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Th17 and Th1 Responses Directed Against the Immunizing Epitope, as Opposed to Secondary Epitopes, Dominate the Autoimmune Repertoire During Relapses of Experimental Autoimmune Encephalomyelitis

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Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease with similarities to multiple sclerosis (MS). It has been suggested that relapses of EAE and MS may be associated with, and even driven by, T cells specific for novel epitopes that are primed during the course of tissue destruction in the target organ or in secondary lymphoid tissues. We show, however, that IFNγ and IL-17 responses against the immunizing epitope remain dominant through out the course of multiphasic EAE. Furthermore, induction of tolerance against a putative secondary epitope did not prevent clinical relapses.

Key words: T lymphocyte; animal model; multiple sclerosis; autoimmunity; epitope spreading

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS). It is widely believed to be mediated by $CD4^+$ T cells reactive against myelin antigens. This theory is supported by striking histopathologic and clinical similarities between MS and the animal model, experimental autoimmune encephalomyelitis (EAE) (Martin and McFarland, 1995). SJL mice immunized with a peptide fragment of proteolipid protein that spans amino acids 139–151 ($PLP_{139–151}$) follow a relapsing remitting course of EAE, reminiscent of the most common presentation of MS (McRae et al., 1992).

Although it is clear that the first episode of neurologic dysfunction in this model is driven by $PLP_{139–151}$-specific T cells, a number of studies suggest that the myelin-specific T cell repertoire expands and diversifies thereafter in a process termed epitope spreading (McRae et al., 1995). It has been argued that T cells reactive against secondary and tertiary epitopes govern successive relapses (Vanderlugt et al., 2000). Similarly, an "epitope spreading cascade" has been observed in some individuals with MS (Tuohy et al., 1997). The phenomenon of epitope spreading holds important theoretic implications for the monitoring and treatment of MS. Drugs that target specific myelin determinants or T cell receptors might be rendered ineffective as the effector T cell pool diversifies (Fontoura et al., 2005). A number of researchers have questioned the prevalence as well as the physiologic significance of epitope spreading during the development of chronic or multiphasic autoimmune disease (Takacs et al., 1997; Lindsey, 1998; Takacs and Altmann, 1998). In fact, multiphasic EAE can be mediated by an immune response restricted to a single myelin epitope with no opportunity for the auto reactive T cell repertoire to evolve, as in SCID mice that express a transgenic T cell receptor specific for a peptide of myelin basic protein (Jones et al., 2003).

Another important issue involves the biologic properties assumed by autoimmune T cells that are generated in the context of spreading. Previous studies exclusively used tritiated thymidine incorporation, delayed type hypersensitivity (DTH) reactions, and IFNγ production as measures of T cell reactivity against secondary and tertiary myelin epitopes. However, the recent recog-

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nition that Th17, rather than Th1 cells are the key encephalitogenic effectors in EAE, suggests that the cells detected by the above assays might represent innocuous bystanders that are not engaged directly in the pathogenic process (Billiau et al., 1998; Segal et al., 1998; Langrish et al., 2005; Park et al., 2005; Chen et al., 2006). Such considerations raise the prospect that, even when epitope spreading occurs during autoimmune disease, it may simply represent an epiphenomenon. Indeed, spreading between myelin epitopes has been detected in MS and EAE during periods of clinical stability (Kumar, 1998; Ristori et al., 2000; Vergelli et al., 2001). It is even possible that spreading could give rise to the generation of regulatory cells that actually limit inflammation and trigger remissions. In support of this notion, IL-10-producing cells reactive against secondary epitopes prevent EAE relapses (Yin et al., 2001; Wildbaum et al., 2002).

In light of the unresolved issues discussed above, we re-examined the phenomenon of epitope spreading by carrying out highly sensitive ELISPOT assays against a broad panel of cytokines, including IL-17 and IL-10, with T cells harvested from the CNS, spleen, and draining lymph nodes of SJL mice at multiple time points after immunization with PLP peptides. Based on our findings, we conclude that in our model, memory Th17/Th1 CD4+ T cells responsive to the primary epitope targeted during the initiation of EAE comprise the immunodominant autoreactive effector cell population throughout the disease course.

MATERIALS AND METHODS

Mice

SJL/J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in microisolator cages. All animal protocols were approved by the University of Rochester Committee on Animal Resources.

Peptides

Peptides with the following sequences were obtained from Bio-Synthesis (Lewisville, TX): $PLP_{139–151}$ HSLGKWLGHPDKF; $PLP_{178–191}$ NTWTTCQSIAFPSK; $MBP_{84–104}$ VHFFKNIVTPRTPPPSQGKGR; and influenza nucleoprotein $(NP)_{260–283}$ RSALILRGSVAHKSCLPACVYG. Purity of >95% was confirmed by mass spectrometry. Peptides were suspended in PBS and sterile filtered (0.22 μm low protein binding Millex-GV filters; Millipore, Bedford, MA) before use.

