65% show specificity for MOG in the micropipette assay (14). In this study in NOD mice, at all time points, the majority of the CD4 T cells in the CNS were reactive to MOG (Fig. 3D), whereas analysis of the spleen of chronically symptomatic animals indicates this increased frequency of
MOG-reactive cells is specific to the CNS (Supplemental Fig. 1). This high frequency observed in the CNS is starkly different when compared with ex vivo MOG42–55/I-A^b tetramer staining (87% versus <1% of CNS-infiltrating cells at acute time points are MOG reactive by micropipette and tetramer, respectively). Of note, the same monomer is used for both techniques. Our findings in this study suggest I-A^b tetramers are even more inefficient than I-Ab tetramers at detecting MOG-specific CD4 T cells from the CNS, and that the micropipette assay is the only reliable way to identify these cells.

Although tetramer negative, the frequency of high-affinity cells was quantified throughout disease using the geometric mean of cells isolated during acute disease (2.34 ± 10^{-2} mm^4) as the threshold for definition of higher-affinity cells. Higher-affinity cells were only detected during symptomatic disease time points, and their frequency in the CNS declined throughout disease. Despite not being high affinity, nearly 84% of the CNS-infiltrating CD4 T cells in the CNS remain reactive to MOG during disease remission. The presence of CD4 T cells with higher affinities for MOG during symptomatic disease indicates that symptomatic disease is predominantly, if not exclusively, initiated and maintained by MOG-reactive cells. Additionally, this preponderance of MOG-specific cells limits any role for epitope spread, as nearly all T cells are specific for MOG.

FIGURE 2. MOG42–55 is the immunogenic I-A^b–restricted epitope within MOG35–55. 

(A and B) MOG-primed lymph node cells from male mice were activated in culture on MOG35–55 for 10 d. Live cells were stimulated with indicated peptide epitopes for 5 h. Brefeldin A was added for the latter 4.5 h of this incubation. Cells were stained for flow cytometry and gated on lymphocytes (identified by forward/side light scatter) and CD4^+ cells. Statistical significance was determined using Sidak's multiple comparison test and one-way ANOVA. In (A), significance of staining as compared with no stimulation control is indicated with ***p = 0.0007 and *p = 0.02. In (B), significance is indicated with ***p = 0.0006 and **p = 0.007. (C) Lymph node cells from four to five male mice were activated in culture for 10 d on MOG35–55. Live cells were stained with MOG-I-A^b tetramer, and OVA-I-A^b tetramer was used as a negative control. Representative flow cytometry data plots shown are gated on lymphocytes as identified by forward/side light scatter, and CD4-positive, CD11b/CD11c/CD19-negative gates. (D) Summary tetramer-staining data from three experiments of four to five male mice each. Each pair of data points represents control and MOG tetramer staining from a single experiment. Statistical significance was determined with a one-tailed, paired Student t test (**p = 0.0044).

IL-17–producing CD4 T cells infiltrate CNS during symptomatic disease

Cytokine production profiles were investigated to examine the functional differences of CD4 T cells isolated from acute, remission, relapse, and chronic disease time points. Specifically, CD4 T cells in the CNS were examined for their ability to produce IFN-γ and IL-17. Pathogenic roles have been demonstrated for both Th1 and Th17 cells in EAE, and more recent work has investigated the nature of IFN-γ/IL-17–double-producing cells, or Th1-like Th17 cells. When looking at CD4 T cells that produce IL-17 in the absence of IFN-γ production, we found significantly lower frequencies of these cells at disease remission compared with disease relapse and chronic progression time points (Fig. 4B). Moreover, when comparing the frequency of IL-17–producing CD4 T cells in the CNS with those in the spleen, we found a significant 10-fold enrichment in the CNS at acute and a 6-fold enrichment at relapse time points (p = 0.004 and p = 0.012, respectively, with two-tailed paired Student t test) and a 4-fold enrichment of IL-17–producing CD4 T cells in the CNS during chronic disease (p < 0.0001). The frequency of IL-17–producing CD4 T cells was not significantly greater in the CNS compared with the spleen during symptom remission, indicating Th17 cells play a role in symptomatic disease.

