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IL-10 Plays an Important Role in the Homeostatic Regulation of the Autoreactive Repertoire in Naive Mice[1]

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We have previously shown that naive SJL (H-2^s) mice, which are highly susceptible to myelin proteolipid protein (PLP)-induced experimental autoimmune encephalomyelitis (EAE), have a very high frequency (1/20,000 CD4 T cells) of $PLP_{139-151}$-reactive T cells in the naive repertoire. In this study, we examine the function of this endogenous $PLP_{139-151}$-reactive repertoire in vivo and find that this repertoire encompasses the precursors of pathogenic T cells. Because SJL mice do not develop spontaneous EAE, we have explored the mechanisms that keep this autopathogenic repertoire in check and prevent the development of spontaneous autoimmunity. We crossed IL-4 and IL-10 deficiency onto the SJL background and analyzed the roles of these two immunoregulatory cytokines in regulating the size and effector function of the endogenous $PLP_{139-151}$-reactive repertoire and development of autoimmune disease. We find that IL-10 is important in the homeostatic regulation of the endogenous $PLP_{139-151}$-reactive repertoire in that it both limits the size of the repertoire and prevents development of effector autoaggressive T cells. SJL IL-10$^{-/-}$ mice with high numbers of $PLP_{139-151}$-specific precursors in the repertoire did not develop spontaneous EAE, but when they were injected with pertussis toxin, they showed atypical clinical signs of EAE with small numbers of typical mononuclear cell infiltrates predominantly in the meninges. EAE could be inhibited by prior tolerization of the mice with soluble $PLP_{139-151}$ peptide. These findings indicate that IL-10 may contribute to the regulation of the endogenous autoimmune repertoire. ***The Journal of Immunology,*** **2004, 173: 828–834.**

Unmanipulated SJL (H-2^s) mice do not develop spontaneous CNS autoimmunity. However, SJL mice are highly susceptible to experimental autoimmune encephalomyelitis (EAE),[4] an animal model of multiple sclerosis induced by immunization with myelin proteolipid protein (PLP). We have previously demonstrated that SJL mice have a very high frequency (1/20,000 CD4 T cells) of $PLP_{139-151}$-reactive T cells in the naive repertoire (1). This represents the only murine model of autoimmune disease in which autoreactive T cells have been detected in healthy, naive animals. This is particularly significant given that myelin-reactive T cells are readily observed in the peripheral blood of normal healthy humans (2–4). Thus, SJL mice may provide a model in which the endogenous factors that alter the balance between tolerance and disease in autoimmune susceptible individuals can be addressed.

The balance between Th1 cells (secreting IL-2, IFN-γ and/or lymphotoxin-α) and Th2 cells (secreting IL-4, IL-5, and IL-10) plays a central role in EAE and many other organ-specific autoimmune diseases. To date most encephalitogenic myelin-reactive T cell lines or clones have been shown to exhibit a Th1 phenotype (5–7). In addition, Th1 cytokines as well as other proinflammatory cytokines such as TNF-α and IL-12 are present in inflammatory CNS lesions during the early phase of EAE, whereas Th2 cytokines are absent (8, 9). Furthermore, Th2 cytokines and TGF-β are present in the CNS during disease remission (10). These observations have led to the hypothesis that myelin-reactive T cells that have a Th1 phenotype are encephalitogenic, whereas myelin-reactive T cells that have a Th2 phenotype are protective.

Indeed, there is substantial evidence to support a protective role for myelin-reactive Th2 cells in EAE. We have previously shown that Th2 T cell lines and clones reactive to an encephalitogenic epitope of PLP are protective, if given at the time of immunization, and reverse the course of EAE if administered upon the first signs of disease (11, 12) Moreover, the administration of IL-4 after adoptive transfer of encephalitogenic myelin basic protein-specific T cell lines has been shown to reduce both clinical and histological disease (13). In these experiments, the protective effect of IL-4 was associated with the induction of myelin basic protein-specific Th2 cells and inhibition of proinflammatory cytokine gene expression (TNF-α) in the CNS, demonstrating that factors that promote Th2 activity can be effective in treating EAE even in the presence of primed encephalitogenic Th1 cells. In studies in which myelin-reactive T cells have been engineered to deliver Th2 cytokines directly to the CNS, both IL-10 and IL-4 have been shown to ameliorate EAE (14, 15).

More recent studies have specifically addressed the relative contributions of IL-4 and IL-10 in the development of EAE. IL-4 transgenic mice exhibit a reduced incidence of disease but IL-10 transgenic mice are completely resistant (16, 17). Although studies

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[4] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; PTX, pertussis toxin; TMEV, Theiler's murine encephalomyelitis virus; PLP, myelin proteolipid protein; LNC, lymph node cell; 7-AAD, 7-aminoactinomycin D.