Monoclonal Antibodies

The following hybridomas were used as capture antibodies in ELISPOT assays: JES6-1A12 (IL-2; eBioscience, San Diego, CA), A11B11 (IL-4; ATCC), TRFK5 (IL-5; eBioscience), JES5-2A5 (IL-10; eBioscience), TC11-18H10.1 (IL-17; Pharmingen, San Diego, CA), and AN18 for IFN-γ (ATTC). The following hybridomas were used as detection antibodies: JES6-5H4 (IL-2; eBioscience), BVD6-24G2 (IL-4; eBioscience), TRFK4 (IL-5; eBioscience), SXC-1 (IL-10; Pharmingen), TC11-8H4.1 (IL-17; Pharmingen), and XMG1.2 (IFN-γ; eBioscience). All detection antibodies were biotinylated by the manufacturer.

Induction and Rating of EAE

SJL/J mice (6–8 weeks old) were immunized subcutaneously (s.c.) with 100 μg of $PLP_{139–151}$ or $PLP_{178–191}$ emulsified in CFA containing 250 μg *Mycobacteria tuberculosis* H37Ra (Difco, Detroit, MI). Mice were monitored daily and disability scored according to the following scale: 0, no clinical signs; 1, flaccid tail; 2, poor righting ability; 3, obvious hind limb weakness; 4, hind limb paralysis; 5, moribund state. To account for the severity and duration of neurologic deficits, a "cumulative disease score" was calculated for each mouse by summing daily disease scores over the entire observation period.

Induction of Peptide-Specific Tolerance

SJL mice were injected with 300 μg of $PLP_{178–191}$ peptide emulsified in IFA by the intraperitoneal (i.p.) route. Control mice were sham-tolerized with an equal volume of PBS in IFA. Two weeks later, mice were challenged with $PLP_{139–151}$ or $PLP_{178–191}$ emulsified in CFA for induction of EAE as described above.

Cell Isolation and Culture

Spleen and draining lymph nodes (axillary, inguinal, and brachial) were passed through a 70-μm cell strainer (BD Falcon, Bedford, MA) to create single cell suspensions. Red blood cells were removed with ACK lysing buffer (Quality Biologic, Gaithersburg, MD). The remaining cells were then washed twice and resuspended in HL-1 media (Cambrex Bio Science, Walkersville, MD) supplemented with 1% L-glutamine.

Spinal cord fragments were digested with DNase and collagenase for 1 hr at 37°C. Mononuclear cells were isolated via Percoll gradient centrifugation. On average, we isolated between 3×10^4–1×10^5 mononuclear cells per spinal cord from $PLP_{139–151}$ primed mice during clinical episodes. In some cases, CD4 T cells were purified from whole spinal cord mononuclear cells using APC-conjugated anti-CD4 antibody (eBioscience) and MACS anti-APC beads (Miltenyi, Auburn, CA). T-depleted splenocytes were prepared by complement-mediated lysis of T cells with anti-Thy1.1 monoclonal antibody (clone J1J).

ELISPOT Assays

Filtration plates (96-well) (MAIP N4550; Millipore, Bedford, MA) were coated with capture antibody (3 μg/ml; 100 μl/well) and incubated overnight at 4°C. Plates were washed three times with sterile PBS and blocked for 1 hr at room temperature with 1% BSA in PBS (Atlanta Biologics, Lawrenceville, GA). After three additional washes with PBS, lymph node cells were plated in triplicate (5×10^6 cells/ml in HL-1 media; 200 μl/well) with or without peptide (50 μg/ml). After a 24–48 hr incubation at 37°C/5% CO_2, plates were washed before addition of detection antibodies (2 μg/ml in PBS-T containing 1% BSA;100 μl/well) and incubation at 4°C for 12 hr. Plates were

washed with PBS-T ×3, loaded with streptavidin-alkaline phosphatase (1:1,000 dilution in PBS-TB) and incubated for another 2 hr at room temperature. Plates were then washed 3 times with PBS and developed with alkaline phosphatase substrate Vector Blue (Vector Labs, Burlingame, CA). Spots were counted using an automated ELISPOT counter (CTL ImmunoSpot Analyzer with ImmunoSpot software, version 2.08; Cellular Technology, Cleveland, OH). Counts are shown as the mean ± SD for each set of triplicate wells.

Lymphoproliferation Assays

Splenocytes or draining lymph node cells were cultured in triplicate at 37°C with 5% CO_2 in 96-well plates (5×10^5 cells in 200 μl/well) with or without peptides at the concentrations designated in the figure legends. Each well was pulsed with ^{3}H thymidine (1 μCi/well) at 72 hr and harvested 16 hr later (TomTec). Incorporated radioactivity was read with a β-scintillation counter (Wallac MicroBeta Trilux).

Statistical Analysis

Differences in cytokine response, proliferation, and clinical score were assessed using the Student's *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

Th1 and Th17 Responses Are Directed Against the Immunizing Epitope $PLP_{139–151}$, But Not Putative Secondary and Tertiary Epitopes, Throughout the Course of Relapsing EAE

Previous analyses of T cell responses against the immunizing or spreading myelin epitopes during the course of relapsing EAE either failed to distinguish between different functional subsets (by simply measuring lymphoproliferation) or focused on Th1 responses. Consequently, pathogenic Th17 responses or regulatory Tr1 or Th2 responses might have been overlooked. Therefore, we carried out ELISPOT assays to measure the frequency of myelin-specific T cells producing IL-17, IL-10, IL-4, IL-5, and IFNγ in the CNS, draining lymph nodes, and spleens of SJL mice after immunization with $PLP_{139–151}$ in CFA. In initial experiments mice were sacrificed on Day 35 post-immunization based on previous reports that epitope spreading is readily apparent by that time point (Vanderlugt et al., 2000). Lymph nodes and splenocytes were pooled from mice with the same clinical scores (n = 5–12).