We also investigated the role of CD4 T cells that produce IFN-γ in the absence of IL-17 (Th1 cells) during relapsing-remitting disease (Fig. 4C). We found these cells were most frequent in the CNS at acute and remission time points, with diminishing frequencies at relapse and chronic time points. This downward trend of IFN-γ production over the course of disease supports the idea of its neuroprotective role and that the loss of IFN-γ in chronicity allows for symptom-eliciting mechanisms to dominate...
the response. A 4- to 5-fold increase in frequencies of Th1 cells was observed in the CNS compared with the spleens of mice during acute disease ($p = 0.0002$) and disease remission ($p = 0.0005$), whereas no difference in frequencies was observed during relapse and chronic time points.

We next looked at CD4 T cells producing both IFN-γ and IL-17 and found the frequency of these cells in the CNS to be highest at disease remission and significantly higher than those found during relapse or chronic disease ($p = 0.0005$) (Fig. 4D). When comparing the frequency of IL-17/IFN-γ producers in the CNS with that observed in the spleen, we found a significant enrichment in the CNS for acute ($p = 0.0005$) and remission ($p = 0.0003$) time points and no significant difference at relapse and chronic time points. The implication from these data is that IFN-γ producers and IL-17/IFN-γ double producers begin to enter the CNS during acute disease, and the increased production of IFN-γ, especially with the exodus or death of Th17 cells, has an overall protective effect during disease remission. Furthermore, it is possible that the absence of IFN-γ production later in disease facilitates the development of symptomatic disease during relapse and chronic time points.

**Increased Treg frequency at symptom remission**

To further investigate the cellular factors involved in the transition from acute disease to symptom remission, we analyzed Tregs in the spleen and CNS of NOD mice induced with MOG peptide. At the remission time point, significantly more Tregs were found in the CNS than were found during acute disease (Fig. 5). Interestingly, an enrichment of Tregs in the CNS was observed, as these cells were nearly two times more frequent in the CNS than in the spleens of remitting mice ($26.06 \pm 2.154\%$ versus $11.47 \pm 0.6541\%$, respectively).

We next evaluated the ratio of Tregs (CD4+Foxp3+CD25+) to non-Treg CD4 T cells (CD4+Foxp3−) during acute disease and symptom remission. We found that, in the CNS, this ratio significantly increases at remission time points compared with acute disease (Fig. 5C), whereas the ratio was not significantly different between the two time points in the spleen. This further supports the hypothesis that an increased frequency of Tregs in the CNS contributes to symptom remission in a relapsing-remitting disease model (26) and that this effect is most pronounced in the CNS.

**Discussion**

In this study, we have investigated the phenotype of CD4 T cells from acute disease, through symptom remission, relapse, and chronic progression in the NOD model of secondary progressive demyelinating disease. We used the micropipette adhesion frequency assay to quantify the affinity of these cells because it allows for the detection of MOG-reactive cells below the avidity threshold of detection of MHC class II tetramers (14, 18, 27). Several studies have demonstrated the increased sensitivity of 2D
affinity measurements as compared with 3D (SPR) data with regard to biologically meaningful measures (19–21, 28). The micropipette assay measures affinity of TCR for pMHC in a cell membrane–anchored, physiologically relevant context, and steric restrictions imposed by the cell membrane have been suggested to be the reason for the lack of correlation of 3D measurements with functional output (20, 21). In this study, we quantify the affinities of CNS-infiltrating CD4 T cells ex vivo throughout the course of relapsing-remitting disease. Although SPR requires the generation of T cell clones, making ex vivo measurements difficult, micropipette allows for ex vivo measurement of a polyclonal T cell response, and thus, this technique is essential to enumerate the frequency and affinity of the entire CD4 T cell response in autoimmune demyelinating disease (14, 29, 30).