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in IL-4$^{-/-}$ mice have yielded conflicting results, the majority of studies have shown that EAE is not exacerbated in the absence of IL-4 (16, 18–21). In contrast, IL-10$^{-/-}$ mice develop severe chronic EAE (16, 19, 20). In addition, treatment with anti-IL-10 before disease onset exacerbates EAE (22). Therefore, IL-10 appears to be more critical than IL-4 in regulating established EAE, however, whether IL-10 also plays a role in regulating the naive endogenous autoreactive T cell repertoire is not known.

In this study we have directly examined the role of the endogenous $PLP_{139-151}$ repertoire in the development of EAE and the role of IL-10 and IL-4 in controlling this repertoire. We find that IL-10, rather than IL-4, plays a critical role in the homeostatic regulation of this large autoreactive repertoire in vivo in normal SJL mice. Our results suggest that lack of spontaneous disease in SJL mice is due in part to the ability of IL-10 to limit the size of this autoreactive repertoire and maintain the nonencephalitogenic phenotype of these cells in vivo.

Materials and Methods

Animals

Female SJL/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Both IL-4$^{-/-}$ (23) and IL-10$^{-/-}$ (24) mice on the 129 background were obtained from Dr. R. Kühn (Institute for Genetics, University of Cologne, Cologne, Germany). IL-4$^{-/-}$ and IL-10$^{-/-}$ mice were backcrossed for at least 10 generations onto the SJL background.

Antigens

$PLP_{139-151}$ (HSLGKWLGHPDKF), alanine substituted peptides on the $PLP_{139-151}$ backbone and $NASE_{101-120}$ (EALVRQGLAKVAYVYK PNNT) were synthesized by Dr. R. Laursen (Boston University, Boston, MA) on a Milligen model 9050 synthesizer using F-moc chemistry. Peptide purity was determined by HPLC and peptide identity was confirmed by mass spectroscopy.

In vitro proliferation assays

Lymph nodes were harvested from naive mice. Lymph node cells (LNCs) (4×10^5 per well) were cultured in serum-free medium (HL-1) supplemented with L-glutamine (2 mM), (BioWhittaker, Walkersville, MD) in triplicate in 96-well round-bottom plates in the presence of various concentrations of peptide for 48 h and pulsed with 1 μCi/well of [^{3}H]thymidine for the last 16 h. [^{3}H]Thymidine incorporation was determined in a Wallac scintillation counter (model 1250; Gaithersburg, MD).

In vivo experiments

Adoptive transfer of EAE. LNCs were harvested from naive SJL mice and cultured at 5×10^6 per well in 24-well plates in the presence of $PLP_{139-151}$ (100 μg/ml) or Con-A (1 μg/ml) in serum-free medium (HL-1) supplemented with L-glutamine (2 mM), (BioWhittaker) and 1% T cell growth factor (BD Biosciences, San Diego, CA). After 4 days, cells were harvested and either run over a Ficoll-Hypaque gradient or enriched by negative selection using a CD3 enrichment column (R&D Systems, Minneapolis, MN). Cells were then resuspended in PBS and injected i.v. (1–3×10^7/mouse) into mice. Recipient mice also received 100 ng of pertussis toxin (PTX; List Biological Laboratories, Campbell, CA) i.v. on day 0 and day 3. Mice were scored daily for clinical signs as follows: 0, no clinical signs; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb paralysis; and 5, moribund or dead. When animals were moribund or at the end of the experiment (generally day 30) they were sacrificed and brains and spinal cords were fixed in 10% formalin, processed for histologic analysis, and evaluated as described (25) by an observer blinded to the clinical status of the mice.

Induction of disease and tolerance in IL-10$^{-/-}$ SJL mice

For inducing tolerance, SJL-IL-10$^{-/-}$ mice and wild-type littermate controls were injected with 300 μg of soluble $PLP_{139-151}$, $PLP_{178-191}$ peptides or PBS i.p. before injection of PTX. Mice were given two injections of 100 ng of PTX on day 0 and 2 and were observed for signs of EAE over a period of one month. Brains and spinal cords were obtained at the peak of the disease or at termination of the experiment and processed for histologic analysis as previously described.

In vitro cytokine assays

Supernatants were collected from LNCs 40 h after activation in vitro. The concentrations of IL-2, IL-4, IL-10, IFN-γ, and TNF-α were measured by quantitative capture ELISA according to the guidelines of the manufacturers. In brief, purified rat mAb to mouse IL-2 (clone JES6-1A12), IL-4 (clone BVD4-1D11), IL-10 (clone JES5-2A5), IFN-γ (clone R4-6A2), and TNF-α (clone MP6-XT22) were obtained from BD Biosciences and used to coat ELISA plates (Immulon 4; Dynatech Laboratories, Chantilly, VA). Recombinant mouse cytokines (IL-2, IL-4, IL-10, IFN-γ, and TNF-α; BD Biosciences) were used to construct standard curves and biotinylated rat mAb to mouse IL-2 (clone JES6-5H4), IL-4 (clone BVD6-24G2), IL-10 (clone SXC-1), and IFN-γ (clone XMG1.2; BD Biosciences) were used as the second Ab. Detection of TNF-α was with biotinylated polyclonal rabbit IgG (BD Biosciences). Plates were developed with TMB Microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and read after the addition of stop solution at 450 nm using a model 3550 Microplate Reader (Bio-Rad, Hercules, CA).

