The Journal of Immunology

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J Immunol 1998; 161:3299-3306; ;
http://www.jimmunol.org/content/161/7/3299

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IL-10 Is Critical in the Regulation of Autoimmune Encephalomyelitis as Demonstrated by Studies of IL-10- and IL-4-Deficient and Transgenic Mice[1]

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Experimental autoimmune encephalomyelitis (EAE) and other organ-specific autoimmune diseases are induced by autoantigen-specific Th1 cells. In contrast, transfer of autoantigen-reactive Th2 cells that produce IL-4 and IL-10 can prevent and/or reverse EAE. The relative roles of these two Th2 cytokines in the regulation of EAE has not been evaluated. Utilizing IL-4 and IL-10 knockout mice deficient for these cytokines and IL-10 and IL-4 transgenic mice overexpressing these cytokines, we demonstrate that IL-10-deficient mice (IL-10$^{-/-}$) are more susceptible and develop a more severe EAE when compared with IL-4-deficient mice (IL-4$^{-/-}$) or wild-type mice. T cells from IL-10$^{-/-}$ mice exhibit a stronger Ag-specific proliferation, produce more proinflammatory cytokines (IFN-γ and TNF-α) when stimulated with an encephalitogenic peptide, and induce very severe EAE upon transfer into wild-type mice. In contrast, while IL-4 transgenic mice develop similar disease compared with their nontransgenic littermates, mice transgenic for IL-10 are completely resistant to the development of EAE. Taken together, our data suggest that IL-10 plays a more critical role in the regulation of EAE by regulating autopathogenic Th1 responses. ***The Journal of Immunology,*** **1998, 161: 3299–3306.**

Experimental autoimmune encephalomyelitis (EAE)[4] is an animal model of the human disease multiple sclerosis, which can be induced by immunization with self Ags derived from central nervous system (CNS) myelin. The disease is characterized by the generation of autoreactive T cells that traffic to the brain and spinal cord and initiate injury to CNS myelin, resulting in a chronic or relapsing-remitting paralysis. Direct evidence for the role of CD4$^+$ T cells in EAE induction has come from adoptive transfer studies, in which myelin Ag-reactive CD4$^+$ T cell lines or clones were shown to induce encephalomyelitis and paralysis following transfer (1).

Subpopulations of CD4$^+$ Th cells produce distinct patterns of cytokines; this has led to the concept of functional heterogeneity among Th cells (2). Th1-type cells produce IL-2 and/or IFN-γ and TNF-β, elicit delayed-type hypersensitivity responses, and activate macrophages. Th2-type cells, on the other hand, produce IL-4, IL-5, and IL-10, are especially important for IgE production and eosinophilic inflammation, and may suppress cell-mediated immunity. These two Th cell populations cross-regulate one another because their respective cytokines act antagonistically. IL-4 and IFN-γ show reciprocal inhibition, and IL-10 inhibits the production of IFN-γ and other Th1 cytokines by interfering with Ag presentation and activation of macrophages (3). Conversely, IL-4 causes Th2 differentiation and inhibits the development of IFN-γ-secreting cells (4). Cytokines play a pivotal role in the initiation, propagation, and regulation of tissue-specific autoimmune injury. Cellular and cytokine changes in the CNS have been described in several studies of myelin basic protein-induced EAE. Th1 cytokines are present in inflammatory EAE lesions in the CNS, whereas Th2 cytokines are absent, strongly suggesting that Th1 cytokines play a role in the pathogenesis of the disease (5). Conversely, recovery from EAE in mice and rats is associated with an increase in the presence of Th2 cytokines and TGF-β in the CNS (6). In other studies, examination of brains and spinal cords from diseased animals revealed the presence of mRNA for IFN-γ and TNF-α during clinical episodes, whereas mRNA for IL-10 appeared at the time of clinical remissions (7, 8). These findings, along with the observation that Th2 cytokines can inhibit the actions of inflammatory Th1 cytokines, suggest that the induction and activation of Th2 cells may potentially prevent EAE and other autoimmune diseases mediated by Th1 cells. In support of this hypothesis, Racke et al. have shown that IL-4-induced immune deviation can be used as a therapy in EAE (9). However, IL-4-deficient mice did not show any increased susceptibility to EAE, suggesting that the role of this Th2 cytokine in the regulation of EAE is complex (10).

To date, most encephalitogenic clones examined have been Thl cells (11, 12), although a recent report suggested that in immunocompromised animals myelin Ag-reactive Th2 cells may also induce EAE (13). We previously reported that proteolipid protein (PLP)-specific IL-4- and IL-10-producing Th2 clones could inhibit EAE if they were given at the time of immunization, and reverse

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Received for publication March 5, 1998. Accepted for publication May 29, 1998.

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[1] This work was supported by grants from the National Multiple Sclerosis Society, New York (RG2571A2 and RG230B3) and the National Institutes of Health (R01NS30843, R01NS35685, PO1AI39671-01A1) to V.K.K. and NS 26773 to R.A.S. E.B. is a Fellow of the Ministère de la Recherche et de la Technologie (MRT).

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[4] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; ELISPOT, enzyme-linked immunospot; MOG, myelin oligodendrocyte glycoprotein; IL-10$^{-/-}$, IL-10-deficient; IL-4$^{-/-}$, IL-4-deficient; LN, lymph node; CNS, central nervous system; PLP, proteolipid protein; WT, wild type.

0022-1767/98/$02.00

disease if given at the first signs of EAE (14). Based on these data, we suggested that Th2 cells may function as regulators of EAE, and the predominant and preferential production of Th2 cytokines in response to autoantigen may confer resistance to autoimmune diseases. In support of this hypothesis are recently published results in which myelin Ag-reactive T cells genetically transduced with a retroviral plasmid containing the IL-4 gene or T cells transfected with IL-10 cDNA could prevent and/or reverse EAE (15, 16). However, direct administration of Th2 cytokines or anti-Th2 cytokine-blocking Abs to animals has produced conflicting effects on the clinical course of EAE (9, 17, 18). To address the issue of which of the two Th2 cytokines (IL-4 or IL-10) plays a more critical role in the disease process, we used IL-4- and IL-10-deficient (IL-$4^{-/-}$ and IL-$10^{-/-}$) mice and transgenic mice overexpressing these cytokines. The results presented in this study demonstrate that IL-10-deficient mice develop a more severe clinical EAE than IL-4-deficient or wild-type (WT) mice and that IL-10-overexpressing transgenic mice are resistant to the development of disease, suggesting that IL-10 plays a more critical role in the regulation of EAE.

