An Interleukin (IL)-10/IL-12 Immunoregulatory Circuit Controls Susceptibility to Autoimmune Disease

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Summary

Cells of the innate immune system secrete cytokines early in immune responses that guide maturing T helper (Th) cells along appropriate lineages. This study investigates the role of cytokine networks, bridging the innate and acquired immune systems, in the pathogenesis of an organ specific autoimmune disease. Experimental allergic encephalomyelitis (EAE), a demyelinating disease of the central nervous system, is widely used as an animal model for multiple sclerosis. We demonstrate that interleukin (IL)-12 is essential for the generation of the autoreactive Th1 cells that induce EAE, both in the presence and absence of interferon γ. The disease-promoting effects of IL-12 are antagonized by IL-10 produced by an antigen nonspecific $CD4^+$ T cell which, in turn, is regulated by the endogenous production of IL-12. This unique immunoregulatory circuit appears to play a critical role in controlling Th cell differentiation and provides a mechanism by which microbial triggers of the innate immune system can modulate autoimmune disease.

T cells are classified into Th1/Th2 subsets based on the array of cytokines they produce upon antigen stimulation, which, in turn, dictates the nature of the immune response (1). Studies in infectious disease and autoimmunity models have demonstrated that immune responses to both self- and foreign antigenic challenges are frequently dominated by induction of a particular Th subset, with profound consequences for clinical outcome (2, 3). Although the inflammatory effector function of Th1 cells is essential for the clearance of intracellular pathogens, it is also responsible for tissue damage typical of organ-specific autoimmunity (4–13). Th2 cells, on the other hand, are critical for the clearance of many helminthic infections and have been implicated in the pathogenesis of systemic autoimmune diseases driven by the production of autoreactive antibodies (14, 15). However, they are generally depicted as suppressor cells or ineffectual bystanders in organ-specific autoimmune diseases (16–20).

Experimental allergic encephalomyelitis (EAE)[1] is a demyelinating disease of the central nervous system (CNS) induced either by active immunization with myelin proteins or by the adoptive transfer of myelin protein–reactive T cells. The $CD4^+$ T cell lines and clones that transfer EAE invariably produce IFN-γ and/or TNF-α/lymphotoxin-α (LTα) on antigenic challenge in vitro (21–23). Lines and clones with the same peptide/MHC specificities that have been manipulated to produce Th2 rather than Th1 cytokines generally lose their encephalitogenic potential and, in certain circumstances, can act to suppress disease (24–28). Furthermore, mRNA encoding Th1 cytokines is found in the CNS during peak disease with levels falling at the time of remission (29–31). Interventions to block the activity of the Th1 cytokine, TNF-α, by use of neutralizing antibodies (32, 33), soluble TNF I receptors (34, 35), or type 1 phosphodiesterase inhibitors (36, 37), lead to reversal of EAE, whereas injection of recombinant TNF-α triggers relapses (38). Conversely, EAE has been treated successfully by the administration of exogenous Th2 cytokines either infused systemically or delivered locally by genetically engineered myelin-reactive cells (39–42). Endogenous production of IL-4 and IL-10, which counterregulate and antagonize Th1 cytokines, has been implicated in the initiation of spontaneous remissions (29–31).

The prevailing dogma, shaped by such observations, depicts the inducers of EAE as polarized Th1 cells and causally links their Th1 phenotype with encephalitogenicity (4). However, the regulation and function of individual cytokines in EAE is more complex than the above studies imply. Alteration in the systemic expression and/or activity of cytokines believed to be important in the pathogenesis of EAE has yielded paradoxical results with respect to clinical outcome. For example, the injection of neutralizing antibodies to IFN-γ exacerbated disease in five independent

[1]*Abbreviations used in this paper:* CNS, central nervous system; EAE, experimental allergic encephalomyelitis; iNOS, inducible nitric oxide synthase; LTα, lymphotoxin-α; MBP, myelin basic protein.

studies (43–47) and administration of recombinant IFN-γ has repeatedly been found to have a protective effect (43, 47, 48). EAE has also been successfully induced in IFN-γ knockout (−/−) and IFN-γ–receptor knockout mice, and in at least two cases the disease was more severe in the knockouts than in wild-type counterparts (49–51). Taken together, these results suggest that IFN-γ can actually act to suppress the development of EAE, either during the evolution of encephalitogenic effectors or at a downstream event critical to the formation of demyelinating plaques. The application of the Th1/Th2 paradigm to EAE was most recently brought into question by the successful induction of the disease in double knockout mice deficient in expression of the other two Th1 cytokines, TNF-α and LTα (52), and by the failure to induce a more severe form of EAE in IL-4−/− mice in comparison to their wild-type counterparts (53). To further complicate matters, a recent study demonstrated that T cells that express a TCR transgene specific for myelin basic protein (MBP) induce EAE in immunodeficient recipients after culture under either Th1 or Th2 polarizing conditions (54). This study, as well as one illustrating a similar phenomenon in an animal model of diabetes (55), suggests that T cells that do not fall into the classic Th1 subset can nevertheless act as mediators of organ-specific autoimmunity.

