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IL-4, IL-10, IL-13, and TGF-β from an Altered Peptide Ligand-Specific Th2 Cell Clone Down-Regulate Adoptive Transfer of Experimental Autoimmune Encephalomyelitis

Deborah A. Young,1* Leslie D. Lowe,* Susan S. Booth,* Matthew J. Whitters,* Lindsay Nicholson,† Vijay K. Kuchroo,‡ and Mary Collins*†

Experimental autoimmune encephalomyelitis (EAE) is induced in the SJL/J mouse by adoptive transfer of activated proteolipid protein peptide (PLP) 139-151-specific Th1 cells. T cells responding to altered peptide ligands (APL) of PLP, previously shown to induce Th2 differentiation and regulate disease in PLP-immunized mice, do not transfer EAE. However, the exact mechanism of disease regulation by APL-specific T cells has not been elucidated. In this report, we show that 1F1, a Th2 clone specific for an APL of PLP139-151 can prevent adoptive transfer of EAE when cocultured with PLP-encephalitogenic spleen cells (PLP-spleen). Cytokines from activated 1F1 cells were detected by hybridization of mRNA to oligonucleotide arrays (DNA chip) and by ELISA. The Th2 cytokines found to be present are IL-4, IL-13, and TGF-β. The Th2 cytokines found to be present are IL-4, IL-13, and TGF-β. The Th2 cytokines found to be present are IL-4, IL-13, and TGF-β. The Th2 cytokines found to be present are IL-4, IL-13, and TGF-β. The Th2 cytokines found to be present are IL-4, IL-13, and TGF-β. The Th2 cytokines found to be present are IL-4, IL-13, and TGF-β.

IL-4 and TGF-β may modulate disease severity and onset and may partially eliminate disease (15, 16). A recent study showed that Th2 cells may also induce EAE when transferred into T cell-deficient animals (16). Induction of Th2 responses concordant with the Th1 response has been shown to successfully suppress EAE. Expression of Th2 cytokines by activated bystander cells, at the induction of disease, will effectively inhibit the Th1 response by immune deviation, where the cytokine profile is shifted from an encephalitogenic Th1 type to Th2 to alter disease severity (18). SJL mice that were preimmunized with keyhole limpet hemocyanin in IFA to generate a memory Th2 population did not develop EAE when challenged with keyhole limpet hemocyanin and encephalitogenic peptide (18). In another system, immunization of mice with LR, an APL of PLP peptide 139-151, protected mice from EAE induced with PLP peptide as well as unrelated encephalitogenic peptides. The T cell population that is induced by LR is of a Th2/Th0 phenotype, cross-reacts with PLP 139-151, and can down-regulate actively induced EAE through bystander effects (19, 20).

We have used an adoptive transfer model of EAE to examine the potential contributions of Th2 cytokines in the regulation of Th1-mediated disease. We show here for the first time that a Th2 cell

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2 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; APL, altered peptide ligand; MBP, myelin basic protein; LR, altered peptide ligand of PLP119-137; A25Fc, IL-13 receptor α extracellular fusion protein; m, murine.

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responding to an APL can regulate EAE mediated by an encephalitogenic Th1 population. In these experiments, primed encephalitogenic lines are used to transfer disease after coinoculation with a Th2 clone specific for LR. We have systematically examined the effects of Th2 cytokines, individually and in combinations, from this clone for their effects on disease. Our data indicate that particular combinations of Th2 cytokines are most effective at modulating disease. Interestingly, IL-4 and IL-13 were synergistic in suppression of disease, suggesting that effects mediated through the IL-4 receptor on T cells are complemented by effects on other cell types expressing the IL-4/IL-13 receptor.