Although a direct correlation exists between responding T cell affinity and extent of immune response (31, 32), we instead observed the highest-affinity CD4 T cells infiltrate the CNS during the initiation/acute phase of disease. I-A^b tetramers, as an avidity-based detection method, failed to detect MOG-specific CD4 T cells in the CNS of sick mice, whereas the micropipette assay as
FIGURE 5. Tregs are more frequent in the CNS at disease remission than during acute disease. Tregs were identified in the CNS and spleens of MOG-induced mice as CD25+Foxp3+ CD4 T cells by flow cytometry. (A) A representative flow plot showing the identification of Tregs from the spleen of a mouse experiencing symptom remission is shown. Bars have been gated on lymphocytes (forward/side light scatter) and CD4-positive, CD11b/CD11c/CD19-negative cells. (B) The frequency of Tregs in the CNS and spleen is shown as a percentage of CD4+ T cells. Bars are plotted at the mean frequency ± SEM. Acute time point data were collected from eight mice from three independent experiments. Remission data are representative of six mice in two independent experiments. One-way ANOVA was used to determine significance. ****p < 0.0001. (C) Ratios of the number of infiltrating Tregs to the number of non-Tregs (CD4+Foxp3−) in the CNS and spleen are shown.
to be the cause of relapse in other relapsing-remitting models of EAE (9, 10, 35), and both initiation and progression of epitope spread are strongly dependent upon MHC class II haplotype in animal models and patients (7, 25, 36–38). Furthermore, intra- and intermolecular epitope spread have been demonstrated in the context of I-A\(^{\beta}\) in the autoimmune diabetes model in NOD mice (2, 3). Although other peptides have been identified as encephalitogenic in I-A\(^{\beta}\)-expressing mice, EAE experiments in Biozzi (I-A\(^{\beta}\)) mice also demonstrated that the T cell response to the priming epitope was dominant throughout a relapsing EAE course as opposed to alternate CNS Ags (8). Additional studies in the NOD model will determine whether any non-MOG–reactive CD4 T cells emerge during disease course, but their low frequency makes it unlikely that this population would contribute substantially to disease pathology.

In addition to quantifying affinity and frequency of MOG-reactive cells, the cytokine profile of CD4 T cells was assessed throughout disease. Although early publications of EAE models demonstrated that IFN-\(\gamma\)–producing Th1 cells elicit disease, data also demonstrate a neuroprotective role for IFN-\(\gamma\) (39–45). Clear roles have also been demonstrated for IL-17–producing Th17 cells in eliciting EAE (23, 46, 47). In this study, we show that IL-17–producing CD4 T cells are enriched in the CNS during periods of symptomatic disease, whereas disease remission is accompanied by an increase in IFN-\(\gamma\)–producing cells, as well as Tregs. Interestingly, the increase in frequency of IL-17–producing cells accompanying relapse and chronicity coincides with the observation of a second wave of higher-affinity cells in the CNS at these time points. Although we did not formally address the differences in affinity among CD4 T cell subtypes, ongoing studies are investigating whether Th17 cells have higher affinity for MOG than Th1 cells. Of further interest is the possibility that a population of IL-17–producing cells in acute disease seems to gain the ability to produce IFN-\(\gamma\), which may neutralize the pathologic effect of the Th17 cells (Fig. 4B, 4D). The plasticity of Th17 cells and their ability to convert in vivo to IFN-\(\gamma\)-producing cells have been documented in both mice and humans (48, 49); however, protective effects of this double-cytokine–producing population have not been published. Moreover, we demonstrate a decline in frequency of IFN-\(\gamma\)–producing cells in the CNS throughout disease course, with the lowest frequency observed during chronic disease. We suggest that the absence of IFN-\(\gamma\)–producing cells during chronic disease renders the CNS vulnerable to pathogenic processes and allows for the establishment and maintenance of chronic symptoms. Although this study shows that, in the CNS, IL-17 production correlates with symptom relapse and chronicity, it suggests a neuroprotective effect of IFN-\(\gamma\) during disease remission.