Thus far, most studies have been designed to investigate the role of highly differentiated, polarized Th1/Th2 effector cells and their signature cytokines in EAE. An alternative strategy is to examine autoreactive cells during formative stages in their development in order to gain insight into the factors that promote the evolution of naive autoreactive precursors into proinflammatory autoimmune effectors. Among the factors currently known to influence patterns of Th cell development, cytokines produced by cells of the innate immune system are the most important. The production of IL-12 by monocytes and dendritic cells results in Th1 differentiation, whereas the production of IL-10 by macrophages and a subset of B cells antagonizes the activities of IL-12 (56–59). Hence, cells populating the innate component of the immune system, stimulated by conserved microbial products and structural elements, can secrete cytokines early in immune responses that can potentially guide maturing Th cells along appropriate lineages (60, 61). In autoimmune disease, this interplay between the innate and adaptive responses may be subverted to promote the development of autoreactive effectors. We have previously demonstrated that MBP-reactive cells can be converted from a quiescent state into autoimmune effectors by exposure to exogenous IL-12 or to microbial products that induce IL-12 production by macrophages (62, 63). Other studies have also demonstrated a disease-promoting effect of IL-12 in EAE as well as other models of organ-specific autoimmunity (7, 64, 65). Conversely, anti–IL-12 was found to delay the onset of disease when administered short term to recipients of primed Th1 EAE effectors, but severe disease ensued immediately after withdrawal of therapy. More prolonged administration of the neutralizing antiserum resulted in protective effects that persisted after cessation of treatment, but the treated animals were not followed for a long enough period to determine whether they would experience relapses. No effort was made in these studies to analyze the role of IL-12 in the induction of the Th1 effectors and the authors postulated that the protective effects of anti–IL-12 at the late stage of disease pathogenesis may have involved an interference with the ability of the mature autoreactive cells to home to the CNS (64).

This study focuses on the contribution of cytokine production by cells of the innate immune system to the generation and differentiation of Th1 autoreactive effector cells. Initially, we use cytokine-deficient mice to demonstrate that IL-12 is absolutely essential for the pathogenesis of EAE, both in the presence and absence of IFN-γ. In a parallel approach involving the neutralization of IL-12 in cytokine-sufficient mice, we characterize a unique immunoregulatory circuit in which endogenous production of IL-12 suppresses IL-10 production by an antigen nonspecific $CD4^+$ T cell. This latter cell, which may represent a new member of the innate immune system, appears to play a critical role in regulating Th cell differentiation.

Materials and Methods

Mice. SJL/J, C57BL/6, C57BL/10, BALB/c, BALB/c *nu/nu*, and C.B-17 SCID mice were all obtained from the National Cancer Institute (Frederick, MD). Breeding pairs of C57BL/6 IL-12−/− (N6) and C57BL/6 IFN-γ−/− (N7) mice were originally provided by J. Magram (Hoffman LaRoche, Nutley, NJ) and Dyana Dalton and T. Stewart (Genentech Inc., South San Francisco, CA), respectively. Breeding pairs of IL-4−/− and IL-10−/− mice were originally obtained from R. Kuhn and W. Muller (University of Koln, Koln, Germany) and backcrossed in our facilities onto the C57BL/6 (N13) and C57BL/10 (N7) backgrounds, respectively. All mice were housed under specific pathogen-free conditions. They were exclusively female and between 8 and 12 wk of age when experiments were initiated.

Induction of EAE. Bovine MBP was obtained from Sigma Chemical Co. (St. Louis, MO). $MBP_{87–106}$, corresponding to residues 87–106 of murine MBP (VVHFFKNIVTPRTPPPQGK), was synthesized by the Laboratory of Molecular Structure, Peptide Synthesis Laboratory (NIAID, NIH, Bethesda, MD) and analyzed and purified by high pressure liquid chromatography. For induction of EAE by active immunization, mice were injected with bovine MBP (400 μg) emulsified in CFA (1:1) by the subcutaneous route over the left flank on day 0 and over the right flank on day 7. Mice were examined daily and rated for severity of neurological impairment as previously described (62, 63). For disease induction by adoptive transfer, donor mice were immunized with $MBP_{87–106}$ (100 μg) or bovine MBP (400 μg) emulsified in CFA (1:1) by subcutaneous injection at four sites over the flanks. 10–14 d later, draining LN cells (axillary and inguinal) were removed and processed as previously described (62). Cells were cultured for 96 h with $MBP_{87–106}$ (50 μg/ml) or bovine MBP (50 μg/ml) in RPMI 1640 containing 10% FCS and standard supplements (TCM). Recovered cells (5×10^7) were injected intraperitoneally into naive syngeneic recipients that were examined daily for signs of EAE. In certain studies, the mice were injected intraperitoneally with polyclonal goat anti–mouse IL-12 (0.5 μg per injection; a gift of Drs. D. Presky and M. Gately, Hoffman-La Roche), normal goat IgG (0.5 mg per injection;

Sigma Chemical Co.), rat anti–mouse IL-10 (1 mg each of mAbs SXC-1 and SXC-2 per injection; reference 66), or control rat IgG (2 mg; Sigma Chemical Co.).

Cell Cultures. Single-cell suspensions of spleen or LN tissue were prepared by passage through wire mesh and red blood cells lysed with ACK buffer (NIH Media Unit, Bethesda, MD). For detection of cytokine production during primary culture, cells (4×10^6/ml) were cultured in TCM in 24-well plates (Costar Corp., Cambridge, MA) for 96–144 h. In some experiments, spleens were depleted of various subpopulations before culture using sheep antifluorescein biomag beads (Perspective Bioresearch Products, Cambridge, MA) together with FITC-conjugated mAbs specific for CD4, CD8, B220, and Mac-1 (PharMingen, San Diego, CA). In other experiments, purified $CD4^+$ T cells and $B220^+$ B cells were cocultured (2×10^5 of each in 200 μl) in 96-well microtiter plates (Costar Corp.). The $CD4^+$ T cells were isolated using CD4 subset enrichment columns (R & D Systems, Inc., Minneapolis, MN) with 90–95% purity achieved. B cells were positively selected to 93–98% purity by incubation with anti-B220 microbeads followed by magnetic separation using VS+ columns (Miltenyi Biotec, Inc., Auburn, CA).