Materials and Methods

Adoptive transfer cocultures and 1F1 cells were activated in proliferation medium: DMEM with 10% FBS, 2 mM glutamine, 1000 U/ml penicillin-streptomycin (Life Technologies, Gaithersburg, MD), 50 μg/ml gentamicin (Life Technologies), 600 μg/ml anti-PPLO agent (Life Technologies), and 5 × 10⁻⁷ M 2-ME, 0.1 mM nonessential amino acids (Life Technologies), and 1 mM sodium pyruvate. After restimulation, 1F1 cells were maintained in the above medium with the following added (clone medium): 1× MEM essential vitamins (all from Life Technologies), 0.2 mM arginine, 10 mM aspartic acid, 0.1 μg/ml folic acid, and 1% human T-Storm without PHA (Collaborative Biomedical Products, Bedford, MA). ELISAs for IL-2, IL-4, IL-5, IL-6, and IL-10 were done with pairs of Abs purchased from PharMingen (San Diego, CA). Assays for the following cytokines used commercial kits: IL-13, IL-17, and GM-CSF (R&D Systems, Minneapolis, MN), TNF-α (Genzyme, Boston, MA), and TGF-β (Promega, Madison, WI). Neutralizing Abs for adoptive transfer experiments were purchased from the following sources: IL-4 (catalogue no. 18190D; PharMingen), IL-10 (catalogue no. 1996-01; Genzyme), and TGF-β (catalogue no. AB-101NA; R&D Systems). IL-13 was neutralized with a human Fe fusion protein of the extracellular domain of the high-affinity mu- rine IL-13 receptor (AS25Fc) (21) made at Genetics Institute (Cambridge, MA). PLP139-151 wild-type (HSLGKWLGHPDKF) and LR (HSLGKWLGHPDKF) peptides were synthesized, purified and characterized at the Genetics Institute. SJL/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Cytokines were obtained from the following sources: Genzyme, murine (m) IL-4 (MIL-4-C); mIL-5 (MIL-5); mIL-9 (240-B). One microgram of 1F1 cytoplasmic RNA was extracted from 4- and 16-h stimulated every 3 wk in proliferation medium with 10 μM LR peptide and 10:1 irradiated (3000 rads) male SJL spleen cells. The LR 1F1 T cell clone was generated from lymph node cells from LR and PLP-immunized mice as described (20) and is maintained in clone medium. It does not cross-react with PLP139-151 (20). 1F1 cells are restimulated every 3 wk in proliferation medium with 10 μM LR peptide and 10:1 irradiated (3000 rads) male SJL spleen cells.

1F1 conditioned medium

Supernatants were harvested from 1F1 cells cultured at 10⁶ cells/ml with 10 μM LR peptide after 72 h and tested in ELISAs. Control conditioned media were generated from cultures containing no LR peptide (nonactivated 1F1) and no 1F1 cells (peptide pulsed spleen cells).

DNA chip analysis

One microgram of 1F1 cytoplasmic RNA was extracted from 4- and 16-h anti-TCR-stimulated cells (1 μg H57-597 Pharmingen 01300D/ml coated tissue culture dishes). 1F1 cells (10⁵/ml) and poly(A⁺) RNA were isolated, amplified, and converted to cDNA and then to biotin-labeled RNA in an in vitro transcription reaction as described by Lockhart et al. (22). Samples were hybridized to 114 gene arrays (Affymetrix, Santa Clara, CA) in a self-contained flow cell and imaged with a scanning confocal microscope also as described (22). Data are expressed as the relative message frequency per 10⁶.

Adoptive transfer of EAE

Spleen cells from female SJL mice immunized for 8–10 days with 100 μg PLP139-151 in CFA injected s.c. were harvested and cultured at 3 × 10⁶ cells/ml with 10 μM PLP in proliferation medium (PLP-spleen). A total of 20 U/ml recombinant COS human IL-2 was added on day 2 of culture. Cultures were harvested after 3 days and washed, and 5 × 10⁻⁷–1 × 10⁻⁷ cells were injected i.p. into naïve 8–12-wk-old SJL females. Some experiments were designed to test the effect of including 1F1 Th2 cells in the culture. 1F1/PLP-spleen cell cocultures typically included 3 × 10⁵ resting 1F1 cells/ml and 10 μM LR peptide added at the same time as the PLP-spleen cells. To neutralize Th2 cytokines in 1F1 cocultures, 10 μg/ml of the indicated neutralizing Ab was added at the start of culture. A25Fc (21) at 2 μg/ml was included in some cultures to neutralize IL13. Control cultures without 1F1 cells contained PLP-spleen cells with PLP peptide and the indicated Ab. Culture supernatants from PLP-spleen cultures and 1F1 cocultures were harvested after 3 days before adoptive transfer to measure cytokines. Mice were scored for EAE using the following scale: 1, limp tail; 2, unsteady gait; 3, hind limb paralysis; 4, hind and forelimb paralysis; and 5, death.

Results

Coculture with a Th2 clone regulates adoptive transfer of EAE

Typically, immunization of SJL mice with the CNS peptide PLP139-151 in CFA results in an inflammatory and demyelinating disease characterized by ascending paralysis and CNS mononuclear cell infiltrates (23–25). Adoptive transfer of lymph node or splenic T cells derived from mice primed with PLP139-151 also results in induction of disease in naive mice. Previous work has shown that coinmunization of mice with PLP139-151 and LR peptide, an altered peptide derived from PLP139-151 in which the TCR contact residues Trp144 and His147 are changed to Leu144 and Arg147, results in disease suppression (20). These results suggested that T cells primed by immunization with the LR peptide act to regulate disease.