Materials and Methods

Mice

Female C57BL/6 and IL-4- and IL-10-deficient mice on the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). The generation of IL-4- and IL-10-deficient mice used in the present study has been described previously. These mice have been backcrossed for >10 generations on the C57BL/6 background (19, 20). Transgenic mice on the FVB background (H-2^q), which express IL-4 and IL-10 in T cells, were obtained from Dr. Robert Tepper (Department of Genetics, Harvard Medical School and Massachusetts General Hospital, Boston). The IL-4 T cell transgenic mice (UD) have been described (21). Although homozygous IL-4 transgenic mice have altered T cell development, the heterozygous mice used in the present study have normal T cell development. For the generation of IL-10 transgenic mice (UR), IL-10 cDNA was cloned under the CD_2 promoter (22). Activation of T cells from IL-10 transgenic mice leads to the production of significantly high amounts of IL-10. The transgenic mice were crossed with SJL, and the (SJL × FVB)F_1 mice were used for the experiments described in this study. All mice were used at 6 to 7 wk of age.

Antigens

Bovine myelin PLP was prepared from a washed total lipid extract of bovine white matter (23). The protein was extracted by the chloroform-methanol method, converted to an aqueous phase, dialyzed overnight against double distilled water, and then used immediately. Peptides used in this study include myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (MEVGWYRSPFSRVVHLYRNGK), or as control, MOG_{92-106} peptide (DEGGYTCFFRDHSYQ). The peptides were synthesized by Dr. David Teplow at the Biopolymer Facility, Center for Neurologic Diseases, on an Applied Biosystems 430A peptide synthesizer (Foster City, CA) using F-moc chemistry.

Proliferative response

Lymph node (LN) cells of mice immunized with 100 μg of MOG_{35-55} in CFA (Difco Laboratories, Detroit, MI) were removed 10 days after immunization and cultured for 72 h in 96-well plates in HL-1 medium (BioWhittaker, Walkersville, MD) in the presence of various concentrations of peptide. They were pulsed with 1 μCi of [^{3}H]thymidine for the last 16 to 18 h, and the mean incorporation of thymidine in insoluble DNA in the triplicate wells was determined in a scintillation counter (model LS 5000; Beckman Instruments, Fullerton, CA).

Cytokine ELISA and ELISPOT

LN cells derived from immunized mice were activated in vitro with the peptide. Culture supernatants were harvested at 24 or 48 h, and the ELISAs were performed as described previously (14). Briefly, 96-well plates were coated overnight with the capture Ab (1 μg/ml) specific for a particular cytokine. The plates were washed and incubated with a blocking solution (Kirkegaard and Perry, Gaithersburg, MD). Culture supernatants and standards were incubated overnight at 4°C. The plates were then washed and incubated with biotinylated anti-cytokine-detecting mAb (1 μg/ml) for 1 h. The plates were developed by adding avidin peroxidase and its substrate. The Ab pairs used were: IL-10, JES5-2A5 and SXC-1; IL-5, TRFK5 and TRFK4; IFN-γ, R4-6A2 and XMG1.2; TNF-α, MP6-XT22 and Rbt a-Ms/Rt TNF-α (all from PharMingen, San Diego, CA).

For detection of cytokine by ELISPOT, LN cells derived from mice immunized with MOG_{35-55} were stimulated for 8 h with the peptide. Cells (8×10^4) were then incubated overnight at 37°C in a sterile nitrocellulose-based 96-well microplate (Millipore, Bedford, MA) coated with the capture Ab. The development of ELISPOT was performed as for ELISA except that the spots were revealed by avidin-alkaline phosphatase and its substrates (Sigma, St. Louis, MO, and Life Technologies, Gaithersburg, MD). The number of spots for each cytokine was counted under a dissecting microscope and expressed as the number of positive spots/10^6 cells.

Preparation and maintenance of T cell lines

C57BL/6, IL-$4^{-/-}$, and IL-$10^{-/-}$ mice were immunized s.c. in the flank with 100 μg of MOG_{35-55} in CFA. LN cells were removed 10 days after immunization and cultured with MOG_{35-55} in complete RPMI 1640 medium (L-glutamine, 5×10^{-5} M β-mercaptoethanol, 100 U/μg penicillin/streptomycin) (BioWhittaker) supplemented with 10% FCS. The cells were subsequently stimulated with syngenic spleen cells as APCs and MOG_{35-55} every 10 to 15 days and maintained in 10% FCS complete RPMI supplemented with 0.6% T-stim (Collaborative Biomedical Products, Bedford, MA).

Induction and assessment of EAE

By active immunization. C57BL/6, IL-$10^{-/-}$, and IL-$4^{-/-}$ mice were injected s.c. in the flank with an emulsion containing 200 μg of the peptide MOG_{35-55}, which is the encephalitogenic epitope in C57BL/6 (H-2^b) mice (24) and CFA supplemented with 400 μg of *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories). In some experiments, mice also received 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) i.v. on day 0 and day 2. IL-10 and IL-4 transgenic mice and (FVB × SJL)F_1 nontransgenic mice were injected as described above with 100 μg of the whole PLP, which induces potent EAE in SJL mice (25). Although MOG_{35-55} peptide can induce very potent disease in B6 mice, this encephalitogenic peptide does not induce disease in the (FVB × SJL)F_1 (H-2^{qxs}) mice; therefore, whole PLP was used for inducing disease in the F_1 mice.