Enumeration of the frequency of I-A^s tetramer-reactive cells by flow cytometry

The description and the validation of I-A^s tetramers for $PLP_{139-151}$ and Theiler's murine encephalomyelitis virus $(TMEV)_{70-86}$ are described elsewhere (26). Briefly, the soluble MHC class II monomers constituting I-A^s α-chain and β-chain with $PLP_{139-151}$ or $TMEV_{70-86}$ nucleotide sequences covalently tethered to the β-chain were generated in a baculovirus expression system. The monomers were then biotinylated and multimerized using streptavidin-conjugated with PE to obtain the IA^s tetramers. Single cell suspensions were obtained from the peripheral lymph nodes of IL-10$^{-/-}$ or IL-4$^{-/-}$ SJL mice and their littermate wild-type controls. CD3$^+$ T cells were then enriched by negative selection using mouse T cell columns (R&D Systems). Approximately 1×10^7 cells/ml were used for neuraminidase treatment at a concentration of 0.7 U/ml (neuraminidase Type X from *Clostridium perfringens*; Sigma-Aldrich, St. Louis, MO) in serum-free DMEM medium at 37°C for 1 h. After washing once with 1× PBS, the cells were incubated with I-A^s tetramers in DMEM medium supplemented with IL-2 at 37°C for 3–4 h at a concentration of 30 μg/ml. Cells were washed with 4 ml of FACS buffer containing 1× PBS, 2% FCS and sodium azide (0.1%) and stained with anti-CD4 Ab (clone RM4.5, CD4-APC) and 7-aminoactinomycin D (7-AAD; BD Biosciences). After incubating at room temperature for 20 min, the cells were washed as previously described and 5×10^5 events were acquired using a FACSort flow cytometer (BD Biosciences). Triple color staining of FACS analysis allowed us to exclude the dead cells (7-AAD-positive fraction) and the tetramer-PE ($PLP_{139-151}$ or $TMEV_{70-86}$)-positive cells were then determined in 7-AAD-negative, CD4-positive populations.

Statistics

Comparison of histological evaluation of EAE in wild-type SJL and IL-10$^{-/-}$ SJL mice tolerized with $PLP_{139-151}$ or $PLP_{178-191}$ was done by Fisher's exact test. The $PLP_{139-151}$-reactive CD4 T cells in IL-10$^{-/-}$, wild-type and IL-4$^{-/-}$ SJL mice were compared with those of $TMEV_{70-86}$ tetramer-positive cells in each group. The data were analyzed by Student's *t* test. Values of $p \leq 0.05$ were considered significant.

Results

Fine specificity of $PLP_{139-151}$ recognition by endogenous $PLP_{139-151}$-reactive cells

Our previous studies indicated that naive SJL mice (H-2^s) have a high frequency of $PLP_{139-151}$-reactive T cells. Indeed, limiting dilution analysis revealed that the precursor frequency of these cells might be as high as 1/20,000 CD4 T cells. We have also shown that encephalitogenic (Th1) clones and protective (Th2) clones specific for $PLP_{139-151}$ differ in their recognition of the $PLP_{139-151}$ peptide (27) (Fig. 1*A*). Encephalitogenic clones express a wide range of different TCRs (28), but they all use the tryptophan at position 144 as the primary TCR contact. Leucine at position 141, lysine at position 143 and histidine at position 147 are all used as secondary TCR contacts (29). In contrast, protective (Th2) clones, which also express a wide range of different TCRs, use the leucine and glycine at positions 141 and 142, respectively, as primary TCR contacts (26). To determine whether the endogenous $PLP_{139-151}$-reactive cells in the naive repertoire of SJL mice

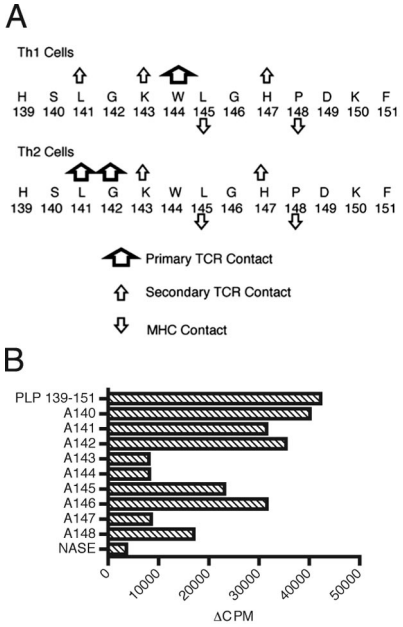

FIGURE 1. Recognition and determination of critical residues for activation of endogenous $PLP_{139-151}$-reactive cells. *A*, Recognition of $PLP_{139-151}$ peptide by pathogenic Th1 and protective Th2 clones. This is based on previously published data (26, 27). *B*, LNCs were harvested from naive SJL mice and tested in triplicate for reactivity to $PLP_{139-151}$, a panel of $PLP_{139-151}$ peptide analogues bearing alanine substitutions at each position, and the control Ag, NASE, over a dose response of 0.1–100 μg/ml peptide. [^{3}H]Thymidine was added at 48 h and plates were harvested 16 h later. Data are shown as mean Δcpm of triplicate wells at 100 μg/ml peptide in which Δcpm = [mean cpm in test wells] − [mean cpm in wells with medium only].