LR 1F1 (1F1) is a T cell clone derived from SJL mice immunized with the LR peptide (20). To determine whether this T cell clone could regulate disease induced by primed encephalitogenic T cells, we compared the encephalitogenic potential of T cell populations from mice immunized with PLP139-151 with similar populations cocultured and coactivated with 1F1 T cells. Immune spleen cells from mice immunized 8–10 days earlier with PLP139-151 were cocultured for 3 days with resting 1F1 T cells and the appropriate peptides. After 3 days, cells from cocultures were adoptively transferred into naive SJL mice, and the mice were monitored for disease. As shown in Fig. 1, mice that received cells from cocultures containing only PLP139-151 immune spleen cells activated with their corresponding peptide (PLP-spleen/PLP) developed EAE, whereas cultures containing both PLP-spleen cells and 10% 1F1 cells with their corresponding peptides (PLP-spleen/PLP + 1F1/LR) did not transfer disease. LR peptide itself was unable to affect the disease transferred by PLP-spleen cells since cells from cultures containing both PLP and LR peptides transferred disease as well as PLP-stimulated spleen cells alone. Additionally PLP-spleen/PLP cultures that included 10% 1F1 cells without the LR peptide also transferred EAE, suggesting that the 1F1 cells required activation with APL to suppress transfer of disease by PLP-spleen cells. Transfer of 1F1 cells activated with normal spleen cells and LR peptide did not result in EAE. We examined the levels of Th1 and Th2 cytokines in the conditioned medium from cocultures containing increasing concentrations of 1F1 cells. Cells from these activated cocultures were then transferred to naive mice to evaluate their potential to transfer disease. As shown in Table I, cultures containing as few as 2% 1F1 cells with the PLP-spleen cells transferred less severe EAE than the PLP-spleen cells alone. Disease transfer from cocultures containing 20% 1F1 cells was completely inhibited. Cytokine analysis of coculture supernatant revealed 5-fold lower levels of the Th1 cytokines IL-2 and IFN-γ, and increasing levels of Th2 cytokines in proportion to the number of 1F1 cells included in the culture.
cells with 1F1 cells (3% cells and 10% 1F1 cells were separated by a 0.4-

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FIGURE 1. Cocultures of PLP-spleen and 1F1 cells. Mice were adoptively transferred with cells obtained from the above listed 3-day cultures and monitored for clinical signs of EAE. Cultures contained either PLP-

mimic membrane. Transwell cocultures in which PLP-spleen and 1F1 cells were separated by a 0.4-

m membrane did not transfer disease in 90% of cases. Transwell cocultures in which PLP-spleen cells and 10% 1F1 cells were separated by a 0.4-

m membrane. Transwell cocultures in which PLP-spleen and 10% 1F1 cells were separated by a 0.4-

m membrane did not transfer disease when both peptides were included in the culture, whereas control cultures without LR peptide did transfer disease (data not shown), suggesting that the 1F1 inhibition is mediated by soluble factors.

1F1 cells secrete high levels of Th2 cytokines upon activation

To identify candidate proteins made by 1F1 cells that might regulate EAE, we used oligonucleotide arrays to examine the expression of 114 different genes known to be expressed in T cells, including cytokine- and lymphocyte-specific genes. RNA was isolated from resting 1F1 cells and from 1F1 cells that had been activated for 4 and 16 h with plate-bound anti-TCR Ab. The RNA was amplified, labeled, and hybridized to the oligonucleotide array as described above (see Materials and Methods). Message levels for 27 cytokines and 6 chemokines were examined. As depicted in Fig. 2, mRNA levels for several cytokines were significantly up-regulated, 3-fold or greater, compared with 

Data from Table I. Adoptive transfer of EAE is inhibited by increasing concentrations of 1F1 cells in cocultures

| Culture† | No. with EAE | Average Day Onset | Mean High Score | p* | IL-2 | IL-3 | IL-4 | IL-6 | IL-10 | IL-13 | GM-CSF | IFN-γ | TNF-α |
|----------|--------------|-------------------|-----------------|----|------|------|------|------|-------|-------|--------|-------|-------|
| PLP-spleen/PLP | 15 (15) | 10.2 | 3.8 | 0.58 | 1.11 | 0.02 | 0.22 | 0.05 | 1.00 | 0.53 | 1.44 | 0.11 |
| PLP-spleen/PLP + 2% 1F1/LR | 14 (15) | 13 | 2.2 | 0.05 | 0.12 | 1.10 | 0.08 | 15.00 | 3.45 | 81.25 | 0.32 | 0.12 | 0.15 |
| PLP-spleen/PLP + 10% 1F1/LR | 12 (15) | 19 | 1.2 | 0.001 | <0.05 | 2.65 | 0.14 | 21.75 | 14.70 | 301.00 | 0.64 | 0.02 | 0.23 |
| PLP-spleen/PLP + 20% 1F1/LR | 1 (15) | 22 | 0 | 0.001 | <0.05 | 4.05 | 0.17 | 80.50 | 15.95 | 371.00 | 0.16 | <0.01 | 0.30 |

*Cells from 15 ml of culture set up as described in Materials and Methods were adoptively transferred into mice (five mice per group). Results are from a total of three experiments.

†Significance from control group was determined by Student’s t test.

‡Cytokines were measured by ELISA from cultures used for adoptive transfer from one representative experiment.