By adoptive transfer of T cell lines. T cell lines derived from C57BL/6 WT, IL-$10^{-/-}$, and IL-$4^{-/-}$ mice were stimulated with 20 μg/ml of MOG_{35-55}. Four days later, cells were washed and resuspended in PBS, then, 10^7 cells were injected i.v. into C57BL/6 WT mice. Where indicated, mice also received an injection of 200 ng of pertussis toxin immediately after cell transfer and 2 days later.

Mice were observed daily and assessed for clinical signs of disease according to the following criteria: 0, no disease; 1, limp tail; 2, hind leg weakness or partial paralysis; 3, complete hind leg paralysis; 4, front and hind limb paralysis; 5, moribund state. Mean clinical score was calculated as follows: individual score were added and divided by the total number of mice in each group for each day of observation; this included the animals that did not develop any disease. Animals were sacrificed at the termination of the experiment (experiment with pertussis toxin) or at the peak of disease (experiment without pertussis toxin). Brains and spinal cords were removed and fixed in 10% formalin. Paraffin-embedded sections were stained with Luxol fast blue-hematoxylin and eosin for light microscopy. Inflammatory foci were counted in meninges and parenchyma as described previously (26).

Results

IL-$10^{-/-}$ mice develop more severe disease than IL-$4^{-/-}$ mice

To investigate the relative roles of IL-10 and IL-4 in the development of EAE, we immunized IL-$10^{-/-}$ and IL-$4^{-/-}$ mice with the encephalitogenic MOG_{35-55} peptide in CFA plus pertussis toxin. We first compared EAE development in IL-$10^{-/-}$ and C57BL/6 WT mice. As shown in Figure 1*A* and Table I, IL-$10^{-/-}$ mice were highly susceptible to disease in that all these mice developed EAE and the disease was significantly more severe than in the control WT mice. In this experiment, WT mice showed a complete remission of disease after the first acute episode, but the IL-$10^{-/-}$ mice developed a chronic and persistent disease with no evidence of remission. The severe and persistent disease observed in mice lacking IL-10 suggests that IL-10 is important for limiting EAE

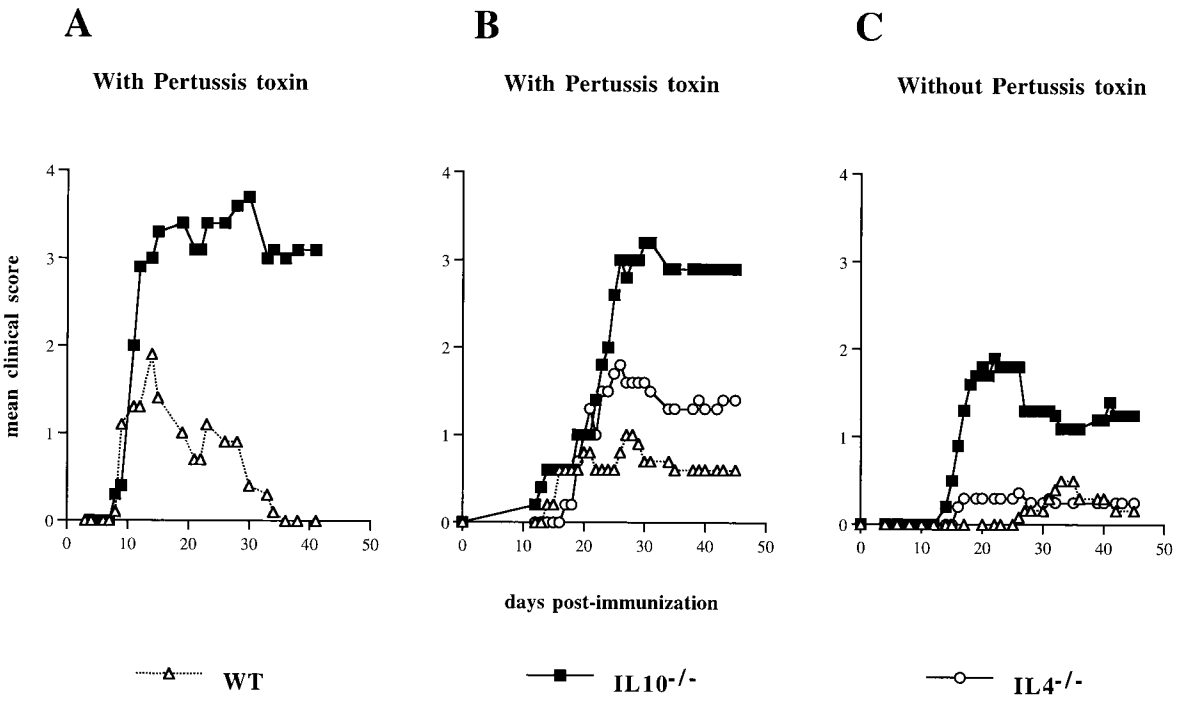

FIGURE 1. Clinical course of EAE in C57BL/6 WT, IL-10$^{-/-}$, and IL-4$^{-/-}$ mice. Groups of IL-10$^{-/-}$, IL-4$^{-/-}$, and WT mice were immunized with 200 μg of MOG_{35-55} in the flanks and were either i.v. injected with pertussis toxin on the day of immunization and 2 days later (*A* and *B*) or not injected with pertussis toxin (*C*) following immunization. The data are presented as the mean disease score in each group during the course of observation.

progression. We repeated the experiment with the addition of IL-4$^{-/-}$ mice. The IL-4$^{-/-}$ mice developed more severe disease than WT mice (Fig. 1*B* and Table I). However, there was no significant difference in the incidence of disease between these two groups (Table I). IL-10$^{-/-}$ mice developed more severe disease than IL-4$^{-/-}$ and WT mice (Fig. 1*B* and Table I). In these experiments, pertussis toxin was used as an adjuvant in the induction of EAE. To further test the differences in susceptibility between the groups, mice were immunized with the encephalitogenic peptide alone, without the toxin (Fig. 1*C* and Table I). With this protocol, the majority (92%) of IL-10$^{-/-}$ mice developed EAE with a mean clinical score of 2.7. In contrast, only 17% of either WT or IL-4$^{-/-}$ mice developed EAE. The two mice in the WT group that developed EAE also showed a very late onset of disease. Histopathologic analysis of the brains and spinal cords of these mice was performed (Table I). We did not see any differences in the number of inflammatory foci between groups of mice immunized with MOG_{35-55} plus pertussis toxin. However, brains and spinal cords from IL-10$^{-/-}$ mice immunized with the peptide without addition of toxin showed higher numbers of inflammatory foci compared with WT or IL-4$^{-/-}$ mice. The greater susceptibility and severity of disease in IL-10$^{-/-}$ mice suggests that IL-10 probably plays a central role in the regulation of EAE.