As shown in Figure 1, we detected high frequencies of IL-17, IFNγ, and IL-2 producing cells in the spleen and draining lymph nodes on in vitro challenge with $PLP_{139–151}$. We also detected significant Th1 and Th17 $PLP_{139–151}$-specific responses in cervical lymph nodes, although at an approximately 10-fold lower magnitude (data not shown). $PLP_{139–151}$ specific IL-4 responses were elicited routinely from splenocytes, but at approximately one-third to one-fifth the frequency of Th1 and Th17 cells. By contrast, we did not detect production of any of the cytokines in our panel in response

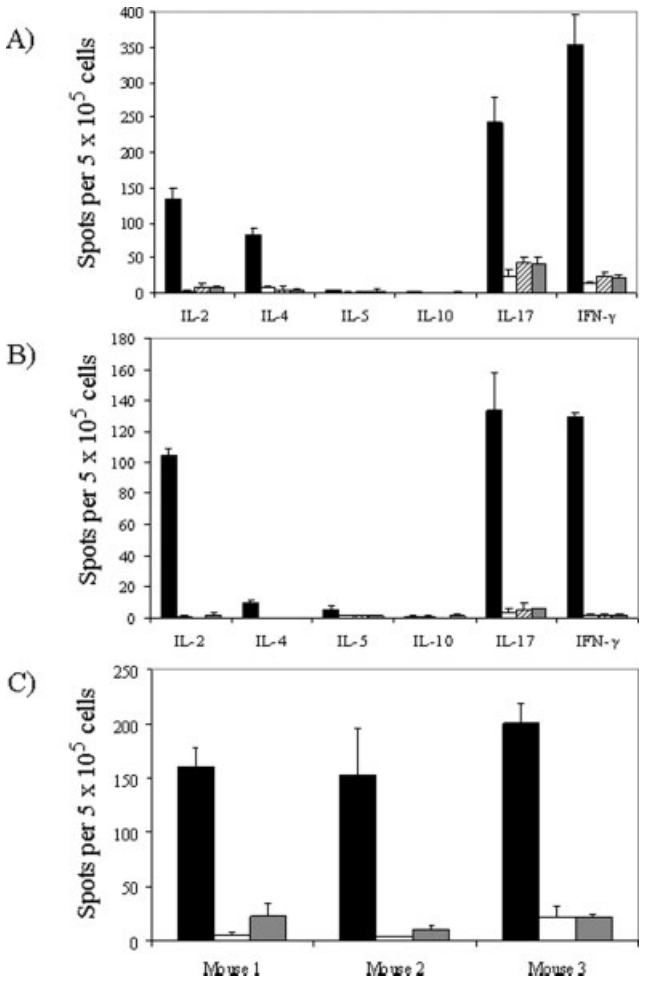

Fig. 1. Cytokine responses to myelin epitopes at Day 35 post-immunization with $PLP_{139–151}$. **A,B:** Splenocytes (A) and draining lymph node cells (B) were pooled from five mice on Day 35 post immunization (mean clinical score = 2.5 ± 0.80) and cultured with either $PLP_{139–151}$ (black), $PLP_{178–191}$ (white), $MBP_{84–104}$ (diagonal lines), or media alone (gray) for ELISPOT analysis. The data shown is representative of five independent experiments. **C:** In parallel experiments, splenocytes from individual mice were analyzed by IL-17 ELISPOT on Days 35–39 post-immunization. Clinical scores at sacrifice were 1, 3, and 3 for mice 1, 2, and 3, respectively. Similar data were obtained with lymph node cells from the same animals (not shown). In all instances, the frequencies of IL-2, IL-4, IL-17, and IFN-γ producing cells in response to challenge with $PLP_{139–151}$ were significantly higher than background with media alone ($P < 0.005$) or on challenge with alternative myelin peptides ($P < 0.005$).

to stimulation with $PLP_{178–191}$ or $MBP_{84–104}$. A similar pattern of antigenic responsiveness and cytokine production was observed when splenocytes and lymph node cells were analyzed from individual mice (Fig. 1C; unpublished observations).

Next we measured cytokine responses timed specifically to the first clinical relapse of EAE. Once again robust $PLP_{139–151}$ specific IL-17 and IFNγ responses were