For measurement of cytokine production by MBP-reactive LN cells during secondary cultures, the cells were harvested and washed extensively after 96 h of primary in vitro stimulation. Then they were resuspended in fresh media (1×10^6 cells/ml) and supplemented with irradiated syngeneic splenocytes (3,000 rads; 4×10^6 cells/ml) with or without $MBP_{87–106}$ (50 μg/ml). Supernatants were assayed 48 h later.

Cytokine ELISA. IFN-γ, IL-4, and IL-10 were quantified using a sandwich ELISA technique based on noncompeting pairs of antibodies as previously described (62). The lower limit of detection for each assay was as follows: IFN-γ, 12–24 pg/ml; IL-4, 8–16 pg/ml; and IL-10, 16–32 pg/ml.

Results

Induction of EAE in Cytokine-deficient Mice. Previous studies have attempted to address the contribution of individual cytokines to the pathogenesis of EAE by examining the susceptibility of cytokine-deficient mice to disease induction. Unfortunately, a comparison of these studies was difficult as different myelin-derived antigens were used and the genetically deficient mice were only backcrossed onto "susceptible" backgrounds for two to four generations. To avoid these problems, we used a single antigen to examine the susceptibility of wild-type mice and several different cytokine-deficient mice that had been backcrossed more completely to either the C57BL/6 or the C57BL/10 backgrounds. C57BL/6 wild-type mice were relatively resistant to EAE, with only 31% of actively immunized individuals exhibiting clinical symptoms (Fig. 1). Among the affected cohort the mean peak clinical score was 2.67 ± 0.8 and the average day of onset was 8.2 ± 0.4. On the other hand, IL-12−/− mice were completely resistant to disease whereas IFN-γ−/− animals exhibited severe EAE at 100% incidence (mean peak clinical score, 4.5 ± 0.68; average day of onset, 9.1 ± 1.6). Surprisingly, the induction of EAE in the IFN-γ−/− animals was completely prevented by the administration of neutralizing antibody to IL-12, but not isotype control antibody, during the course of active immunization (Fig. 1, *top*). This result suggested that IL-12 promotes the development of EAE by an IFN-γ–independent mechanism. The failure of IL-12−/− and anti–IL-12–treated IFN-γ−/− mice to develop disease was not secondary to the induction of a Th2 response as neither IL-4 nor IL-10 was detected after stimulation of LN cells from these animals with MBP in vitro (data not shown). Interestingly, the incidence and severity of disease in IL-4−/− mice was comparable to that of the C57BL/6 wild-type controls (Fig. 1, *bottom*; mean peak clinical score, 1.95 ± 0.7) with slightly delayed onset (13 d ± 3). By contrast IL-10−/− mice exhibited enhanced disease incidence and severity in comparison to C57B/10 wild-type controls (Fig. 1, *bottom*; mean peak clinical scores, 2.4 ± 0.6 vs. 1 ± 0.1 in IL-10−/− and wild-type mice, respectively) with similar kinetics (average day of onset, 11.5 ± 0.5 and 11 ± 1.3, respectively). LN cells from both the IL-4−/− and IL-10−/− strains produced elevated levels of IFN-γ after stimulation with MBP in vitro compared to LN cells from wild-type mice (data not shown). A deficiency of MBP-specific Th2 cells could, theoretically, be responsible for the increased incidence of EAE, as well as the propensity for Th1 cytokine production, in IL-10−/− mice. However, this explanation is unlikely since the IL-4−/− mice did not differ significantly from controls with regard to those characteristics and IL-4 is required for Th2 development (67).

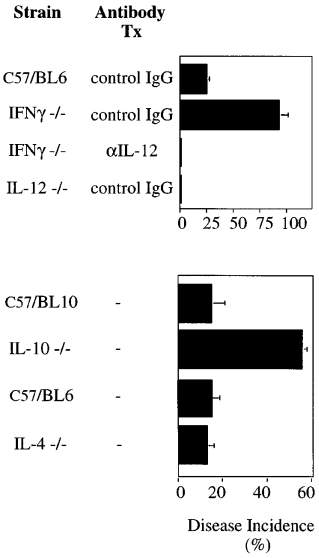

Figure 1. (*A*) Induction of EAE in both wild-type and IFN-γ−/− mice is dependent on the presence of IL-12. C57BL/6 wild-type ($n = 66$), IFN-γ−/− ($n = 89$) and IL-12−/− ($n = 33$) mice were immunized with bovine MBP (400 μg) emulsified in CFA (1:1) on days 0 and 7. IFN-γ−/− mice were injected intraperitoneally with 0.5 mg of neutralizing anti–IL-12 antibody ($n = 30$) or control IgG ($n = 59$) on days 0, 3, 6, and 12. Mice were examined for signs of neurologic impairment from days 0 to 50. Mice with clinical scores of 1 (indicating a limp tail) or higher were considered symptomatic. Data was pooled from five independent experiments; standard deviation reflects the variability between individual experiments. (*B*) IL-10−/− mice, but not IL-4−/− mice, exhibit heightened susceptibility to EAE. C57BL/10 wild-type ($n = 32$) and C57BL/10 IL-10−/− ($n = 39$) mice, and C57BL/6 wild-type ($n = 20$) and C57BL/6 IL-4−/− ($n = 15$) mice, were immunized with MBP and examined for clinical signs according to the protocol described above. Data was pooled from three independent experiments.