To determine whether this inhibitory effect was mediated by a soluble factor or cell-cell contact, we established a transwell system in which PLP-spleen cells and 1F1 cells were separated by a 0.4-

m membrane. Transwell cocultures in which PLP-spleen cells and 10% 1F1 cells were separated by a 0.4-

m membrane did not transfer disease when both peptides were included in the culture, whereas control cultures without LR peptide did transfer disease (data not shown), suggesting that the 1F1 inhibition is mediated by soluble factors.

To examine the role of Th2 cytokines in the modulation of the adoptive transfer model of EAE by 1F1 cells, neutralizing Abs or reagents to IL-4, IL-10, or IL-13 were included in the 1F1/PLP-

spleen cocultures as indicated in Fig. 4. ELISA (data not shown) confirmed neutralization of each cytokine in the coculture supernatant. Neutralization of either IL-4 or IL-13 alone partially abrogated the protective effect of the 1F1 cells. In each case, the disease observed was less severe than that of the activated spleen-

PLP controls, suggesting that other factors made by 1F1 could also contribute to the suppression of EAE. Anti-IL-10 did not neutralize any of the protective effects of 1F1 cells and, interestingly, anti-

IL-10 in combination with anti-IL-4 appeared to counteract the previously observed neutralization effect of anti-IL-4 alone. Animals transferred with PLP-spleen/1F1/LR cocultures containing combinations of anti-IL-4, anti-IL-10, and anti-IL-13 or anti-IL-4 and anti-IL-13 had significantly more disease than mice receiving untreated cocultures. Cultures containing all three Abs however transferred slightly less disease (less severity and delayed onset) compared with PLP-spleen control cultures not containing 1F1 cells, suggesting that not all down-regulatory effects had been neutralized. Interestingly, inclusion of all three Abs in control PLP-

spleen cocultures resulted in the transfer of significantly less...
disease (Fig. 4, bottom left panel) when compared with PLP-spleen/PLP cultures with no Abs. These results suggested that the combination of these three cytokines participate in the maximal EAE response transferred by the PLP-spleen/PLP cultures alone. Role of TGF-β in suppression of EAE by Th2 clone 1F1

TGF-β was detected both in activated 1F1 supernatants (670 pg/ml) and at low levels of mRNA by DNA chip analysis. As shown in Fig. 5, addition of anti-TGF-β to PLP-spleen-1F1 cocultures partially neutralized the protective effect of 1F1, suggesting that TGF-β plays a role in suppressing transfer of EAE by coculture with the 1F1 cells. Since neutralization of IL-13 alone (Fig. 4) removed a significant amount of 1F1 suppression, we tested whether the combination of anti-IL-13 and anti-TGF-β would be additive. Interestingly, neutralization of the combination of IL-13 and TGF-β was no more effective than neutralization of either cytokine alone. This effect was also observed with the combination of anti-IL-4 and anti-TGF-β (data not shown). Neutralization of the four cytokines (IL-4, IL-13, IL-10, and TGF-β) however not only abrogated the protective effect of the 1F1 cells but actually exacerbated disease severity when compared with PLP-spleen cells cultured without 1F1 cells.

Combinations are more effective than individual Th2 cytokines

To determine whether individual Th2 cytokines suppress adoptive transfer of EAE, each cytokine was added separately to cultures of PLP-spleen cells at the approximate concentrations that were measured in cultures containing activated 1F1 cells that did not transfer disease. As shown in Fig. 6, both IL-4 (25 ng/ml) and TGF-β alone were able to significantly inhibit EAE, whereas IL-13 (100 ng/ml) was only partially inhibitory. Addition of IL-9 (10 ng/ml) or IL-10 (50 ng/ml) did not significantly affect the disease course whereas GM-CSF appeared to slightly enhance the severity of EAE. Fig. 7 shows that the combination of a suboptimal dose of IL-4 (5 ng/ml) with either IL-10 or IL-13 was synergistic in suppressing disease and was as effective as the combination including the four cytokines IL-4, IL-10, IL-13, and TGF-β. Levels of the cytokines IL-2, IL-3, IL-4, IL-10, IL-17, GM-CSF, IFN-γ, and TNF-α from supernatants of cytokine-treated cultures were measured by

FIGURE 2. DNA chip expression analysis of cytokines and cell surface molecules of 1F1 cells. 1F1 cells were activated with plate-bound anti-TCR as described. Frequency of cytokine (top) or cell surface molecule (bottom) mRNA per 10⁶ messages was determined by hybridization to oligonucleotide arrays as described. Genes were considered present if ≥10 copies were detected. Gene expression was considered significantly induced if the frequency was increased >3-fold compared with the t = 0 time point. This experiment is representative of two separate experiments. One lot of in vitro transcription material from stimulated and nonstimulated 1F1 was applied to three different lots of DNA chips with similar results.