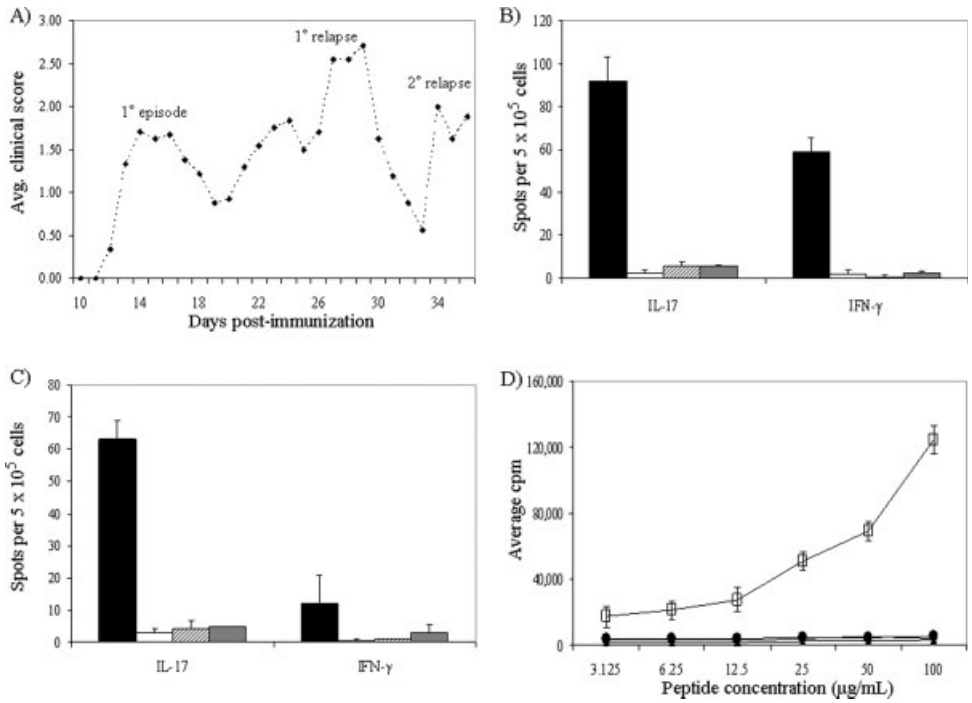

Fig. 2. Cytokine responses to myelin epitopes during the first relapse of $PLP_{139–151}$-induced EAE. **A:** Typical clinical course of $PLP_{139–151}$ -immunized mice. **B,C:** Splenocytes (B) and draining lymph node cells (C) were pooled from five mice at the peak of the first relapse (Day 28 post-immunization; mean clinical score = 2.8 ± 1.2) and subjected to ELISPOT analysis. The frequency of cytokine producing cells was determined after challenge with $PLP_{139–151}$ (black), $PLP_{178–191}$ (white), or $MBP_{84–104}$ (diagonal lines). Background counts of cells cultured with media alone are depicted in gray. The frequencies of IFNγ or IL-17 producing cells in response to challenge with $PLP_{139–151}$ were significantly higher than background counts with media alone ($P < 0.05$) or on challenge with alternative myelin peptides ($P < 0.05$). **D:** Lymphoproliferative responses were measured in parallel. Pooled lymph node cells were stimulated across a range of concentrations with either $PLP_{139–151}$ (open squares), $PLP_{178–191}$ (closed triangles), $MBP_{84–104}$ (closed circles), or $NP_{260–283}$, an $I\text{-}A^s$-restricted peptide of influenza nucleoprotein (broken line). Wells were pulsed with tritiated thymidine at 72 hr and harvested 16 hr later. Proliferation in response to $PLP_{139–151}$ was significantly higher than proliferation in response to $NP_{260–283}$ ($P < 0.05$) or alternative myelin peptides ($P < 0.05$) across all concentrations. This experiment was repeated three times with similar results.

readily evident in spleen and lymph nodes as was a lower frequency IL-4 response in the spleen (Fig. 2B,C and data not shown). We did not, however, find any response to $PLP_{178–191}$ or $MBP_{84–104}$ irrespective of the cytokine measured. Published studies that have shown epitope spreading at relapse used tritiated thymidine incorporation as the read-out measure of antigen reactivity. Therefore, we measured lymphoproliferative responses to $PLP_{139–151}$, $PLP_{178–191}$, and $MBP_{84–104}$ in parallel to the cytokine assays. Consistent with the ELISPOT data, only $PLP_{139–151}$ elicited significant thymidine incorporation by lymph node cells above background levels (Fig. 2D).

We considered the possibility that, epitope spreading might emerge relatively late in the disease process. It was also possible that the anti-myelin cytokine response changes over time, with new Th lineage commitments arising in the aftermath of the first relapse and remission. Therefore, we repeated the above studies with lymphoid tissues harvested at the time of the second relapse as well as during remission. Once again, only responses against $PLP_{139–151}$ were measurable (Fig. 3; data not shown). A similar pattern of cytokine production was observed at all time points, characterized by robust IL-17, IFNγ, and IL-2 responses in draining and cervical lymph nodes and spleen, a lower frequency IL-4 response in the spleen, and no antigen-specific IL-5 or IL-10 in any of the lymphoid tissues examined.

IFNγ and IL-17 Responses Targeting the Primary Epitope Remain Dominant During Relapses of $PLP_{178–191}$-Initiated EAE

Because SJL mice preferentially express the DM2O isoform of PLP (that lacks residues 116–150) in the thymus (Klein et al., 2000), T cells specific for $PLP_{139–151}$ evade negative selection and occur at an unusually high frequency in the naive peripheral repertoire (Anderson et al., 2000; Reddy et al., 2004). It was possible that the absence of epitope spreading documented in Figures 1–3 was particular to the model we used, consequent to an unparalleled immunodominance of $PLP_{139–151}$ in the SJL strain (Whitman et al., 1991). We speculated that spreading might be conspicuous in SJL mice if $PLP_{139–151}$