Proliferative response and cytokine production in response to the encephalitogenic peptide in IL-4- and IL-10-deficient mice

To study the mechanism, we tested whether the absence of IL-10 affected the induction and expansion of MOG_{35-55}-specific T cell responses. We immunized groups of IL-10$^{-/-}$, IL-4$^{-/-}$, and WT mice with MOG_{35-55}, and 10 days later we measured the proliferative responses of the LN cells to the immunizing MOG peptide and control MOG_{92-106} peptide. As shown in Figure 2*A*, LN cells derived from WT and IL-4$^{-/-}$ mice showed similar proliferation

Table I. *EAE in IL-10- and IL-4-deficient mice*

	Clinical Disease[a]			Histologic Lesions[a]	
	Incidence	Day of onset	Mean maximal score[b]	Incidence	Mean No. of foci in mice
With Pertussis toxin[c]					
C57BL/6 WT	15/18 (83%)	11.9 ± 1.3	2.3 ± 0.1	14/16	55.8 ± 17.0
C57BL/6 IL-10$^{-/-}$	18/18 (100%)	13.2 ± 1.1	3.7 ± 0.2[d]	14/14	37.5 ± 9.3
C57BL/6 IL4$^{-/-}$	9/11 (82%)	15.3 ± 1.3	2.9 ± 0.4	7/8	22.0 ± 5.7
Without Pertussis toxin[e]					
C57BL/6 WT	2/12 (17%)	28.5 ± 2.5	1.5 ± 0.5	0/6	0 ± 0
C57BL/6 IL-10$^{-/-}$	11/12 (92%)[f]	20.6 ± 2.5	2.7 ± 0.3	3/5	89.3 ± 5.2
C57BL/6 IL-4$^{-/-}$	2/12 (17%)	20 ± 4.0	2 ± 0.0	1/6	6 ± 0

[a] Data are presented as mean ± SE.
[b] Data are presented as mean maximal clinical scores for the animals showing clinical disease.
[c] IL-10$^{-/-}$, IL-4$^{-/-}$, and WT mice were injected with 200 μg MOG_{35-55} in CFA plus 200 ng of pertussis toxin on day 0 and on day 2.
[d] Statistically significant when compared with WT mice; $p < 0.001$ by Wilcoxon rank sum test.
[e] Mice were injected with 200 μg MOG_{35-55} in CFA without pertussis toxin.
[f] Statistically significant when compared with WT and IL-4$^{-/-}$ mice; $p < 0.001$ by Fisher's exact test.