FIGURE 3. Cytokines secreted by 1F1 cells. Cytokines were measured by ELISA as described from supernatants of 1F1 cultures stimulated with LR peptide and irradiated splenocyte feeder cells for 72 h. Results are expressed as the means ± SD of three separate experiments.
Table II shows each measured cytokine expressed as a percentage of the control culture. The single cytokines which had the greatest effect in modulating transfer of disease, 1.0 ng/ml TGF-β, 25 ng/ml IL-4, 300 ng/ml IL-13, and IL-4 plus IL-13, also contained significantly lower levels of IFN-γ compared with control PLP-spleen cultures. Cells from these cultures also transferred significantly less disease (Figs. 6 and 7). IL-4 and IL-10 were present at low levels in some cultures, but none of the cytokine treatments significantly altered these levels from controls (data not shown). TGF-β suppressed the levels of all measured cytokines with the exception of IL-17, whereas IL-4-treated cultures had increased levels of IL-2, IL-3, GM-CSF, and TNF-α and decreased levels of IL-17. Although the combination of IL-4 and IL-13 was more effective in suppressing transfer of disease than IL-13 alone, both cultures showed reduced levels of IFN-γ but only the IL-4 and IL-13 combination showed reduced levels of IL-17.

FIGURE 4. Neutralization of cytokines in 1F1 spleen cocultures. Mice were monitored for clinical signs of EAE after adoptive transfer of PLP-spleen cultures (◦) or PLP-spleen cells plus 1F1 cells stimulated with both PLP and LR peptides (■). The indicated neutralizing Abs (upper left of each panel) were added to PLP-spleen plus 1F1 cells with both peptides (Δ) or PLP-spleen (X) before adoptive transfer. Each experiment contained 20 mice with five mice per group. Listed for each curve are incidence of EAE, average highest clinical score, and the mean day onset. Significance was determined with Student’s t test. The p value represents the comparison between cultures containing 1F1 with (Δ) and without (X) Ab. The p’ represents the comparison between PLP-spleen cultures with (X) and without (◦) Ab. Each panel is representative of at least three experiments.
Th2 cytokines inhibit adoptive transfer of EAE

Day after adoptive transfer

FIGURE 5. Role of TGF-β in suppression of adoptive transfer of EAE by 1F1 cells. Mice were monitored for clinical signs of EAE after adoptive transfer of PLP-spleen cultures with PLP peptide (●) or PLP-spleen cells plus 1F1 cells stimulated with PLP and LR peptides (■). The indicated neutralizing Abs (upper left of each panel) were added to PLP-spleen/PLP (X) and PLP-spleen plus 1F1 cells with both peptides (▲). Each experiment contained 20 mice with five mice per group. Listed for each curve are incidence of EAE, average highest clinical scores, and the mean day onset. Significance was determined with Student’s t test. The p value represents the comparison between cultures containing 1F1 with (▲) and without (■) Ab. The p’ represents the comparison between PLP-spleen cultures with (X) and without (●) Ab. Each panel is representative of at least three experiments.

Discussion

Disease course in EAE is determined by the number and activation state of encephalitogenic T cells, as well as the cellular context in which the T cells are activated (15–17). Th2 cells have been proposed as a regulatory cell type in EAE, and introduction of Th2 cytokines or cells during the initial priming phases of the immune response can result in disease suppression (11, 13, 18, 19, 20). However, Khoruts et al. (15) found that adoptive transfer of Ag-specific polarized Th2 populations did not inhibit EAE induced with Th1 cells. In addition, EAE was induced after adoptive transfer of Th2 cells into RAG-1-deficient mice (16). Thus, the precise mechanisms by which Th2 cells and cytokines can modulate disease remain to be defined. Immunization of mice with analogues of encephalitogenic peptides such as LR can protect mice from actively induced EAE. A previous report shows that LR induces a cross-reactive Th2/Th0 T cell population which protects mice from EAE in a bystander fashion (20). The experiments presented in this report demonstrate that a Th2 cell clone, responding exclusively to an APL of a known encephalitogenic peptide, can regulate EAE mediated by an encephalitogenic PLP139-151-specific Th1 population. We have conducted experiments to evaluate the effects of individual Th2 cytokines and combinations of Th2 cytokines made by a LR-responsive clone on primed, encephalitogenic T cells and EAE.

We have used an adoptive transfer model of EAE induced with PLP139-151 in the SJL mouse to examine the potential role of Th2 cells and cytokines in modulating the disease induced by PLP-primed T cells. We demonstrated that coculture of a Th2 cell line, 1F1, with PLP-primed encephalitogenic Th1 cells prevented adoptive transfer of EAE. Disease prevention was dependent upon activation of the Th2 cells, since cocultures containing 1F1 cells without the activating LR peptide transferred EAE. The inhibition by the 1F1 cells was due to soluble bystander effects since the 1F1 cells only responded to a non-cross-reactive analogue peptide included in the culture and were still inhibitory if activated on separate sides of a transwell membrane (data not shown).