are the precursors of pathogenic or protective cells, we tested the proliferative response of lymph node cells from naive SJL mice to the $PLP_{139-151}$ peptide and a panel of peptides bearing alanine substitutions at each position along the $PLP_{139-151}$ backbone (Fig. 1*B*). LNCs from naive SJL mice proliferated well to all alanine substituted peptides tested, with the exception of A143, A144, and A147, indicating that these residues are important for recognition of the $PLP_{139-151}$ peptide by these cells. Thus, significant numbers of $PLP_{139-151}$-reactive cells present in naive SJL mice recognize $PLP_{139-151}$ in a pattern similar to encephalitogenic T cell clones, suggesting that this population likely encompasses the precursors of pathogenic cells.

Endogenous $PLP_{139-151}$-reactive cells are the precursors of encephalitogenic cells

We then tested directly whether the endogenous $PLP_{139-151}$-reactive cells are the precursors of encephalitogenic cells. We reasoned that if these cells were under dynamic regulation in vivo, stimulation for a short period in vitro might disturb the balance of this process and lead to the emergence of cells with frank pathogenic potential. Our ability to detect the proliferation of $PLP_{139-151}$-specific cells ex vivo without immunizing the mice gave us the ability to expand the naive $PLP_{139-151}$ precursors in vitro and then test their encephalitogenic potential upon adoptive transfer. We also tested whether activation would need to be Ag-specific or whether polyclonal stimulation could affect progression to spontaneous EAE, and whether disrupting the blood brain barrier with PTX would be sufficient to induce disease. LNCs from naive SJL mice were cultured in vitro with $PLP_{139-151}$ peptide or Con-A. Four days later, T cell blasts were harvested and adoptively transferred into naive SJL recipients (Table I). Five of sixteen SJL mice that received LNCs cultured with $PLP_{139-151}$ developed clinical EAE. Four mice that exhibited no signs of clinical EAE had significant histological disease as indicated by the presence of inflammatory foci in the CNS. None of the mice that received cells stimulated with Con-A developed signs of clinical or histological disease, suggesting that specific activation of the endogenous $PLP_{139-151}$-reactive repertoire is necessary for disease induction. Furthermore, none of the mice that received PTX alone developed either clinical or histological EAE, indicating that simply providing the endogenous $PLP_{139-151}$-reactive repertoire with access to the CNS is not sufficient for these cells to induce disease.

Endogenous $PLP_{139-151}$-reactive cells are not fully differentiated in vivo

We have previously shown that the $PLP_{139-151}$ reactive T cells in naive SJL mice are enriched in the CD44 high subset (1) indicating that these cells have divided in vivo (30). Moreover, our present data suggest that these cells may be the precursors of pathogenic cells. Taken together these findings raise an important question: why don't normal SJL mice develop spontaneous EAE? To address this, we examined whether endogenous $PLP_{139-151}$-reactive T cells have acquired an effector phenotype by examining their production of effector cytokines. Although LNCs from naive SJL mice respond well to stimulation with $PLP_{139-151}$ (Fig. 2*A*), they produce only low levels of IL-2, and no effector cytokines, IL-4, IL-10, IFN-γ, or TNF-α (Fig. 2*B*). Thus, endogenous $PLP_{139-151}$-reactive cells do not appear to be differentiated to an effector phenotype in vivo suggesting one explanation for the lack of spontaneous EAE in normal SJL mice.

Immune regulation of the endogenous $PLP_{139-151}$-reactive repertoire

There are many additional possible explanations for the nonencephalitogenic phenotype of the endogenous $PLP_{139-151}$-reactive repertoire in SJL mice. It is possible that these cells are undifferentiated because PLP is sequestered in the CNS and therefore is

Table I. *Induction of classical EAE by adoptive transfer of naive T cells activated with PLP 139–151*[a]

	Clinical Disease			Histological Disease		
Cells	Incidence	Mean day of onset	Mean maximum severity	Incidence	Mean inflammatory foci with clinical disease	Mean inflammatory foci without clinical disease
PLP 139–151[b]	5/16	20.8 ± 3.8	2.7 ± 0.7	8/11	90.5 ± 21.2[c]	57.5 ± 11.7[c]
Con-A	0/7	–	–	0/7	–	0[d]
PTX	0/5	–	–	0/4	–	0[e]

[a] Mice received 1–3 × 10^7 cells i.v. On day 0 and day 3, mice received 100 ng of PTX i.v. Dash represents not applicable.
[b] Mean values ± SE.
[c] Number (n = 4) of mice with histological disease as indicated by presence of inflammatory foci.
[d] Number (n = 7) of mice studied.
[e] Number (n = 4) of mice studied.