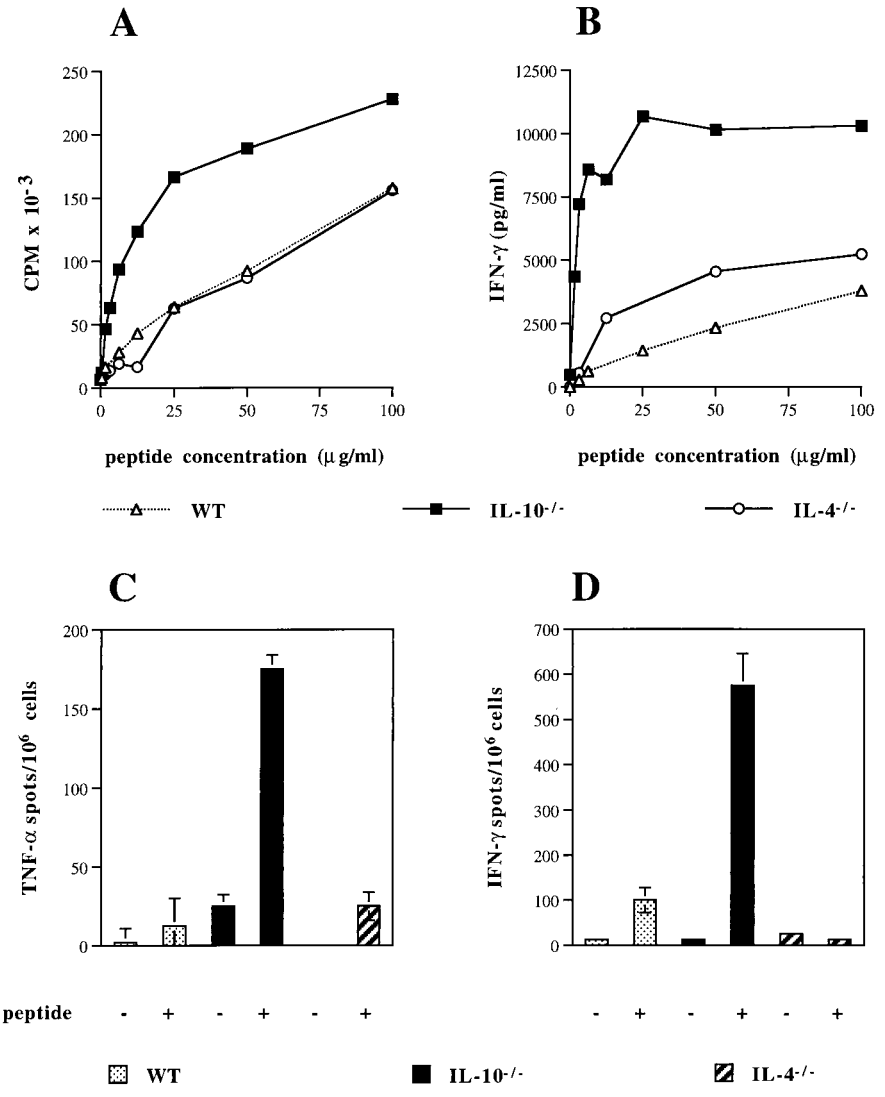

FIGURE 2. *A* and *B*, Proliferation and cytokine production of LN cells from C57BL/6 IL-$10^{-/-}$, IL-$4^{-/-}$, and WT mice immunized with $MOG_{35–55}$. IL-$10^{-/-}$, IL-$4^{-/-}$, and WT mice were immunized with $MOG_{35–55}$. LN cells were harvested 10 days after immunization and were activated with different concentrations of peptide. Proliferation was measured in a 72-h activation assay by [^{3}H]TdR incorporation and presented as mean cpm from triplicate wells. The mean cpm in the presence of 50 μg/ml of the control peptide $MOG_{92–106}$ were 3490 for WT LN cells, 6930 for IL-$4^{-/-}$ LN cells, and 4370 for IL-$10^{-/-}$ LN cells. Levels of IFN-γ were determined by ELISA in 48-h culture supernatants of the LN cells following Ag-specific activation. One representative experiment of four is shown. *C* and *D*, Enumeration of cytokine-producing cells by ELISPOT. The number of IFN-γ- and TNF-α-secreting cells in LN cells from mice immunized with $MOG_{35–55}$ was measured by ELISPOT assay. Then, 5×10^4 cells/well were cultured for 6 h with the peptide or with medium alone. Results are expressed as mean $\pm$ SD of cytokine-positive spots/10^6 cells in duplicate wells. Two independent experiments gave similar results.

to the peptide. By contrast, equal numbers of LN cells from IL-$10^{-/-}$ mice showed significant proliferation at lower doses of the peptide, and proliferation was higher at most of the peptide doses tested. We also analyzed the expression of the CD62L marker—an L-selectin expressed on all leukocytes, which is down-regulated on T lymphocytes upon activation (27)—on the surface of LN and spleen cells derived from mice 6 days following immunization with $MOG_{35–55}$. We found that 59% of LN $CD4^+$ T cells and 71% of splenic $CD4^+$ T cells in IL-$10^{-/-}$ mice have down-regulated the CD62L marker compared with 21% of LN and 23% splenic $CD4^+$ T cells in WT mice; and 23% of LN and 37% of splenic $CD4^+$ T cells in IL-$4^{-/-}$ mice (data not shown). These data suggest that in the absence of IL-10, relatively more T cells become activated and proliferate in response to $MOG_{35–55}$.

Since the proinflammatory cytokines are associated with EAE, we tested the supernatants of LN cells for the production of IFN-γ and TNF-α cytokines and the anti-inflammatory cytokines (IL-4 and IL-10) 48 h after stimulation with different dosages of $MOG_{35–55}$. As shown in Figure 2*B*, cells derived from IL-$4^{-/-}$ and WT mice produced much lower levels of IFN-γ compared with LN cells derived from IL-$10^{-/-}$ mice. The supernatants from the LN cells did not show detectable production of IL-2 or of the anti-inflammatory cytokines IL-4 and IL-10 (data not shown). To determine whether the increased cytokine production was due to an increased expansion of Ag-specific cells, LN cells from mice immunized with $MOG_{35–55}$ were harvested and stimulated in vitro with the peptide for 6 h, then tested for IFN-γ and TNF-α production by individual cells using the ELISPOT technique. The numbers of cells producing TNF-α and IFN-γ were very high in IL-$10^{-/-}$ cells 6 h after stimulation with the peptide, whereas fewer IL-$4^{-/-}$ and WT cells secreted these cytokines (Fig. 2, *C* and *D*). These data indicate that the frequency of IFN-γ- and TNF-α-producing cells is increased by encephalitogenic Ag stimulation in IL-$10^{-/-}$ mice.

To differentiate whether the cytokines were produced by T cells or other cells in the unseparated LN cells, short term $MOG_{35–55}$ T cell lines were generated and reactivated with plate-bound anti-CD3 mAb. We found that, in addition to IFN-γ, T cells derived from WT mice secreted large quantities of IL-10 and IL-5, indicating their ability to produce Th2 cytokines in addition to Th1 cytokines (Fig. 3, *A* and *B*). By contrast, T cells from IL-$4^{-/-}$ and IL-$10^{-/-}$ mice secreted a lower level or no IL-10, respectively, and T cells from both groups produced a lower level of IL-5 (Fig. 3, *A* and *B*). We did not detect significant levels of IL-4 in the supernatants from the three groups. We confirmed that T cells derived from IL-$10^{-/-}$ mice produced a large amount of TNF-α, while comparatively low amounts of this cytokine were secreted by T cells from WT and IL-$4^{-/-}$ mice (Fig. 3*C*). These results