We went on to define the mechanism by which the 1F1 cells mediate disease suppression and have systematically examined the role of cytokines produced by these cells. Both gene chip analysis and ELISA identified potential immunoregulatory cytokines expressed and secreted by the 1F1 cells. By neutralizing and add-back experiments, our results showed that several Th2 cytokines expressed and secreted by the 1F1 cells only responded to a non-cross-reactive analogue peptide included in the culture and were still inhibitory if activated on separate sides of a transwell membrane.

We found that IL-10 alone did not play a major role in down-regulating the induction of disease by adoptive transfer. The described role for IL-10 in the regulation of EAE is complex, having both exacerbating and protective functions depending on the timing of IL-10 administration and, most likely, the types of other cytokines that are present. IL-10 has potent down-regulatory effects during the priming and induction phases of the response to autoantigen (26–28) as well as in the recovery phase. If administered at the same time as PLP, IL-10 delayed disease onset and severity (27). Mice transgenic for IL-10 were resistant to the induction of EAE (9), suggesting that encephalitogenic cells do not develop in the presence of IL-10. In later stages of disease, IL-10 mRNA expression is increased during the recovery phase (8, 10) and IL-10-deficient mice develop a more chronic disease following immunization with myelin oligodendrocyte glycoprotein (9, 29), both suggesting that IL-10 is required for recovery from EAE. In adoptive transfer models however IL-10 is ineffective in inhibiting disease. IL-10 pretreatment of lymph node cells sensitized to MBP before adoptive transfer had no effect on their ability to transfer disease (12). Similarly, treatment of mice with IL-10 at various intervals after adoptive transfer showed no or a worsening effect on clinical signs of EAE. These results suggest that IL-10 alone has little or no effect on primed Th1 cells or established disease. As
shown by the experiments described in this report, addition of as much as 50 ng/ml IL-10 had little or no effect on disease transferred by activated T cells. We have also been unable to inhibit proliferation of PLP-spleen cells to PLP peptide in vitro (data not shown).

Neutralization of IL-10 in the 1F1 cocultures suggested that this cytokine alone does not play a significant role in suppressing disease and that additional cytokines from 1F1 are involved in mediating suppression. Moreover, 1F1 cocultures with IL-10 present (treated with anti-IL-4 and anti IL-13) transferred more severe disease than cultures without IL-10, implying an enhancing role for IL-10. Removal of only IL-4 and IL-10 from the cocultures (leaving IL-13 and TGF-β) resulted in disease, again suggesting that IL-10 contributes to enhanced encephalitogenicity. In control PLP-spleen cultures as well, neutralization of IL-10 alone resulted in less severe disease and neutralization of the combination of IL-10, IL-4, and IL-13 resulted in significantly less disease, suggesting that IL-10 in combination with other cytokines could enhance encephalitogenicity. These results from the experiments with Abs to IL-10 suggest that the suppressive effects of IL-4 and IL-13 in 1F1 cocultures are more profound in the absence of IL-10. The direct cytokine add-back experiments demonstrate that IL-10 alone neither suppresses disease transfer nor significantly enhances EAE when included at the 10-ng/ml level, but can suppress disease when included with a low level of IL-4. The effect of IL-10 appears to be complex and may enhance or suppress Th1 effector function depending upon the nature of the cytokine milieu.

In contrast to IL-10, we observed that activation of PLP-specific spleen cells in the presence of IL-4 inhibited adoptive transfer of EAE (Fig. 6). These data are in agreement with studies in the

![Diagram of cytokine effects on disease transfer](http://www.jimmunol.org/Downloaded from fig6.jpg)
MBP-induced model of EAE, where Racke et al. (11) have shown that administration of IL-4 after adoptive transfer of MBP-specific lymph node cells limited the severity of disease. Similarly in the MBP model, targeted delivery of IL-4 by retrovirally transduced encephalitogenic T cell hybridomas 10 days after immunization resulted in reduced onset and severity of disease (30). IL-4 plays a less significant role in regulating the induction of EAE and in recovery from actively induced EAE. IL-4-transgenic mice developed more severe EAE with similar recovery times compared with wild-type controls (9, 10).

IL-4 was particularly effective in inhibition of adoptive transfer of EAE in combination with other Th2 cytokines. We observed that IL-4 synergized with IL-10 to inhibit adoptive transfer of EAE.

**FIGURE 7.** Regulation of adoptive transfer of EAE by combinations of cytokines. PLP-spleen cells were cultured with PLP peptide alone (●) or with PLP peptide plus the cytokines indicated in each panel. After 72 h, 1–2 x 10^7 cells were adoptively transferred into naive mice and scored for disease. Each curve represents five mice per group. Incidence of EAE, average highest disease scores and mean day onset are listed in each panel. The p values, determined by Student's t test, represent the comparison of the cytokine-treated cultures with PLP-spleen/PLP (●) cultures. Each panel is representative of three experiments.