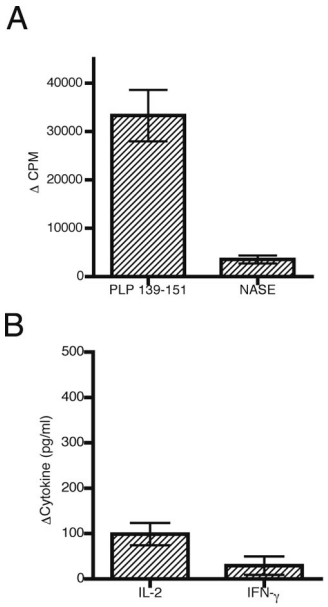

FIGURE 2. Endogenous $PLP_{139-151}$-reactive cells are not differentiated in vivo. *A*, LNCs were harvested from naive SJL mice and tested in triplicate for reactivity to $PLP_{139-151}$ and the control Ag, NASE, over a dose response of 0.1–100 μg/ml peptide. [^{3}H]Thymidine was added at 48 h and plates were harvested 16 h later. Data are shown as mean Δcpm of triplicate wells at 100 μg/ml peptide in which Δcpm = [mean cpm in test wells] − [mean cpm in wells with medium only]. Values shown correspond to activation with 100 μg/ml $PLP_{139-151}$ or NASE peptide. *B*, Supernatants were collected 40 h after activation, and cytokine concentration was determined by specific ELISA. Only values above the limit of detection of the ELISA are shown. Values shown correspond to activation with 100 μg/ml $PLP_{139-151}$. No specific cytokine production was observed in response to NASE or at lower concentrations of $PLP_{139-151}$ and no significant production of IL-4, IL-10, or TNF-α was detected (data not shown). An average of 15 independent experiments is shown.

not available to activate and differentiate them. However, experiments indicating that PLP is expressed outside the CNS make this less likely (31–33). Given that the Ag is available, that these cells are poised to divide, and that they are capable of becoming pathogenic after activation ex vivo (Table I), there may be additional regulatory mechanisms that keep them in check.

One possibility is that regulatory cells and/or immunoregulatory cytokines actively control this repertoire. To address the role of immunoregulatory cytokines, we bred IL-4 and IL-10 deficiency onto the SJL background and analyzed endogenous $PLP_{139-151}$-reactivity in these mice. We found that endogenous $PLP_{139-151}$-reactivity is present in both IL-$4^{-/-}$and IL-$10^{-/-}$ SJL mice (Fig. 3). Interestingly, half of the IL-$10^{-/-}$ SJL mice examined exhibited greater proliferative responses to $PLP_{139-151}$ compared with control and IL-$4^{-/-}$ SJL mice. This suggested that there may be a higher frequency of endogenous $PLP_{139-151}$-reactive cells in IL-$10^{-/-}$ SJL mice or that the absence of IL-10 allows for responding cells to exhibit a greater proliferative potential. To confirm this, we measured directly the frequency of the endogenous $PLP_{139-151}$-reactive repertoire in IL-$10^{-/-}$ SJL mice, IL-$4^{-/-}$ SJL mice and wild-type littermate control mice using a $PLP_{139-151}$/I-A^s tetramer that we have recently generated (26). A $TMEV_{70-86}$/I-A^s tetramer was used as a control. We used three-color flow cytometric analysis for tetramers, CD4 and 7-AAD to determine the frequency of tetramer-positive cells in the live $CD4^+$ population. In the naive repertoire of IL-$10^{-/-}$ SJL mice, the frequency of $PLP_{139-151}$ tetramer-positive cells was significantly higher (0.53% of CD4) when compared with negative control tetramers (0.28% of CD4) and such differences were not observed in either littermate controls or IL-$4^{-/-}$ SJL mice (data not shown). These results supported the hypothesis that the endogenous repertoire was expanded in IL-$10^{-/-}$ SJL mice. To test this further, we immunized groups of IL-$10^{-/-}$ SJL mice and wild-type littermate controls with 100 μg of $PLP_{139-151}$ in CFA. After 10 days, the mice were sacrificed and LNCs were restimulated with 20 μg/ml $PLP_{139-151}$ for 6 days. In the cultures derived from IL-$10^{-/-}$ SJL mice, the frequency of $PLP_{139-151}$ tetramer-positive cells was significantly higher than their littermate controls, as demonstrated by tetramer staining on