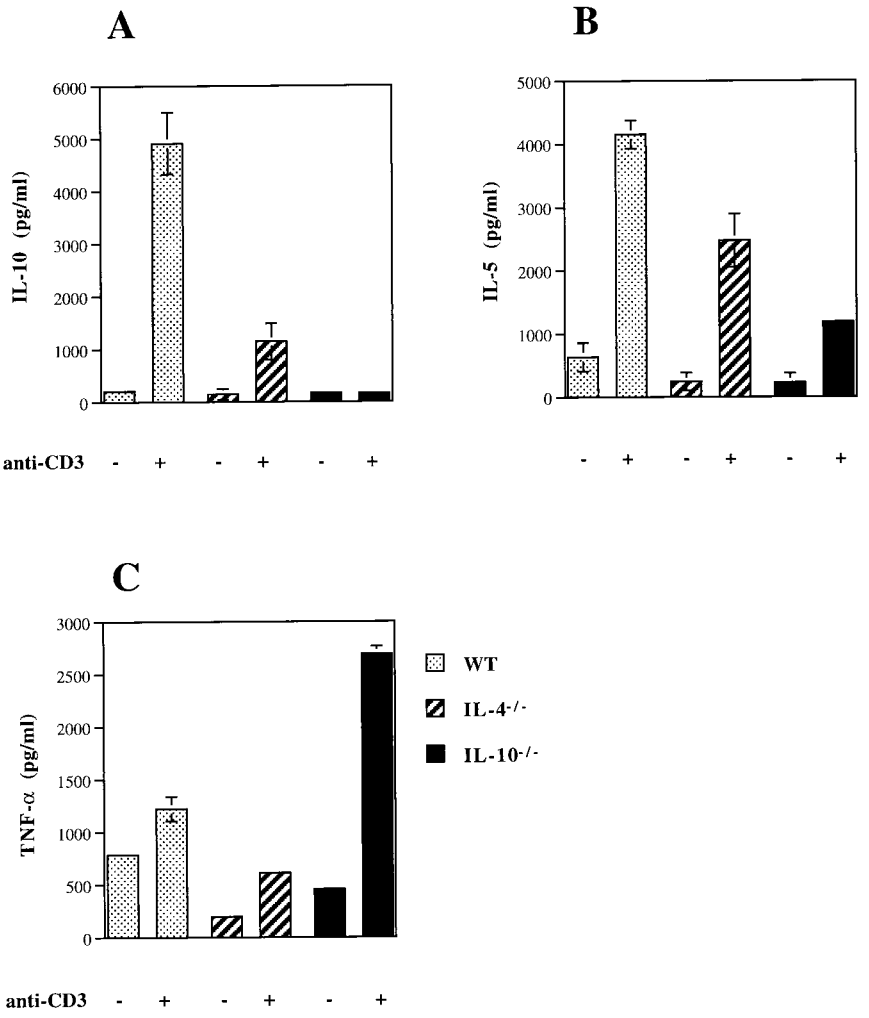

FIGURE 3. Cytokine production by $MOG_{35–55}$-specific T cell lines from C57BL/6 IL-$10^{-/-}$, IL-$4^{-/-}$, and WT mice after activation with anti-CD3 Ab. LN cells from IL-$10^{-/-}$, IL-$4^{-/-}$, and WT mice immunized with $MOG_{35–55}$ were stimulated with this peptide to establish short term T cell lines. Fifteen days later, cells were washed and cultured in the presence of plate-bound anti-CD3 or medium alone. Levels of IL-10, IL-5, and TNF-α were determined by ELISA in 24-h culture supernatants. Data are presented as mean cytokine production ± SD from triplicate wells. One representative experiment of three is shown.

show that IL-10 plays a central role in down-regulating IFN-γ and TNF-α production by autoreactive T cells and that IL-4 does not appear to inhibit production of proinflammatory cytokine to the same extent.

T cells lines derived from IL-$10^{-/-}$ mice transfer severe disease

Since IL-10 is produced by both T cells and APCs, it was not clear whether the enhanced disease in IL-$10^{-/-}$ mice was due to a lack of regulation of T cell responses by IL-10 produced by the APC compartment or whether T cells from the IL-10-deficient mice were more pathogenic. To address this issue, we generated long term T cell lines specific for the $MOG_{35–55}$ from the WT and IL-4- and IL-10-deficient mice. The T cell lines were stimulated three to four times with the peptide, and 4 days after the last stimulation, T cells were transferred into naive C57BL/6 mice that were either left untreated or were further injected with pertussis toxin. As shown in Figure 4*A*, WT mice that received lines derived from C57BL/6 mice (B6.31) developed no disease if the toxin was not injected but developed a mild disease if pertussis toxin was coinjected with the T cells. Lines derived from IL-$4^{-/-}$ mice (4.21) induced a more severe disease in WT C57BL/6 mice that were not injected with the toxin, but all of the mice recovered spontaneously without any signs of relapse (Fig. 4*B*). However, when 4.21 T cell lines were injected together with pertussis toxin, the severity of the disease was enhanced and the disease was persistent (Fig. 4*B*). The mice that received T cell lines derived from IL-$10^{-/-}$ mice (10.22) but without any pertussis toxin developed fulminant disease and died by day 9 after the transfer (Fig. 4*C*). In contrast, the mice that received 10.22 T cell lines together with the toxin developed disease within a week, but these mice recovered spontaneously. These data demonstrate that the encephalitogenic T cell lines derived from IL-10-deficient mice induce a lethal EAE and that pertussis toxin permits recovery of mice, possibly by hyperstimulating and promoting activation-induced cell death in these T cells.

IL-10 transgenic mice are resistant to induction of EAE

Inasmuch as studies with IL-$10^{-/-}$ and IL-$4^{-/-}$ mice demonstrated a role for IL-10 in limiting EAE, we attempted to confirm this by inducing disease in transgenic mice overexpressing IL-4 and IL-10 in T cells (Fig. 5). Since IL-4 and IL-10 transgenic mice were available only on the FVB background, which is not susceptible to EAE, we made an F_1 cross between SJL and FVB mice. The F_1 mice were immunized with whole PLP in CFA to induce EAE. Using this protocol, we induced disease with a mean maximum score of 2.4 in 66% of the (FVB × SJL)F_1 mice. The (FVB × SJL)F_1 UD (IL-4 transgenic) mice developed a disease with a slighly lower incidence (60%) as compared with the (FVB × SJL)F_1 WT mice ($p < 0.03$ calculated with Fisher's exact test) with a mean maximum score of 2.2. However, (FVB × SJL)F_1 UR (IL-10 transgenic) mice were completely resistant to induction of EAE (Fig. 5). The disease incidence of IL-10 transgenic mice was significantly different ($p < 0.005$) when compared with the F_1 control mice. None of the mice showed any clinical sign of disease. These data demonstrate that overexpression of