**Table II.** Cytokine ELISA of TGF-β-, IL-4-, and IL-13-treated PLP-spleen cultures prior to adoptive transfer

| Culture                          | Cytokines as % PLP-Spleen/PLP Response^a |
|----------------------------------|------------------------------------------|
|                                  | IL-2          | IL-3          | GM-CSF       | IFN-γ        | TNF-α        | IL-17        |
| PLP-spleen/PLP                  | 100.00        | 100.00        | 100.00       | 100.00       | 100.00       | 100.00       |
| PLP-spleen/PLP + TGF-β (1.0 ng/ml) | 53 ± 32       | 0.310^b      | 17 ± 01      | 0.006*       | 14 ± 02      | 0.013*       | 35 ± 26      | 0.024*       | 100.00       | 0.046*       | 114 ± 31      | 0.360       |
| PLP-spleen/PLP + TGF-β (0.1 ng/ml) | 56 ± 06       | 0.057        | 33 ± 15      | 0.103        | 44 ± 03      | 0.025*       | 37 ± 42      | 0.100        | 46 ± 06      | 0.052        | 129 ± 19      | 0.050*       |
| PLP-spleen/PLP + IL-4 (25 ng/ml) | 301 ± 82      | 0.048^b      | 330 ± 04     | 0.008*       | 282 ± 25     | 0.060        | 17 ± 02      | 0.009*       | 330 ± 105    | 0.056        | 49 ± 09       | 0.010*       |
| PLP-spleen/PLP + IL-4 (5 ng/ml) | 170 ± 90      | 0.237        | 289 ± 27     | 0.064        | 252 ± 33     | 0.096        | 100 ± 13     | 0.970        | 179 ± 117    | 0.170        | 55 ± 09       | 0.003*       |
| PLP-spleen/PLP + IL-13 (300 ng/ml) | 100 ± 10       | 0.915        | 113 ± 07     | 0.226        | 110 ± 18     | 0.550        | 29 ± 07      | 0.040*       | 140 ± 40     | 0.193        | 89 ± 14       | 0.200        |
| PLP-spleen/PLP + IL-13 (100 ng/ml) | 97 ± 08        | 0.610        | 121 ± 17     | 0.331        | 115 ± 11     | 0.290        | 37 ± 29      | 0.210        | 138 ± 48     | 0.254        | 95 ± 17       | 0.630        |
| PLP-spleen/PLP + IL-4 (5 ng/ml) | 253 ± 43       | 0.024^b      | 329 ± 72     | 0.137        | 243 ± 18     | 0.056        | 25 ± 04      | 0.021*       | 264 ± 155    | 0.156        | 46 ± 10       | 0.002*       |

^a Cytokines were measured by ELISA as described and are expressed as the percentage of the control PLP-spleen + PLP response.

^b Mean ± SD of four separate experiments.

p value as determined by Student's t test. *p ≤ 0.05 is considered significantly different from the control culture.
in experiments using suboptimal concentrations of IL-4, even though IL-10 alone had no effect on disease severity. Surprisingly, neutralization of IL-10 and IL-4 in the 1F1 cocultures had no effect on disease severity, although neutralization of IL-4 alone partially abrogated the suppressive effects of the 1F1 cells. Interference between the suppressive effects mediated by IL-4 and IL-10 was also seen in the direct disease model by Nagelkerken et al. (27). In this experiment IL-4 had no effect on the severity of EAE when injected at the same time as PLP peptide in SJL mice, but abrogated the inhibitory effects of IL-10 when administered with IL-10. In our hands, IL-4, unlike IL-13 or TGF-β alone, also significantly reduced the levels of IL-17 when compared with control PLP-spleen cultures. IL-17 has recently been reported to stimulate the production of IL-1 and TNF-α from macrophages, suggesting a role for IL-17 in promoting an inflammatory response (31).

We found that murine IL-13 can inhibit the adoptive transfer of EAE in PLP-induced disease in SJL mice. Additionally, neutralization of IL-13 in the 1F1-spleen cell cocultures demonstrated a role for IL-13 in suppressing disease transfer. In agreement with our data, expression of human IL-13 by a hybridoma cell line adoptively transferred into rats resulted in suppression of clinical signs of EAE induced by immunization with MBP-CFA (32). The results in the report show that IL-13 synergized with IL-4 to inhibit adoptive transfer of EAE, although both IL-4 and IL-13 were able to independently inhibit adoptive transfer of EAE at high concentrations.

The mechanism for suppression by IL-13 is not clear, although it is likely to be functioning through APC in the coculture. IL-13 has been reported to exert both pro- and anti-inflammatory effects on monocytes. IL-13 up-regulates the capacity of monocytes to present Ag, resulting in increased proliferation of T cells to Ag (33). IL-13 can have a priming immunostimulatory effect on monocytes if added before an inflammatory stimulus and suppressive effect when added after (34). In our hands, proliferation of PLP-specific lymph node or spleen populations in the presence of PLP peptide over a wide range of IL-13 concentrations was not inhibited and was enhanced at higher concentrations of IL-13 (data not shown).