Role of IL-12 in the Evolution of IFN-γ–producing Encephalitogenic Effector T Cells. To define IL-12–dependent stage(s) in the development of EAE effectors, we neutralized IL-12 during individual steps of an adoptive transfer protocol. Susceptible female SJL mice were immunized once with $MBP_{87–106}$ peptide (the immunodominant epitope for $H\text{-}2^s$

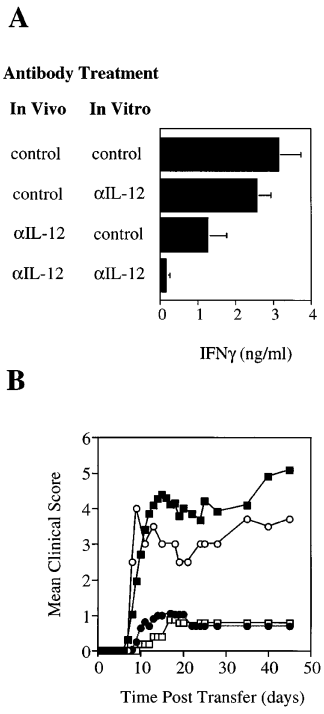

Figure 2. The ability of MBP-reactive LN cells to produce IFN-γ and transfer disease is compromised after neutralization of IL-12 during antigen priming. (*A*) SJL mice were immunized with $MBP_{87–106}$ (100 μg) in CFA and injected intraperitoneally with 0.5 mg of either control IgG or goat anti–mouse IL-12 on days 0, 3, and 6. Draining LN were harvested on day 10 and LN cells were cultured in the presence of $MBP_{87–106}$ (50 μg/ml) with anti–IL-12 (10 μg/ml) or control IgG (10 μg/ml). 96 h later the cells were washed extensively and restimulated for 48 h with antigen and irradiated syngeneic splenocytes to measure IFN-γ production. The values indicated represent the means and standard deviations of five independent experiments using a total of 25–30 donor mice/group. (*B*) Cells (5×10^7) from the four groups described in *A* (exposed to control IgG only [*filled squares*]; anti–IL-12 in vitro [*open circles*]; anti–IL-12 in vivo [*filled circles*]; and anti–IL-12 both in vivo and in vitro [*open squares*]) were injected intraperitoneally into naive syngeneic recipients and recipient animals were evaluated for neurological impairment. The incidence of disease was 90.5, 80, 51, and 29%, respectively. Results were pooled from five independent experiments with a total of 25–35 recipient mice in each group.

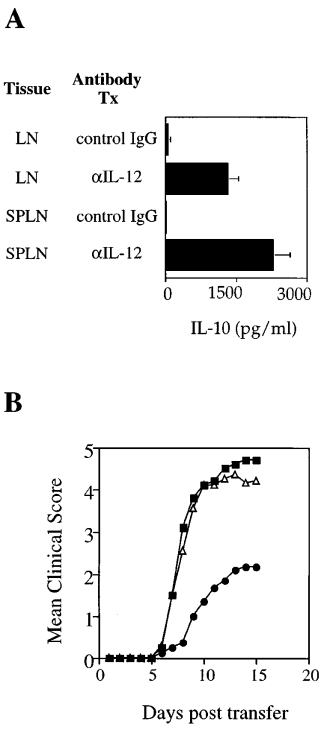

Figure 3. Systemic administration of anti–IL-12 elicits IL-10 production by lymphoid tissue from MBP-primed mice. The protection from EAE mediated by anti–IL-12 is largely reversed by neutralization of IL-10. (*A*) Spleens and draining LN were removed from SJL mice that had been immunized with $MBP_{87–106}$ 10 d earlier and treated with either anti–IL-12 or control IgG according to the protocol described in Fig. 2. Splenocytes and LN cells were stimulated with antigen and supernatants were assayed for IL-10 production at 96 h. Results represent the means and standard deviations of six independent experiments in which cells were pooled from 4–5 mice per group per experiment. (*B*) Draining LN were removed from $MBP_{87–106}$ primed SJL mice that had been injected with control IgG (*filled squares*), anti–IL-12 (*filled circles*) or a combination of anti–IL-12 and anti–IL-10 (*open triangles*) according to the schedule described in Fig. 2. LN cells from each group were stimulated with $MBP_{87–106}$ for 96 h, in the presence (*open triangles*) or absence (*filled symbols*) of anti–IL-10, washed extensively, and then injected (5×10^7) into naive syngeneic recipients. The incidence of disease was 87, 58, and 95% among recipients of control IgG–, anti–IL-12–, and anti–IL-12/anti–IL-10–treated splenocytes, respectively. The results shown in the figure are mean clinical scores obtained from three independent experiments with a total of 20–30 recipient mice per experimental group.

strains) in CFA; 10 d later, draining LN cells were stimulated with antigen in vitro for 4 d and then transferred into naive syngeneic recipients. As both the in vivo immunization and the in vitro boost are required for the generation of encephalitogenic effectors, we treated mice with a polyclonal anti–IL-12 antiserum during the course of priming and/or added the antiserum to the in vitro cultures. MBP-reactive LN cells exposed to anti–IL-12 only during culture were mildly inhibited in their ability to produce IFN-γ on subsequent antigenic challenge in vitro (Fig. 2 *A*), but were unimpaired in their capacity to transfer disease (Fig. 2 *B*). In contrast, LN cells from donor mice treated with anti–IL-12 in vivo exclusively were severely compromised in their ability to produce antigen-dependent IFN-γ on secondary challenge in vitro (Fig. 2 *A*) and their encephalitogenicity was markedly decreased on adoptive transfer (Fig. 2 *B*). Administration of anti–IL-12 both in vivo and in vitro abrogated IFN-γ production in secondary cultures, but did not further impair the ability of cells to transfer disease when compared to cells from animals that were only exposed to anti–IL-12 in vivo (Fig. 2, *A* and *B*).