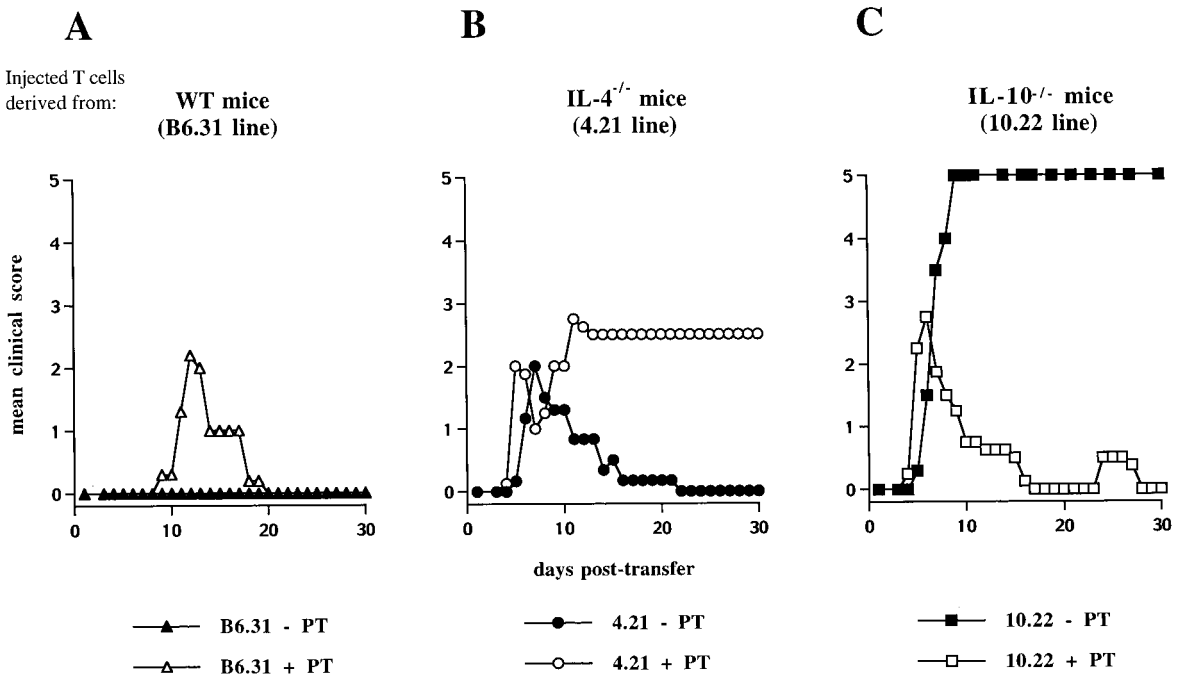

FIGURE 4. Clinical course of EAE in C57BL/6 recipient mice that received $MOG_{35–55}$-reactive T cell lines derived from C57BL/6 WT (B6.31 line) (*A*), IL-$4^{-/-}$ (4.21 line) (*B*), and IL-$10^{-/-}$ mice (10.22 line) (*C*). Naive C57BL/6 mice were injected i.v. with 10^7 $MOG_{35–55}$-reactive T cell from different lines, which had been stimulated 4 days previously with peptide. Groups of mice either received 200 ng of pertussis toxin i.v. immediately after cell transfer and 2 days later (+PT) or were not injected with pertussis toxin when the T cell lines were transferred (−PT). The data are presented as the mean disease score in each group. Each group consisted of five mice. One representative experiment of three is shown.

IL-10 completely inhibits clinical signs of disease and protects the animals from developing EAE. The lack of disease in the IL-10 transgenic mice was not due to the lack of a T cell response to the immunizing protein, since we found that T cells from IL-10 transgenic mice develop a good proliferative response to the PLP (data not shown).

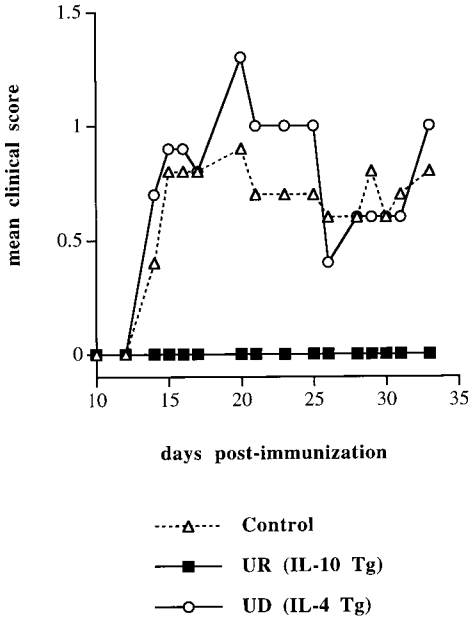

FIGURE 5. IL-10 transgenic mice but not IL-4 transgenic mice are resistant to EAE induction. IL-10 transgenic (UR), IL-4 transgenic (UD), and genetically matched littermates (FVB × SJL)F_1 were immunized in the flanks with 100 μg of whole PLP and injected i.v. with pertussis toxin (200 ng). The data are presented as the mean disease score in each group. Each group consisted of six to eight mice.

Discussion

The Th2 cytokines IL-4 and IL-10 have both been implicated in the regulation of autoimmune diseases. In this study, we have analyzed the relative roles of these two Th2 cytokines in the induction and regulation of EAE. This is particularly important because it has been demonstrated that IL-10 expressed in the pancreatic islet β cells does not inhibit but enhances autoimmune diabetes, suggesting that IL-10 may act as potentiating/enhancing factor for the generation of autopathogenic T cells and for the induction of autoimmunity (28). In this study, using both the IL-4- and IL-10-deficient and transgenic mice, we have addressed this issue. We have demonstrated that 1) IL-10-deficient mice develop more severe EAE when compared with IL-4-deficient or genetically matched WT mice; 2) T cells from IL-10-deficient mice show a stronger Ag-specific proliferative response to $MOG_{35–55}$, with the production of more Th1 cytokines, and induce very severe EAE when transferred into the WT mice; and 3) while IL-4 transgenic mice develop similar disease compared with their nontransgenic littermates, mice transgenic for IL-10 are completely resistant to the development of EAE. Taken together, these data suggest that IL-10 plays a more crucial role in the regulation of EAE than IL-4. Although it has previously been shown that IL-4 can also regulate EAE, the data presented here, along with those of others, raises the possibility that many of the regulatory effects seen with IL-4 may be due to immune deviation and the generation of IL-10-producing T cells that mediate effector regulation in EAE (29, 30). The critical role of IL-10 in regulating EAE was further emphasized by the experiments in which mice were immunized with the encephalitogenic peptide but were not given pertussis toxin. In these experiments, the majority of the IL-$10^{-/-}$ mice developed EAE with

significant lesions in the CNS, whereas the IL-4$^{-/-}$ and the WT mice developed only a limited disease with low incidence, severity, and lesion load in the CNS.