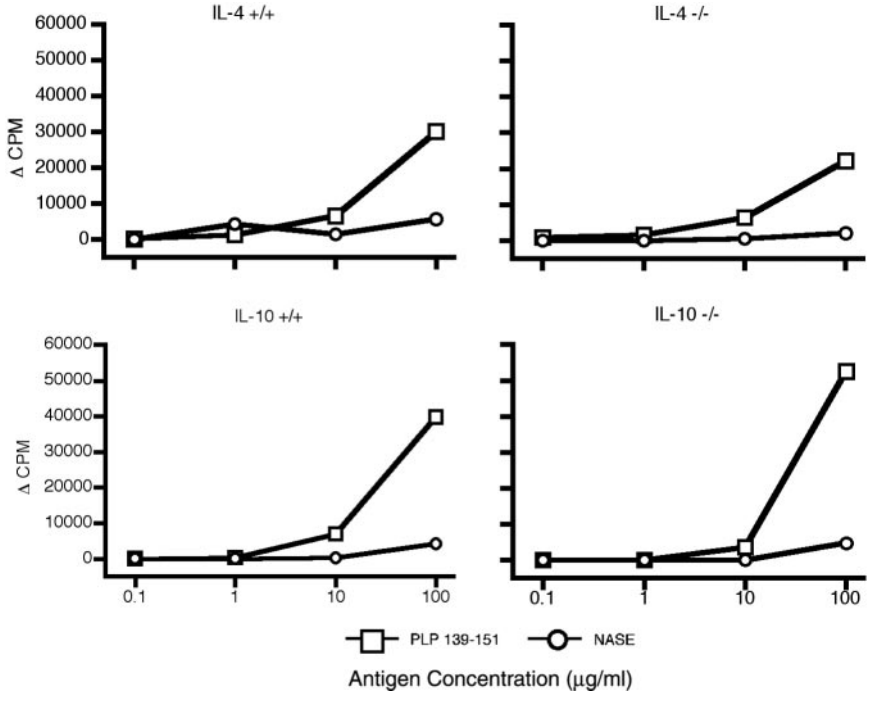

FIGURE 3. Endogenous $PLP_{139-151}$-reactivity in IL-$4^{-/-}$ and IL-$10^{-/-}$ SJL mice. LNCs were harvested from naive IL-$4^{-/-}$ and IL-$10^{-/-}$ SJL mice and tested in triplicate for reactivity to $PLP_{139-151}$ and the control Ag, NASE, over a dose response of 0.1–100 μg/ml peptide. [^{3}H]Thymidine was added at 48 h and plates were harvested 16 h later. Data are shown as mean Δcpm of triplicate wells in which Δcpm = [mean cpm in test wells] − [mean cpm in wells with medium only]. This assay was repeated four times.

day 4 and day 6 postactivation with $PLP_{139-151}$ (Fig. 4 and data not shown). The staining with $TMEV_{70-86}$ tetramers was similar in IL-$10^{-/-}$ SJL mice and wild-type littermate controls, demonstrating that this effect was specific to the endogenous $PLP_{139-151}$ reactive repertoire.

We then determined whether the PLP-reactive cells from IL-$4^{-/-}$ SJL mice and IL-$10^{-/-}$ SJL mice differ in their production of effector cytokines (Fig. 5). We found that the endogenous $PLP_{139-151}$-reactive cells in IL-$4^{-/-}$ SJL mice, like those in wild-type SJL mice, do not produce any IL-4, IL-10, IFN-γ, or TNF-α. Although we were unable to detect any IL-2 from these cells, it is possible that a small amount of IL-2 was produced and consumed by proliferating cells. In contrast, the endogenous $PLP_{139-151}$-reactive cells from IL-$10^{-/-}$ SJL mice produced levels of IL-2 comparable to those observed in wild-type littermates and significant amounts of IFN-γ (Fig. 5). No IL-4, IL-10 or TNF-α was detected from these cells. Therefore it appears that the endogenous $PLP_{139-151}$-reactive cells in IL-$10^{-/-}$ SJL mice have expanded and at least some cells have acquired a Th1 effector phenotype, raising the possibility that these mice may have a lower threshold for disease induction. However, none of the IL-$10^{-/-}$ SJL mice developed spontaneous EAE. We hypothesized that one reason for this is that the endogenous $PLP_{139-151}$ reactive T cells from IL-$10^{-/-}$ SJL mice do not get access to the CNS.

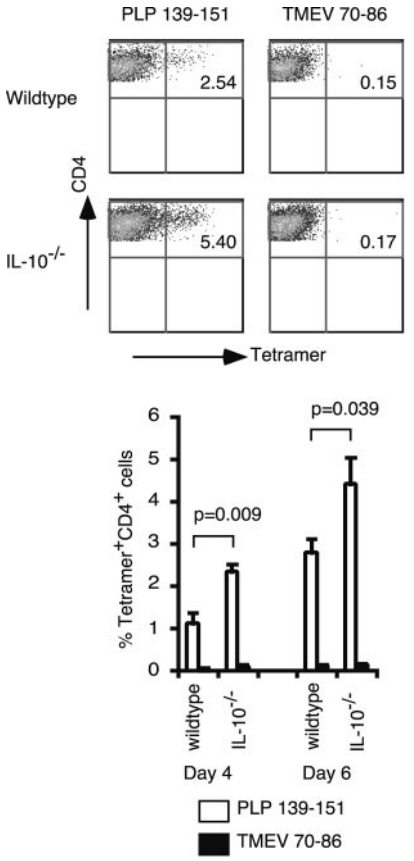

FIGURE 4. Precursor frequency of $PLP_{139-151}$-reactive CD4 T cells in IL-$10^{-/-}$ and their littermate controls. The mice deficient for IL-10 and their littermate controls were immunized with $PLP_{139-151}$ in CFA. Ten days later, LNCs were restimulated with $PLP_{139-151}$ for 4–6 days. Viable lymphoblasts were incubated with $I\text{-}A^s$ tetramers ($PLP_{139-151}$ or $TMEV_{70-86}$) for 3–4 h at room temperature followed by staining with 7-AAD and anti-CD4 Ab. Data were acquired by using flow cytometer and the percentage of tetramer-positive cells were determined in the live CD4 subset. Error bar represents mean ± SEM values for each group ($n = 3$).