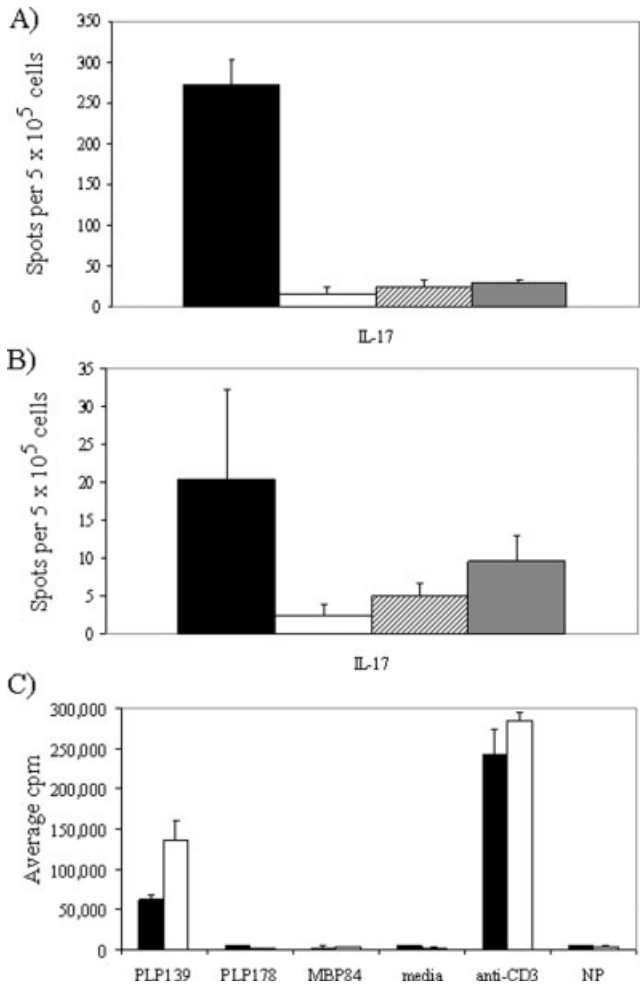

Fig. 3. IL-17 responses to myelin epitopes during the second relapse of $PLP_{139–151}$-induced EAE. **A,B:** Frequency of IL-17 producers among splenocytes (A) and draining lymph node cells (B) pooled from 10 mice (day 36 post-immunization; mean clinical score = 2.6 ± 0.89) was determined after culture with either $PLP_{139–151}$ (black), $PLP_{178–191}$ (white), $MBP_{84–104}$ (diagonal lines), or media alone (gray). The frequency of IL-17 producers among splenocytes or lymph node cells cultured with $PLP_{139–151}$ was significantly higher than the background counts of cells cultured with media alone ($P < 0.005$) or cells cultured with alternative myelin peptides ($P < 0.005$). **C:** Lymphoproliferation assays were carried out using the same splenocytes (black) and lymph node cells (white). In all instances, proliferation in response to $PLP_{139–151}$ was significantly higher than proliferation in response to alternative myelin peptides ($P < 0.005$) or in the absence of antigenic challenge ($P < 0.005$). The data shown is representative of three independent experiments.

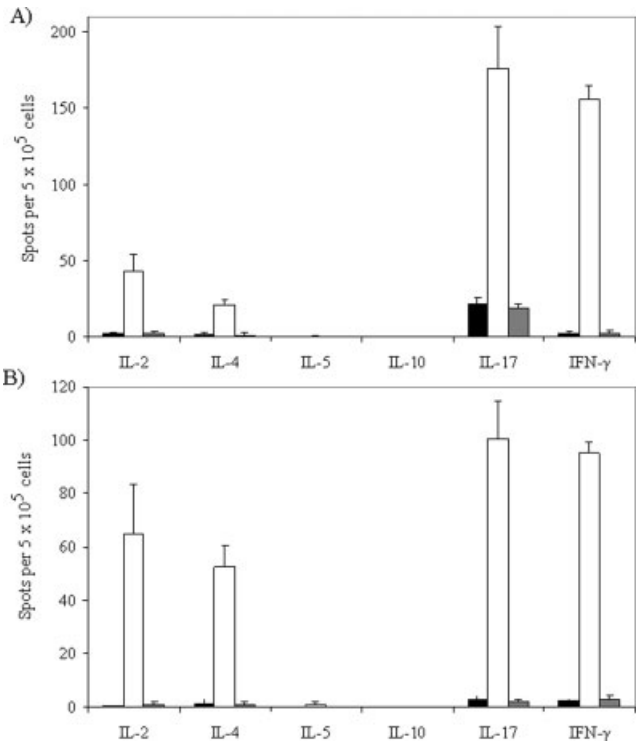

Fig. 4. Cytokine responses to myelin epitopes during the presenting episode of $PLP_{178–191}$-induced EAE. **A,B:** Splenocytes (A) and draining lymph node cells (B) were harvested and pooled from five mice at peak disease (day 13 post-immunization; mean clinical score = 3.8 ± 0.50). Cells were stimulated with either $PLP_{139–151}$ (black), $PLP_{178–191}$ (white), or cultured in media alone (gray) for ELISPOT analysis as described in Figure 1. IL-2, IL-4, IL-17, and IFNγ responses to $PLP_{178–191}$, but not $PLP_{139–151}$, were significantly higher than background. This experiment was repeated three times with similar results.

acted as the secondary as opposed to the primary epitope. To test this hypothesis, we immunized SJL mice with an alternative encephalitogenic peptide, $PLP_{178–191}$, and searched for reactivity against $PLP_{139–151}$ at several time points. $PLP_{178–191}$ immunized mice exhibited a severe relapsing course with mean clinical scores of 3.75 ± 0.5 at the peak of the first episode and 3.33 ± 1.15 at relapse.