The Protective Effect of Anti–IL-12 Is Partially Due to the Induction of IL-10. To investigate whether the MBP-reactive cells from the susceptible SJL strain had assumed a Th2 phenotype after treatment with anti–IL-12 in vivo and/or in vitro, we measured IL-4 in the supernatants of the primary and secondary cultures of the four experimental groups studied in Fig. 2. We were unable to detect IL-4 in any of the supernatants tested. On the other hand, both LN cells and splenocytes from anti–IL-12 treated donors, but not control IgG–treated donors, produced significant quantities of IL-10 during the primary culture (Fig. 3 *A*). This result raised the question of whether the suppression of the encephalitogenic phenotype by anti–IL-12 treatment was secondary, in part, to the production of IL-10 during the activation of MBP-specific T cells. Indeed, neutralization of IL-10 using a mixture of mAbs during both immunization and the in vitro culture largely reversed the protection from disease afforded by the treatment of donor mice with anti–IL-12 (Fig. 3 *B*).

The Suppression of IL-10 Production by IL-12 Is Antigen and IFN-γ Independent. Initially, we postulated that the source of IL-10 was an MBP-specific T cell population induced during priming in the presence of anti–IL-12. However, similar amounts of IL-10 were produced by cultures in the presence or absence of $MBP_{87–106}$ (data not shown). In fact, IL-10 production was not at all dependent on immunization with MBP in CFA as splenocytes from naive SJL mice previously treated with anti–IL-12 produced as much IL-10 as splenocytes from identically treated animals immunized with MBP (Fig. 4 *A*). An anti–IL-12 antiserum from a sep-

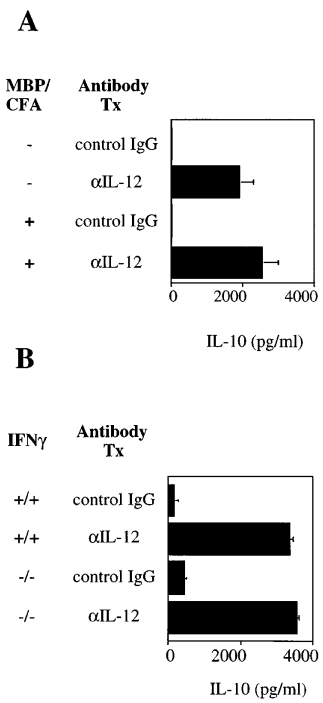

Figure 4. Suppression of IL-10 production by endogenous IL-12 is antigen and IFN-γ independent. (*A*) Spleens were harvested from naive or $MBP_{87–106}$-primed SJL mice that had been injected with anti–IL-12 or control IgG on days −10, −6, and −3 before killing. Supernatants were assayed for IL-10 after 96 h of culture. Results represent the means of four independent experiments with groups consisting of 5–7 mice in each experiment. Standard deviations reflect the variability between individual experiments. (*B*) Naive C57BL/6 wild-type and IFN-γ−/− mice were treated as in *A*. Results represent the means and standard deviations of two independent experiments in which spleens were pooled from four mice in each group.