We also observe an enrichment of Tregs in the CNS during disease remission and a significantly higher ratio of Tregs to non-Treg CD4 T cells compared with acute time points. An essential role for Tregs in mediating the recovery from symptomatic EAE has been demonstrated by several groups (13, 50). We believe Tregs play an important role in eliciting remission; however, these regulatory mechanisms are clearly overcome upon entry into relapse. This could be explained by the observation that Tregs are less effective at regulating the response of Th17 cells compared with Th1 cells (51). If Tregs are not able to effectively regulate this pathogenic population, the symptoms experienced during relapse and chronic progression can be explained by the refiniltration or in situ priming of Th17 cells in the CNS. Additionally, Korn et al. (34) found that Foxp3\(^{+}\) Tregs isolated from the CNS were able to suppress naive MOG-specific CD4 T cells but were unable to suppress CNS-infiltrating T cells from MOG-induced animals. They attributed this observation to the ability of CNS-infiltrating T effector cells to secrete IL-6 and TNF-\(\alpha\), which abrogated the ability of Tregs to suppress T effector responses (34). This functional exhaustion of Tregs might accompany the transition from remission to relapse and chronicity in the NOD model, although further study is needed to answer this question.

In summary, this study demonstrates that CD4 T cell affinity for MOG is highest during acute disease and that the majority of CNS-infiltrating CD4 T cells remain MOG reactive throughout disease. Additionally, symptomatic time points of relapse and chronic disease are accompanied by a small population of higher-affinity MOG-reactive cells infiltrating the CNS, supporting the argument that a second wave of MOG-reactive cells is responsible for relapse and disease progression. Because the vast majority of CD4 T cells in the CNS are reactive to MOG, this severely limits any role epitope spread could play in disease progression, which challenges the widely held belief that relapsing-remitting disease is elicited by epitope spread. The reinfiltration of higher-affinity cells at symptomatic time points of relapse and chronicity coincides with higher frequencies of Th17 cells in the CNS. It is clear that with the changing affinity and cytokine profiles of CNS-infiltrating MOG-reactive CD4 T cells throughout secondary progressive disease, the evolution of symptoms is governed by a balance of many pathogenic and regulatory/protective processes. One great challenge that is faced by those attempting to design Ag-specific therapies is choosing the Ag (and its HLA restriction) to which they wish the patient to be tolerated. This is particularly challenging in that many CNS Ag-specific clones can be grown out of both patient and healthy donor blood, so identifying which Ag-specific T cells are actually pathogenic seems daunting. Our data would suggest that there may in fact be one (or few) CD4 T cell specificity that is responsible for eliciting relapse of disease. Furthermore, the concept of epitope spread may be somewhat limited. Of interest, ongoing Ag-specific therapy trials involved administration of autologous PBMCs chemically coupled with only seven myelin peptides (52), which resulted in a decrease in Ag-specific T cell responses. We propose tolerogenic efforts for relapsing-remitting autoimmune disease may be successful if focused to a single or limited number of epitopes.

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Disclosures

The authors have no financial conflicts of interest.

References

Figure 1. Frequency and affinity of MOG-reactive CD4 T cells is significantly lower in spleen compared to CNS. Adhesion frequencies (A) and calculated 2D affinities (B) for CD4 T cells isolated from both the spleen (SPL) and CNS of chronically symptomatic NOD mice are shown. Each micropipette assay using splenic CD4 T cells was conducted on a single spleen from a NOD mouse with MOG-induced disease. Data from spleen are representative of 2 independent experiments while CNS experiments are representative of 3 independent experiments. (A) 65 splenic CD4 T cells were assessed of which 13 cells bound with a frequency above 0.1. 64 CD4 T cells were assessed from the CNS of chronic mice of which 45 bound with a frequency above 0.1. The affinities of those cells are reported (B) with **** indicating p<0.0001 as calculated by a two-tailed Student’s t-test.