Reminiscent of our findings with $PLP_{139–151}$-induced disease, lymph node cells and splenocytes from mice immunized with $PLP_{178–191}$ mounted cytokine and lymphoproliferative responses exclusively against the primary epitope, both during the presenting episode and relapse (Figs. 4,5; unpublished observations). As before, responses were skewed toward IFNγ and IL-17 producers.

PLP-Specific T Cells in the CNS of Mice With Relapsing EAE Mirror Those in the Periphery With Regard to Antigen Specificity and Th Lineage

Several studies have suggested that epitope spreading is initiated in the CNS during EAE (Targoni et al., 2001; McMahon et al., 2005). Therefore, we next turned our attention to CNS infiltrating cells. $PLP_{139–151}$-specific IFNγ secreting cells occurred at a high frequency among spinal cord mononuclear cells that were isolated from $PLP_{139–151}$ primed mice during the first relapse, exceeding their frequency in the lymphoid tissues by over 100-fold (Fig. 6A). By contrast, IFNγ was

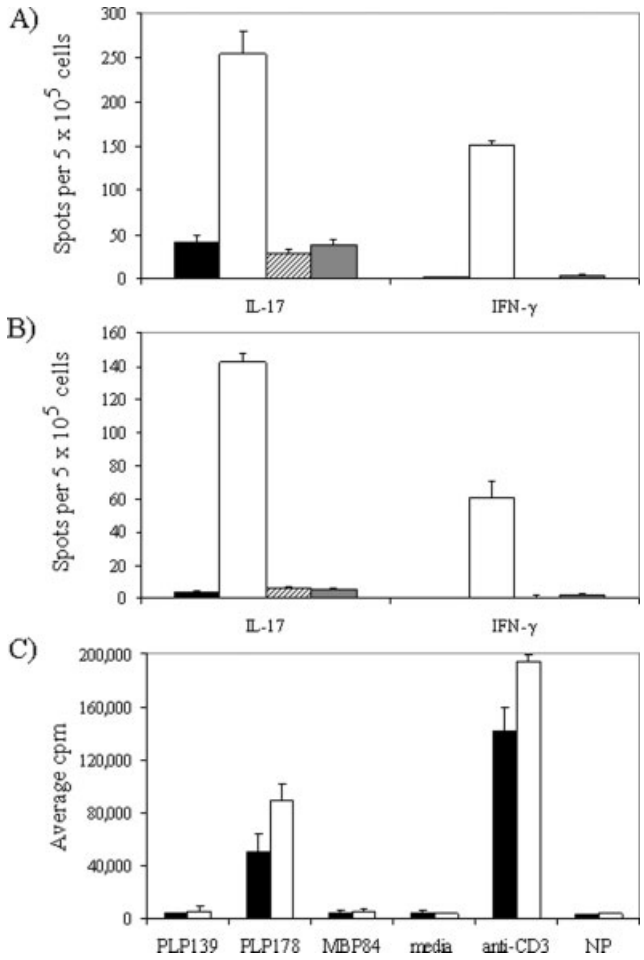

Fig. 5. Cytokine responses to myelin epitopes during the first relapse of $PLP_{178–191}$ induced EAE. **A,B:** Splenocytes (A) and draining lymph node cells (B) were pooled from five mice at the peak of relapse (day 25 post-immunization; mean clinical score = 3.3 ± 1.1) and stimulated with either $PLP_{139–151}$ (black), $PLP_{178–191}$ (white), $MBP_{84–104}$ (diagonal lines), or cultured in media alone (gray). Frequencies of IL-17 and IFNγ producing cells were determined by ELISPOT. Only responses to $PLP_{178–191}$ were higher significantly than background ($P < 0.005$). **C:** Lymphoproliferative responses of pooled splenocytes (black) and lymph node cells (white) were measured in parallel (C). Proliferation in response to $PLP_{178–191}$ was greater significantly than proliferation in response to media alone ($P < 0.005$) or alternative myelin peptides ($P < 0.005$). The data shown is representative of three independent experiments.

not produced above background levels in wells pulsed with $PLP_{178–191}$.

We also detected a high frequency of IL-17 producers, even in the absence of peptide challenge, presumably the result of presentation of endogenous myelin antigens by CNS resident APC (Fig. 6A). In an attempt to unmask PLP-specific IL-17 secretion, we repeated the ELISPOT assays using $CD4^+$ T cells purified from the mononuclear cell fraction combined with T-depleted splenocytes from naive syngeneic donors as APC. Consequently, we were able to measure a significant $PLP_{139–151}$-