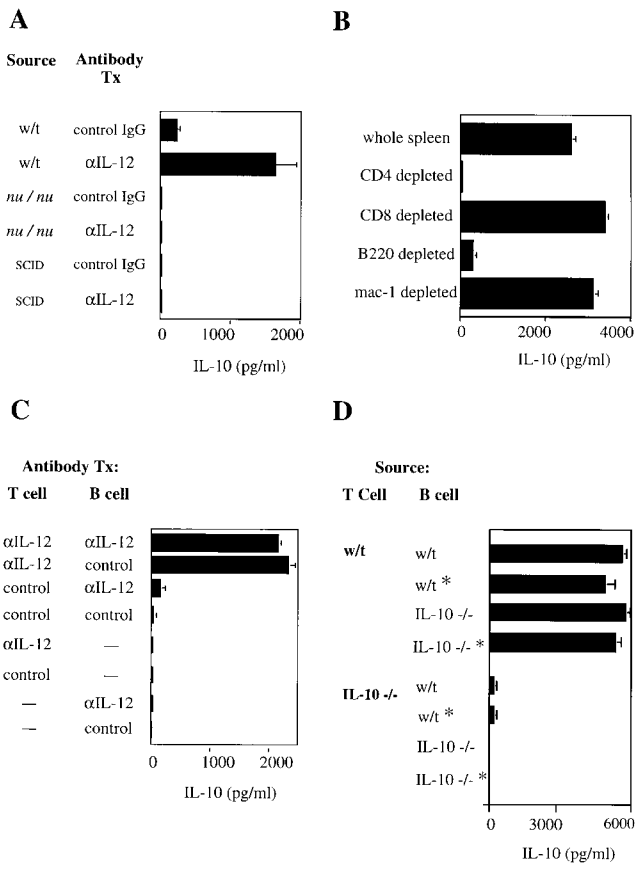

Figure 5. After neutralization of IL-12, IL-10 is produced by antigen nonspecific $CD4^+$ T cells in collaboration with B cells. Spleens were harvested from unimmunized mice that had been injected intraperitoneally with 0.5 mg of control IgG or anti–IL-12 on days −10, −6, and −3 before killing. Supernatants were collected for assay of IL-10 after 96–120 h of culture. (*A*) IL-10 production by splenocytes from BALB/c wild-type (*w/t*), *nu/nu*, and SCID mice at 96 h. Results represent means and standard deviations of two independent experiments in which spleens were pooled from 3–5 mice in each group in each experiment. (*B*) Splenocytes from anti–IL-12 treated SJL mice (n = 5–6) were depleted of various subpopulations using sheep antifluorescein biomag beads and FITC-conjugated mAbs specific for the cell surface markers indicated. Recovered cells (4×10^6 cells/ml) from each group were cultured and supernatants assayed for IL-10 after 120 h. In each case, the recovered cells were 99–100% free of the depleted population as determined by flow cytometry using PE-conjugated mAbs (not shown). Results represent means and standard deviations of three independent experiments. Whole spleen and subpopulation preparations derived from control IgG–treated donors failed to produce detectable IL-10 (not shown). (*C*) $CD4^+$ T cells and $B220^+$ B cells purified from spleens of anti–IL-12 or control IgG–treated SJL mice (n = 4–7/group) were cocultured (2×10^5 of each in 200 μl) in 96-well microtiter plates. Supernatants were collected at 120 h to quantify IL-10 levels. Results represent means and standard deviations of four independent experiments. Levels of purity ranged from 90 to 95% for $CD4^+$ T cells and 93-98% for $B220^+$ B cells as assessed by flow cytometry (not shown). (*D*) $CD4^+$ T cells from spleens of anti–IL-12–treated C57BL/10 wild-type or IL-10−/− mice were cocultured with $B220^+$ B cells purified from spleens of either anti–IL-12 or control IgG–treated donors (2×10^5 of each in 200 μl). An asterisk (*) denotes B cells taken from anti–IL-12–treated mice. Supernatants were collected at 144 h to quantify IL-10 levels. Results represent means and standard deviations of two independent experiments. Levels of purity ranged from 92 to 94% for $CD4^+$ T cells and from 95 to 99% for $B220^+$ B cells. Three to five donor mice were used for each group in each experiment. $CD4^+$ T cells purified from control IgG–treated donors failed to produce IL-10 irrespective of the B cells with which they were combined (not shown).

arate source (sheep anti–mouse IL-12; gift of Genetics Institute, Cambridge, MA) was equally effective (not shown).

Since mRNA encoding IL-12 p40 and p35 has been found in the spleen and lymph node of naive mice (68), these results are consistent with the possibility that a low baseline level of IL-12 tonically suppresses an IL-10 producing cell in lymph node and spleen. IL-12 could act directly on a cell bearing IL-12 receptor β1 and β2 chains or indirectly through the induction of IFN-γ. To clarify the role of IFN-γ in IL-12–mediated suppression of IL-10, C57BL/6 IFN-γ−/− and wild-type mice were treated with anti–IL-12 or control IgG. Splenocytes from IFN-γ−/− mice treated with anti–IL-12, but not control IgG, produced large quantities of IL-10, at levels comparable to that produced by wild-type splenocytes from identically treated donors (Fig. 4 *B*). Thus, IL-12 suppresses IL-10 production by an IFN-γ–independent mechanism.

IL-10 Is Produced by Anti–IL-12–treated $CD4^+$ T Cells, but They Require the Presence of B Cells as Accessory Cells In Vitro. To define which cell type was responsible for IL-10 production after treatment with anti–IL-12 in vivo, we compared IL-10 production by splenocytes from anti–IL-12–treated *nu/nu*, C.B-17 SCID, and wild-type BALB/c mice. Splenocytes from *nu/nu* and SCID mice failed to produce detectable IL-10, whereas spleen cells from normal BALB/c mice produced amounts of IL-10 comparable to those produced by spleen cells from similarly treated SJL mice (Fig. 5 *A*). As these results clearly implicate the T cell as the source of IL-10 production after anti–IL-12 treatment, we depleted various subpopulations from anti–IL-12–treated SJL spleen preparations before culture and measured IL-10 levels in supernatants 96 h later. As expected,

depletion of $CD4^+$ cells abrogated IL-10 production, whereas depletion of $CD8^+$ and $Mac1^+$ cells had no effect. Surprisingly, the $B220^+$-depleted splenocytes produced significantly less IL-10 than the whole splenocyte population (Fig. 5 *B*). Furthermore, neither $CD4^+$ T cells nor $B220^+$ cells purified from spleens of anti–IL-12–treated donors produced IL-10 when cultured separately, whereas IL-10 production was restored when the two subpopulations were recombined (Fig. 5 *C*). $CD4^+$ T cells from anti–IL-12–treated donors produced similar levels of IL-10 whether combined with purified B cells from control IgG or anti–IL-12–treated wild-type donors (Fig. 5 *C*), or with B cells from control IgG–treated IL-10−/− mice (Fig. 5 *D*). Conversely, $CD4^+$ cells from control IgG-treated wild-type donors or anti–IL-12–treated IL-10−/− donors failed to produce detectable IL-10 when combined with B cells from any of the sources mentioned (Fig. 5, *C* and *D*).