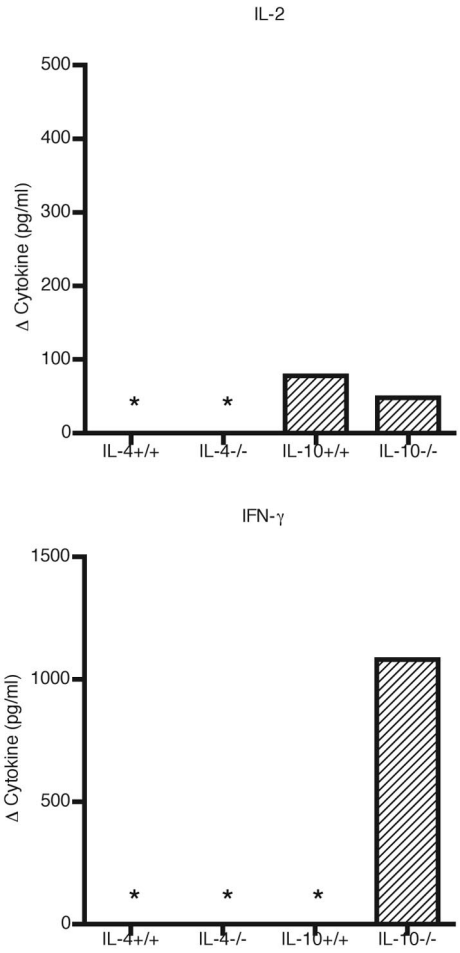

FIGURE 5. Cytokine production of endogenous $PLP_{139-151}$-reactive cells in IL-$4^{-/-}$ and IL-$10^{-/-}$ SJL mice. LNCs were harvested from naive IL-$4^{-/-}$ and IL-$10^{-/-}$ SJL mice and tested in triplicate for reactivity to $PLP_{139-151}$ and the control Ag, NASE, over a dose response of 0.1–100 μg/ml peptide. Supernatants were collected 40 h after activation, and cytokine concentration was determined by specific ELISA. Only values above the limit of detection of the ELISA are shown. Values shown correspond to activation with 100 μg/ml $PLP_{139-151}$ peptide. No specific cytokine production was observed in response to NASE or at lower concentrations of $PLP_{139-151}$. *, Undetectable data.

To test this hypothesis, we administered PTX to IL-$10^{-/-}$ SJL mice and wild-type littermate controls to allow access to the CNS because PTX has been shown to open the blood brain barrier (34) and to have adjuvant like effects on the immune system (35). Furthermore, to determine whether disease is induced by the endogenous $PLP_{139-151}$ repertoire in IL-$10^{-/-}$ SJL, we tolerized mice by injection of high-dose aqueous Ag ($PLP_{139-151}$, $PLP_{178-191}$, or PBS). IL-$10^{-/-}$ SJL mice tolerized with PBS and injected with PTX developed atypical clinical signs characterized by weight loss and hind limb weakness, which did not progress into progressive paralysis. Two of five PBS treated IL-$10^{-/-}$ SJL mice had small numbers of typical mononuclear cell infiltrates located predominantly in the meninges, providing one explanation for the atypical manifestation of disease (Table II). In contrast, none of the wild-type SJL mice injected with PTX developed any signs of disease or lesions. Importantly, none of the IL-$10^{-/-}$ or wild-type SJL mice tolerized with $PLP_{139-151}$ developed lesions. Interestingly, three of eight IL-$10^{-/-}$ SJL mice tolerized with $PLP_{178-191}$ developed classical signs of EAE with lesions both in the meninges and parenchyma. Although the reasons for this are not clear, it is

Table II. *Induction of atypical EAE in IL-10$^{-/-}$ SJL mice by PTX*

Mouse	Tolerization	Classical EAE Incidence[a]	Mean No. of Mice with CNS Lesions[b]	
			Meninges	Parenchyma
SJL wild-type	PBS	0/4	0	0
	PLP 139–151	0/6	0	0
	PLP 178–191	0/7	0	0
SJL IL-10$^{-/-}$[c]	PBS	0/5	7.5[d]	2[e]
	PLP 139–151	0/7	0	0
	PLP 178–191	3/8	26.5 ± 17.7[f]	27.3 ± 22.1[f]