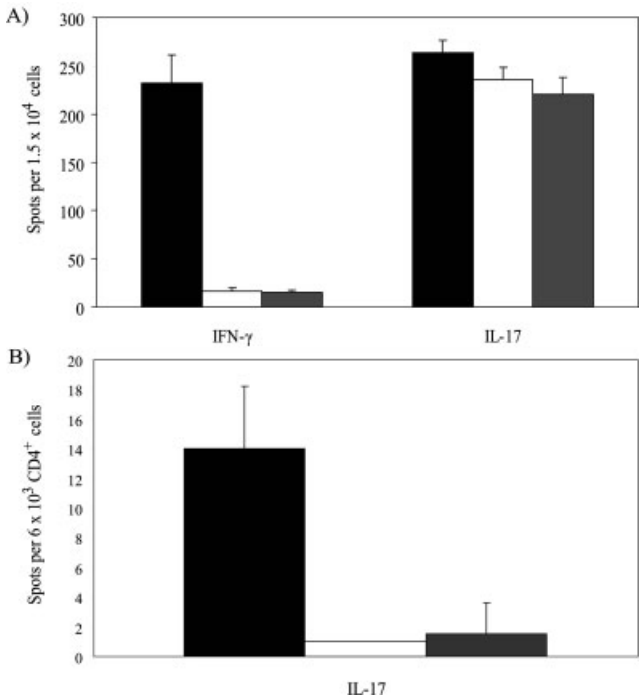

Fig. 6. Myelin-specific IL-17 and IFN-γ responses in the CNS during the first relapse of $PLP_{139–151}$-induced EAE. **A,B:** Mononuclear cells were isolated from the pooled spinal cords of 10–12 relapsing mice (mean clinical score = 2.5 ± 0.76) by Percoll gradient centrifugation. Whole mononuclear cells (A) or purified CD4+ T cells reconstituted with T-depleted naive splenocytes (B) were cultured with either $PLP_{139–151}$ (black), $PLP_{178–191}$ (white), or media alone (gray) for ELISPOT analysis. For whole spinal cord cells, the frequency of IFN-γ producers on challenge with $PLP_{139–151}$ was higher significantly than background counts with media alone ($P < 0.005$) or on challenge with $PLP_{178–191}$ ($P < 0.005$). For purified CD4 T cells, the frequency of IL-17 producers on challenge with $PLP_{139–151}$ was significantly higher than background levels with media alone ($P < 0.05$) or on challenge with $PLP_{178–191}$ ($P < 0.05$).

specific Th17 response (Fig. 6B). We did not, however, detect a $PLP_{178–191}$-specific response.

Tolerance Against $PLP_{178–191}$ Does Not Prevent Relapses of $PLP_{139–151}$-Initiated EAE

To investigate directly the functional role of $PLP_{178–191}$ reactive T cells in relapsing EAE in $PLP_{139–151}$ immunized SJL mice, we used a well-established protocol to induce antigen-specific tolerance. Injection of $PLP_{178–191}$ in IFA by the i.p. route prevents lymphoproliferative and cytokine recall responses as well as clinical EAE on subsequent challenge with $PLP_{178–191}$ in CFA s.c. (Gaur et al., 1992; Marusic and Tonegawa, 1997; Ichikawa et al., 2002). Tolerance is maintained for 40 days or longer past the i.p. injection and it is not broken by s.c. injection with an immunogenic irrelevant peptide or PBS in CFA 2 weeks later (unpublished observations).

Despite pretreatment with $PLP_{178–191}$ in IFA, 89% of mice underwent EAE relapses after active immuniza-

TABLE I. Tolerization to PLP_{178} Does Not Affect Relapse Rate in PLP_{139} Immunized Mice

Pretreatment	PBS + IFA	PLP_{178} + IFA	—	PLP_{178} + IFA	—
Challenge	PLP_{139} + CFA	PLP_{139} + CFA	PLP_{139} + CFA	PLP_{178} + CFA	PLP_{178} + CFA
Relapse incidence	8/8	8/9	3/3	0/3	3/4
Mean cumulative disease score[b]	58 (30)	44 (14)	46 (9)	0 (0)	23[a] (5)

Mice were injected with either PBS or $PLP_{178–191}$ in IFA i.p. 2 weeks before challenge with $PLP_{139–151}$ in CFA. Standard deviations are shown in parentheses.

[a]Mice were sacrificed early (Day 23 post-immunization).

[b]Cumulative scores were calculated by summing daily scores of individual mice over the observation period.

tion with $PLP_{139–151}$ in CFA (Table I). This was comparable to the relapse rate of control mice pretreated with PBS in IFA. Furthermore, cumulative disease scores did not differ significantly between mice pretreated with $PLP_{178–191}$ in IFA and those pretreated with PBS in IFA ($P = 0.24$). Hence, consistent with our earlier data showing the immunodominance of $PLP_{139–151}$ throughout the disease course, this result suggests that clinical relapses in our model are not dependent on T cell reactivity against $PLP_{178–191}$.

DISCUSSION

Recent clinical studies have shown that broadly acting immunosuppressive agents, including drugs that inhibit hematopoiesis, globally deplete T and B lymphocytes, or block lymphocyte and monocyte trafficking, can be of therapeutic benefit in MS (Moreau et al., 1994; Polman et al., 2006). The occurrence of serious and sometimes life-threatening adverse events, such as opportunistic infections and neoplasia, provide a clear incentive for the development of more focused, antigen-specific strategies (Kleinschmidt-DeMasters and Tyler, 2005).