Splenocytes from Anti–IL-12–treated Naive Mice Inhibit the Induction of EAE. The abrogation of the protective effects of anti–IL-12 by coinjection of anti–IL-10 (Fig. 3 *B*) and the increased incidence of EAE in IL-10−/− mice (Fig. 1) strongly suggested that IL-10 plays a downregulatory role in the generation of EAE effector cells. To directly demonstrate that the IL-10–producing $CD4^+$ T cell, generated in the absence of antigenic priming, can downregulate the generation of EAE effectors, we performed an adoptive transfer study. SJL mice were injected with splenocytes from anti–IL-12– or control IgG–treated syngeneic donors and then immunized with bovine MBP according to the schedule shown in Fig. 1. Splenocytes from anti–IL-12–treated, but not control IgG–treated, SJL mice significantly inhibited the induction of EAE (Fig. 6) thereby directly demonstrating the biologic importance of the IL-10–producing $CD4^+$ T cells whose presence is revealed when animals are treated with anti–IL-12.

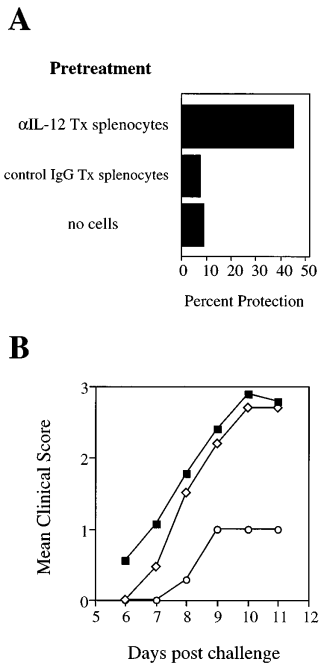

Figure 6. Splenocytes from anti–IL-12-treated naive mice directly suppress the induction of EAE. SJL mice were injected with splenocytes (1×10^8) pooled from 5–7 anti–IL-12–treated or control IgG–treated syngeneic donors, or they were left untreated. Mice from all three groups were subsequently immunized with bovine MBP in CFA 1 and 7 d later. (*A*) Results represent the percent of mice that remained healthy (clinical score of 0) over the 30 d period between the second immunization and killing. The experiment shown consisted of 10 recipients of anti–IL-12–treated splenocytes, 14 recipients of control IgG–treated splenocytes, and 26 mice that were not pretreated. (*B*) The mice described in *A* were rated daily for signs of neurologic impairment according to the scale used in Fig. 2.

Discussion

These studies demonstrate a number of novel mechanisms by which cytokine production by the innate immune system controls an autoimmune response mediated by cells within the adaptive immune system. In the model systems we have used, IL-12 produced by macrophages and/or dendritic cells plays a critical role in the generation of autoimmune effectors, while IL-10 production by antigen nonspecific regulatory $CD4^+$ T cells subserves a counterregulatory or suppressive function. During the induction of EAE in susceptible strains of mice, the homeostatic balance maintained between these antagonistic cytokines is upset to favor IL-12, the production of which is stimulated by mycobacterial components contained in the adjuvant. On the other hand, the enhanced susceptibility to EAE of IL-10−/− mice may be secondary to the loss of the dominant suppressive functions of this cytokine.

Although one might have predicted that IFN-γ would be indispensable for the generation and function of autoimmune effector cells, studies using anti–IFN-γ mAbs and IFN-γ−/− and IFN-γ–receptor−/− strains have uniformly demonstrated that IFN-γ is not required and in many cases exerts a protective effect in EAE (43, 45, 49, 51). It was therefore somewhat surprising that IL-12−/− mice were resistant to EAE and that treatment of IFN-γ−/− mice with anti–IL-12 prevented EAE. Furthermore, the generation of EAE effectors could be markedly inhibited by treatment of highly susceptible SJL mice with anti–IL-12 during the course of priming alone. Taken together, these results demonstrate for the first time that a cytokine implicated in the pathogenesis of EAE, namely IL-12, is indispensable for its manifestation, whereas the effector molecules IFN-γ, TNF-α, and LTα appear to be redundant (50, 52). These results should be contrasted with the requirements for both IL-12 and IFN-γ in mediating resistance to a number of intracellular pathogens including *Toxoplasma gondii*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes* (69-71). Although IFN-γ–independent actions have been attributed to IL-12 in the past (72, 73, 57), in only one other case has such an action been reported to affect the pathogenesis of disease. Taylor and Murray (74) found that treatment of IFN-γ−/− mice infected with *Leishmania donovani* with exogenous IL-12 induced leishmanicidal activity and also partially restored the near-absent tissue granulomatous response. The action of IL-12 against *L. donovani* was TNF-α–dependent and involved upregulation of inducible nitric oxide synthase (iNOS). It was unclear from this study whether the protective effects of IL-12 were mediated by antigen-specific $CD4^+$ T cells or by NK cells. Nevertheless, induction of the TNF-α/iNOS pathway may also be partially responsible for the IFN-γ–independent actions of IL-12 in EAE. We have detected high levels of mRNA encoding both TNF-α and iNOS in the spinal cords of actively immunized C57BL/6 IFN-γ−/− mice and markedly reduced levels after treatment with anti–IL-12 (unpublished

data). Although treatment of mice with anti–TNF-α has been shown to prevent EAE, the contribution of TNF-α to the pathogenesis of demyelination has been difficult to define because the therapeutic effects of anti–TNF-α appeared to have been mediated by prevention of entry of pathogenic T cells into the CNS (32). It would therefore be of interest to assess whether treatment with anti–TNF-α and/or anti–LTα are able to protect IFN-γ−/− mice from disease at a time point when pathogenic effector cells have already entered the CNS. It would also be of interest to determine whether administration of recombinant TNF-α reverses the protection afforded by the anti–IL-12 treatment of actively immunized IFN-γ−/− mice.