Cytokines play a pivotal role in the initiation or regulation of autoimmune disease, and detailed in vivo studies looking at EAE lesions in the CNS implicate Th1 cytokines in the progression of disease and Th2 cytokines in disease remission (7). Kennedy et al. found that mRNA for TNF-α was present in the CNS of mice during clinical episodes (8). Using in situ methods, they also found that IFN-γ peaked before the disease, strongly suggesting that Th1 cytokines play an important role in the pathogenesis of the disease especially in its initiation. However, IL-10 and IL-4 mRNAs were detected only during disease remission. The remission phase of the disease was also associated with increased levels of TGF-β (6). However, conflicting results have been obtained when recombinant cytokines have been administered in vivo. Treatment of mice with IL-4, in an adoptive transfer experiment, resulted in an amelioration of EAE (9); on the other hand, a recent report shows that IL-4 treatment was ineffective in inhibiting disease (29). While Rott et al. found that administration of IL-10 during the induction phase of EAE in rats could suppress the clinical signs of the disease (17), Cannella et al. showed that injection of IL-10 in mice worsened its clinical course (18). Systemic administration of cytokines raises potential problems because of their short half-life and the issue of access to the appropriate organ. Furthermore, because of the complex interplay of cytokines it is difficult to know when, during the establishment of disease, cytokines are produced and where regulation occurs. Thus, administration of cytokines may not mimic their in vivo expression in the tissue microenvironment. Although, cytokine-transgenic and -deficient mice also do not provide a physiologic way of analyzing the overexpression or deficiency of a particular cytokine on the disease phenotype, nonetheless these mice provide the best tools available at the present time. The use of conditional knock out with cell type-specific loss or overexpression of a specific cytokine may, in the future, provide a better model to understand how these cytokines are regulating disease induction and progression.

While analyzing the mechanism by which IL-10$^{-/-}$ mice developed such severe disease, it became clear that LN cells from IL-10$^{-/-}$ mice developed a higher proliferative response with the production of more Th1 cytokines when compared with the LN cells from the IL-4$^{-/-}$ or WT mice immunized identically with the same myelin Ag. This suggests that the increased incidence and severity of disease in the IL-10-deficient mice may be due to more rapid expansion of myelin Ag-reactive T cells and/or enhanced production of Th1 cytokines from T cell lines following Ag-specific activation. Our results cannot differentiate whether increased severity and incidence of disease in the IL-10$^{-/-}$ mice is due to more expansion of pathogenic T cells following active immunization, to a higher frequency of myelin Ag-specific T cells, or to both. Our ongoing studies with the generation of T cell clones from the different mouse strains and adoptive transfer of equal numbers of the T cell clones from each of the lines will directly address this issue. These data also raise an important question of whether IL-10 normally plays a role in regulating the size of the expanded population of T cells and the type and the amount of Th1 cytokines produced following specific immunization? In support of this hypothesis are the data showing that the T cell lines derived from C57BL/6 control mice immunized with the encephalitogenic peptide MOG35–55 besides producing Th1 cytokines also produce IL-10 and IL-5. These T cell lines do not induce as potent EAE when transferred into wild-type mice compared with T cell lines from IL-10$^{-/-}$ mice, suggesting that IL-10 producing T cells are normally induced following immunization, and these T cells probably play a critical role in keeping immune responses under control. Similarly, an immunoregulatory role of IL-10 has been shown in a number of other system; a nonlytic IL-10/Fc fusion protein was found to block autoimmunity and prevent diabetes (31) and administration of rhIL-10 was found to inhibit autoimmune thyroiditis (32). Sundstedt et al. found that repeating injection of SEA in mice results in a T cell hyporesponsiveness characterized by an impaired IFN-γ and TNF-α production and with an increased IL-10 response called "Th10" (33). A recent evidence supports that the inhibitory effects of IL-10 may be due to induction of a regulatory T cell population. Groux et al. reported that IL-10 gives rise to CD4$^+$ T cell clones, which themselves produce high levels of IL-10 with little or no IL-4 or IL-2 (34). These T cells suppress the proliferation of CD4$^+$ T cells and prevent colitis when transferred into SCID mice. The authors designated these IL-10-producing T cells as Tr1, a unique subset of regulatory T cells that have the capacity to suppress Ag-specific immune responses and actively down-regulate autoimmune responses in vivo. It is likely that in IL-10$^{-/-}$ mice this regulatory population of Tr1 cells is not generated, thus leading to enhanced Ag-specific proliferative responses and more intense autoimmunity, as demonstrated by the increased severity and incidence of EAE.

We and others previously reported that myelin Ag-specific IL-4- and IL-10-producing Th2 cells could inhibit EAE if given at the time of immunization and could reverse disease if given at the first signs of EAE (14, 30, 35, 36). These data have been further supported by the results from other groups showing that myelin Ag-reactive T cells transduced with a retroviral vector containing the IL-4 gene or T cells transfected with IL-10 cDNA could both inhibit and/or reverse EAE (15, 16). The higher disease severity and incidence observed in IL-10$^{-/-}$ and IL-4$^{-/-}$ mice compared with WT mice favor a protective role of these Th2 cytokines in the development of EAE. However, our results suggest that both cytokines are not equally protective. IL-10, and to a lesser extent IL-4, seems to be essential for limiting TNF-α and IFN-γ production (Fig. 2, *B–D*). Besides its role in the generation of regulatory Tr1 cells and inhibition of autopathogenic Th1 cell generation, IL-10 may protect from the development of autoimmunity by inhibiting the production of proinflammatory cytokines and the activation of macrophages that have been implicated in the pathogenesis of EAE.

Acknowledgments

We thank Lindsay B. Nicholson for critical review of the manuscript.

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