The literature on myelin reactivity during the course of EAE and MS is controversial. Whereas many studies have detected the emergence of novel T cell autoreactivities after the onset of clinical EAE, others have found that the autoimmune response remains focused on the disease-inducing epitope throughout the disease course. Some of these discrepancies could be attributed to differences in murine strain (or even colony), the immunogenicity of the priming epitope, the method of disease induction (i.e., passive transfer vs. active immunization), environmental conditions (housing, exposure to pathogens), and the timing or location of T cell harvest (Voskuhl et al., 1996). In addition, the use of T cell assays with various degrees of sensitivity and that measure different biologic functions have added to the complexity. A number of investigators have noted that reactivity against the priming, or initially immunodominant myelin epitope begins to wane at the time point when epitope spreading surfaces during the progression of both EAE and MS (Tuohy et al., 1999; Targoni et al., 2001). Conversely, in our study as well as in others that failed to detect spreading, T cell responses against the priming epitope remained robust, and even became stronger, with successive relapses (Takacs and Altmann, 1998). One interpretation of these observations is that spreading is most likely to occur in those instances where the disease-initiating T cell subpopulation either contracts or becomes anergic after the peak of the presenting episode. Alternatively, T cells recruited against novel epitopes might either regulate or out-compete those T cell clones reactive to the primary epitope for access to myelin-bearing APC.

We expanded on previous studies by conducting an exhaustive search for emerging T cell reactivities in lymphoid tissues and the CNS over multiple time points (based on stage of disease as well as absolute time from immunization) using highly sensitive ELISPOT assays that encompassed a broad range of cytokines. Based on our results, we can be numbered among those investigators who find no evidence for recruitment of Th1 cells reactive against secondary or tertiary epitopes during the course of multiphasic disease. In addition, we were unable to detect spreading to alternative Th lineages that have been associated with regulatory subsets (such as Tr1 cells) or pro-inflammatory subsets (i.e., Th17 cells) recognized more recently.

As alluded to earlier, EAE in $PLP_{139–151}$-immunized SJL mice is not a typical model of progressive or relapsing autoimmune demyelinating disease in that T cells reactive against the initiating epitope occur at an unusually high precursor frequency. Nevertheless, we were also unable to detect spreading in SJL mice immunized with the subdominant epitope, $PLP_{179–191}$ (Figs. 4,5). We can not rule out the possibility that epitope spreading is a feature of progressive EAE induced in alternative strains with other encephalitogenic peptides, or in MS, in which the target epitope(s) remain unknown. It is also possible that, in our model, spreading occurred to myelin epitopes that we did not investigate.

Although EAE and MS have been portrayed classically as Th1-driven diseases, a growing body of data suggest that this characterization is overly simplistic. Th17 polarized cells have emerged as critical effector cells in EAE pathogenesis (Park et al., 2005; Chen et al., 2006). In contrast, IFNγ plays a paradoxic protective role in some models of EAE and can inhibit Th17 differentiation (Ferber et al., 1996; Willenborg et al., 1996; Billiau et al., 1998; Segal et al., 1998; Harrington et al., 2005). Such observations suggest that PLP-specific Th1 cells act to regulate the pathogenic Th17 effector response, thereby limiting, rather than amplifying, neuroinflammation. In vitro stimulation of myelin-specific T cells from

innately resistant or tolerized mice with recombinant IL-12 enhances their encephalitogenic properties (Segal and Shevach, 1996; Ichikawa et al., 2002). Thus IL-12 induces characteristics, distinct from IFNγ production, that facilitate the CNS migration or effector functions of auto reactive T cells. Future experiments will determine the role of myelin-specific Th1 cells in our model, which we recognize might vary based on location and the stage of disease.

We found that mice pretreated with $PLP_{178–191}$ in a tolerogenic context (i.e., injected at a high dose i.p. without adjuvants), nevertheless succumbed to relapsing EAE after challenge with $PLP_{139–151}$ in CFA at an incidence comparable to controls (Table I). Mice that received the same pre-treatment were protected from EAE induced by active immunization with $PLP_{178–191}$ in CFA. Therefore, the elimination of measurable T cell responses against a putative secondary epitope was not sufficient to abrogate EAE relapses. Conversely, other investigators were able to suppress EAE relapses partially by injecting SJL mice in remission with splenocytes coupled chemically to PLP peptides distinct from the immunizing epitope (Vanderlugt et al., 2000). They interpreted their results as showing that relapses are driven by T cells specific for the coupled peptide and that these cells were either deleted or rendered nonfunctional by the therapeutic intervention. An alternative interpretation is that the peptide-coupled splenocytes primed a population of regulatory T cells that, in turn, suppressed effector T cells specific for the primary epitope in a bystander fashion (Yin et al., 2001; Wildbaum et al., 2002). The mechanism of tolerance induction via the protocol that we used in Table I remains to be elucidated fully. The disparate effects of myelin peptide treatment on relapse rate when administered coupled to splenocytes as opposed to emulsified in IFA might reflect the fact that the two protocols target distinct immunoregulatory pathways.

In conclusion, the experimental data illustrated in this study complement a growing body of literature indicating that, at least in some instances of autoimmune demyelination, Th17 cells specific for a single epitope dominate the myelin-specific T cell response through multiple relapses. Longitudinal clinical studies show that some individuals with MS maintain a strikingly focused and stable epitope response against MBP over many years despite continuing disease activity (Goebels et al., 2000). By extension, therapies that target specific T cell reactivities might be useful in some cases of relapsing-remitting MS and other chronic autoimmune diseases. Therapeutic modalities that promote the development of regulatory T cells against novel neuroantigens could be effective in disease driven by a focused response if they are capable of mediating bystander suppression.

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