Because it has previously been reported that IL-12−/− mice mount a Th2 response when infected with *L. major*, whereas wild-type mice mount a Th1 response (75), we intensively investigated whether anti–IL-12–treated SJL mice develop a Th2 response when immunized with MBP in CFA. Although no evidence for antigen-specific IL-4 production was observed, we consistently observed antigen-independent IL-10 production. The IL-10–producing population implicated has several unique properties that distinguish it from conventional Th2 memory cells. After anti–IL-12 treatment, it was as readily obtained from naive mice as from mice that had been previously immunized. The tonic inhibition of IL-10 production by the constitutive presence of IL-12 in vivo is an IFN-γ–independent activity as upregulation of IL-10 production was also seen when IFN-γ−/− mice were treated with anti–IL-12. Cell purification studies demonstrated that the IL-10 producing cell was a $CD4^+$ T cell, but required coculture with B cells for induction of IL-10 production in vitro; we have not yet evaluated other antigen-presenting cell types for their potential to activate IL-10 production by these $CD4^+$ T cells. The $CD4^+$ IL-10–producing cell resembles the IL-4 producing $CD4^+$, $NK1.1^+$ cell in that it produces cytokines in the absence of priming and therefore appears to be a member of the newly recognized category of unconventional "natural" T cells in the innate immune system that guide adaptive responses through the production of Th1/2-modulating cytokines (76, 77). The induction of IL-4 production by $CD4^+NK1.1^+$ T cells in response to CD1d stimulation (78, 61) raises the possibility that a nonclassical MHC molecule may also serve as the ligand for the IL-10 producer and that the IL-10 producing T cell is responding to an autoantigen presented by the B cell. Currently we are conducting experiments to clarify the nature of the interaction between the B and T cells responsible for IL-10 production. It should be noted that we did not observe enhanced IL-10 production by spleen cells from IL-12−/− mice, but this may be secondary to adaptive processes that have taken place in the animal in the absence of IL-12.

The production of IL-10 by the $CD4^+$ T cells is highly significant biologically as coinjection of anti–IL-10 largely reversed the autoimmune suppressive effects of anti–IL-12. Most importantly, IL-10 producing cells from anti–IL-12–treated naive mice markedly suppressed the induction of EAE after transfer into sensitized recipients. It is also likely that the increased incidence of EAE that we observed in IL-10−/− mice is due to the functional absence of this immunoregulatory cell. The concept that exposure of developing autoimmune effector cells to IL-10 hinders their development is supported by an earlier study in which EAE was suppressed by the administration of recombinant IL-10 to rats during priming (40). It remains to be seen if this $CD4^+$ IL-10–producing T cell plays a broader role in immune surveillance by preventing organ-specific autoimmunity. The high incidence of spontaneous autoimmune phenomena in IL-10−/− mice suggest that this might be the case (79). The innate immune system appears to be biased towards the development of inflammatory immune responses as IL-10 production is constitutively suppressed by IL-12. Therefore, $CD4^+$ IL-10–producing T cells may be needed to curtail overzealous responses to acute infections that would otherwise lead to immunopathology. The slightly delayed expression of IL-10, as compared to IL-12 and other proinflammatory cytokines, in several infectious disease models make it a particularly effective downregulator of the IL-12 response (58). The importance of this negative regulatory role of IL-10 is highlighted by the emergence of a lethal immune response, characterized by overproduction of IL-12, IFN-γ, and TNF-α, in IL-10−/− mice infected with *T. gondii* (80).

Thus far efforts to manipulate cytokine production to alter the course of autoimmune disease have been focused on modification of the adaptive immune response. Although positive results have been reported, these interventions were mainly effective when administered early in the evolution of the disease before the expression of clinical signs (39, 42, 64). Furthermore, recent reports of Th2-mediated autoimmunity suggest that approaches to induce immune deviation of Th1 effector cells may also ultimately result in immunopathology (54, 55). Collectively, our studies strongly suggest that manipulation of the cytokine milieu, the IL-12/IL-10 balance, maintained by cells of the innate immune system, can have profound effects on the incidence of autoimmune disease. In chronic autoimmune diseases that progress as a result of the continuous reactivation of autoantigen-specific memory T cells, it would be unlikely that attempts to block IL-12 production or enhance IL-10 production would be therapeutically useful. On the other hand, relapsing remitting autoimmune disease may provide a unique opportunity for effective intervention employed during periods when new effectors, capable of initiating relapses, are recruited from a naive precursor pool. Studies documenting the phenomenon of determinant spreading in EAE and multiple sclerosis suggest that relapses are attributable in large part to the activation of naive T cells specific for cryptic epitopes during or after the peak of the initial episode (81–83). We have previously suggested that IL-12 production in response to an infectious insult during disease remission may lead to recruitment of Th1 cells (63); consideration should be given to the prophylactic use of IL-12 antagonists and/or IL-10 in patients with autoimmune diseases during epidemic outbreaks that have been associated with autoimmune sequelae.

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