IL-4 and IL-13 use a shared receptor on monocytes and other APC (35). Both IL-4 and IL-13 can antagonize the effects of IFN-γ and down-regulate inflammatory cytokines (33). We observed decreased levels of IFN-γ in cocultures containing 1F1 cells and in PLP-spleen cultures containing the combination of IL-4, IL-13, and IL-10, suggesting that IFN-γ made by the Th1 population was down-regulated in these cultures. Both IL-13 alone and IL-4 alone also decreased IFN-γ levels in these studies. In contrast, we observed increased levels of TNF-α in PLP-spleen activation cultures containing IL-13 alone or IL-4 and IL-13 in combination. IL-4 exhibits comparable anti-inflammatory effects to IL-13, but IL-4 and IL-13 do not exhibit synergistic effects on monocytes (33). IL-4 unlike IL-13 can enhance proliferation of activated T cells (33), and the synergy we observe between IL-13 and IL-4 may be due to the actions of IL-13 and IL-4 on distinct APC and T cell populations, respectively.

As with IL-13, TGF-β can have both proinflammatory and anti-inflammatory effects, depending on concentration, the presence of other cytokines, and activation status of the T cell (36). Neutralization of TGF-β in the 1F1/PLP-spleen cocultures showed that this cytokine contributed significantly to the inhibition of EAE by 1F1 cells. Addition of as little as 100 pg/ml of TGF-β to the culture before adoptive transfer also inhibited development of EAE. Of all of the cytokines made by the 1F1 cells, we found that only TGF-β also inhibits the proliferation of PLP-specific LNC or spleen cell populations in vitro (data not shown). Previous reports have described the in vivo inhibitory role of TGF-β in EAE (37–40) and have shown that addition of anti-TGF-β exacerbates EAE, suggesting a regulatory role for endogenous TGF-β (41, 42). We found that addition of low levels of TGF-β alone inhibited adoptive transfer of EAE by PLP-spleen cells and suppressed levels of Th1 cytokines in the culture supernatant. Two additional experiments supported inhibitory roles for TGF-β in our adoptive transfer model of EAE. Neutralization of TGF-β in cocultures of 1F1 cells with PLP-spleen restored disease transfer and addition of anti-TGF-β in combination with Abs to IL-4, IL-10, and IL-13 would sometimes increase disease severity, supporting the concept that TGF-β was itself an endogenous regulator of the Th1 population.

TGF-β in combination with IL-4, IL-10, and IL-13 showed the most profound inhibitory effect on both the transfer of EAE and the production of Th1 cytokines in the culture supernatant, suggesting that the presence of additional Th2 cytokines are important for a maximum inhibitory effect.

Other 1F1 cytokines evaluated in this adoptive transfer model were IL-5, IL-9, and GM-CSF. Not unexpectedly, IL-5, with reported activities on eosinophils and B cells, and IL-9, with activities on mast cells and some T cells (43, 44), were found to have negligible effects on the adoptive transfer of EAE in this report. Preliminary results with weakly encephalitogenic PLP-spleen populations however suggest that IL-9 may have a subtle inhibitory effect on adoptive transfer of EAE. We have found that IL-9 enhances proliferation of activated T cell populations, and it is formally possible that the IL-9 treatment allows one to transfer lower numbers of encephalitogenic T cells for disease induction. GM-CSF on the other hand enhances Ag presentation and secretion of inflammatory cytokines in macrophages (45). In this report, addition of a low level of GM-CSF to PLP-spleen cultures had no effect whereas inclusion of higher levels led to a marginal enhancing effect in the adoptive transfer of EAE.

Other cytokines made by 1F1 cells such as IL-6 and IL-3, not evaluated in adoptive transfer experiments presented in this report, have been implicated to play a role in encephalitogenicity. EAE cannot be induced in IL-6-deficient mice (46, 47) nor can IL-6-deficient myelin oligodendrocyte glycoprotein-specific T cells transfer disease, suggesting that IL-6 is essential for the development of disease (46). IL-3 has also been reported to enhance the growth of encephalitogenic T cells (48), and we have found that IL-3 enhances the proliferation of Th1 cells as well (data not shown).

In summary, our results indicate that the most effective means of modulating EAE by immune deviation results from the induction of certain combinations of Th2 cytokines, which can modulate EAE induced by the adoptive transfer of primed, encephalitogenic T cells. The most effective combinations included TGF-β, IL-4, and IL-13. This combination of cytokines is similar to that observed in the previously described Th3 subset of regulatory cells (13) and is predicted to have effects on both the APC and T cell populations. Our data support the idea that induction of specific combinations of Th2/Th3 cytokines could be of potential therapeutic benefit, in that suppression of disease mediated by primed, encephalitogenic T cells was observed. Our data also suggest that the variation in results obtained in other studies addressing the role of Th2 cells in modulating EAE may be due to differences in the precise cytokine profiles expressed by these various populations.

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