[a] A score of 1 or higher for clinical signs after i.v. PTX.
[b] Mice with five or more lesions.
[c] Significantly more lesions in SJL IL-10$^{-/-}$ mice compared with SJL wild-type mice ($p = 0.22$; Fisher's exact test). Mean values are ± SE.
[d] Number of mice ($n = 2$) with lesions in the meninges.
[e] Number of mice ($n = 1$) with lesions in the parenchyma.
[f] Number of mice ($n = 4$) with lesions in meninges or parenchyma.

possible that tolerizing with $PLP_{178-191}$ potentiates the ability of the endogenous $PLP_{139-151}$-reactive repertoire to induce disease in IL-10$^{-/-}$ SJL mice. Another possibility is that treatment with $PLP_{178-191}$ is immunogenic rather than tolerogenic in the absence of IL-10. Finally, IL-10$^{-/-}$ SJL mice developed significantly more lesions overall than wild-type SJL mice ($p = 0.022$). Taken together these findings support the notion that the endogenous $PLP_{139-151}$-reactive repertoire is poised for disease induction and that IL-10 is an important regulator of this repertoire.

Discussion

The role of IL-10 has been studied extensively in the development of EAE. Although IL-10 is an important determinant of disease outcome, the effects of IL-10 on regulating the size and effector phenotype of the naive autoreactive T cell repertoire have never been determined. The presence of a very high frequency (1/20,000) of T cells reactive to the encephalitogenic epitope of myelin proteolipid protein, $PLP_{139-151}$, in naive SJL mice (1) provides us with a unique model with which to address this question.

In this study we demonstrate that the endogenous $PLP_{139-151}$-reactive cells in naive SJL mice recognize the $PLP_{139-151}$ peptide in the same manner as encephalitogenic Th1 clones suggesting that they encompass the precursors of encephalitogenic cells. However, these cells are not autoaggressive in vivo, providing one explanation for the lack of spontaneous disease in SJL mice. Most importantly, we show that the differentiation state of these cells is affected by the lack of IL-10, and not IL-4, and that their frequency is increased in IL-10$^{-/-}$ SJL mice. This was shown by a direct examination of the frequency of $PLP_{139-151}$-specific precursors in naive mice and their expansion in response to Ag. Based on these data, we speculate that IL-10, besides acting as an immunoregulatory cytokine, may play an important role in controlling the expansion of self-reactive cells in the naive repertoire. It remains to be determined how IL-10 may perform this function. Thus, the absence of IL-10 may predispose mice to develop a dysregulated autoreactive repertoire. Our data showing that $PLP_{139-151}$ reactive cells are partially differentiated in vivo in IL-10$^{-/-}$ SJL mice and the occurrence of spontaneous inflammatory bowel disease in IL-10$^{-/-}$ C57BL/6 mice (24) are consistent with this view.

Our data showing the expansion and differentiation of the endogenous $PLP_{139-151}$ reactive repertoire in IL-10$^{-/-}$ SJL mice prompted us to examine the sensitivity of IL-10$^{-/-}$ SJL mice to the induction of EAE. Although these mice do not develop EAE spontaneously at a detectable frequency in our colony, they displayed an atypical form of EAE following injection of PTX, suggesting a lower threshold for disease induction. Taken together, our data support the importance of IL-10 over IL-4 in regulating EAE, and further suggest that IL-10 plays a key role not only in the amelioration of actively induced EAE but also in the homeostatic regulation of the naive autoreactive T cell repertoire.

Involvement of IL-10 in the homeostatic regulation of the endogenous $PLP_{139-151}$-reactive repertoire raises the possibility that a naturally occurring IL-10 secreting population probably regulates this repertoire in vivo. It is possible that IL-10 secreting cells such as Tr1 cells (36) regulate this repertoire; however, Tr1 cells are generated after Ag exposure and have not yet been reported to exist in the naive state. Another possibility is that an IL-10 secreting macrophage-dendritic cell population regulates this repertoire (37).

The fact that we have not observed any spontaneous clinical neurological disease in IL-10$^{-/-}$ SJL mice thus far suggests that other regulatory factors and cells may act to keep these cells in check. Other cell populations that have been proposed to play a role in regulating autoimmunity and EAE are $CD4^+CD25^+$ T cells (38) and $CD8^+$T cells (39–41). It will be interesting to determine whether $CD4^+CD25^+$ regulatory cells are operational in SJL-IL-10$^{-/-}$ mice and whether manipulating any of these cell populations will have an affect on the endogenous $PLP_{139-151}$-reactive repertoire and the development of spontaneous CNS autoimmunity in SJL mice.

Myelin-reactive T cells can be readily detected in the peripheral blood of normal healthy humans (2–4). Likewise, $PLP_{139-151}$-reactive cells can be readily detected in naive SJL mice. Thus identifying factors that regulate the activation and differentiation of the endogenous $PLP_{139-151}$-reactive T cells in naive SJL mice provide us with a unique opportunity to gain insight into the factors that regulate the activation of the myelin-reactive T cells in apparently normal humans who develop multiple sclerosis. The data presented in this study point to IL-10 as one of the factors that may play a role in the predisposition of autoimmune prone individuals to multiple sclerosis.

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