Coupling of Peripheral Tolerance to Endogenous Interleukin 10 Promotes Effective Modulation of Myelin-activated T Cells and Ameliorates Experimental Allergic Encephalomyelitis

By Kevin L. Legge, Booki Min, J. Jeremiah Bell, Jacque C. Caprio, Lequn Li, R andal K. Gregg, and Habib Zaghouani

From the Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996

Abstract

Several immune-based approaches are being considered for modulation of inflammatory T cells and amelioration of autoimmune diseases. The most recent strategies include simulation of peripheral self-tolerance by injection of adjuvant free antigen, local delivery of cytokines by genetically altered T cells, and interference with the function of costimulatory molecules. Although promising results have been obtained from these studies that define mechanisms of T cell modulation, efficacy, practicality, and toxicity, concerns remain unsolved, thereby justifying further investigations to define alternatives for effective downregulation of aggressive T cells. In prior studies, we demonstrated that an immunoglobulin (Ig) chimera carrying the encephalitogenic proteolipid protein (PLP)1 peptide corresponding to amino acid sequence 139-151 of PLP, Ig-PLP1, is presented to T cells ~100-fold better than free PLP1. Here, we demonstrate that aggregation endows Ig-PLP1 with an additional feature, namely, induction of interleukin (IL)-10 production by macrophages and dendritic cells, both of which are antigen-presenting cells (APCs). These functions synergize in vivo and drive effective modulation of autoimmunity. Indeed, it is shown that animals with ongoing active experimental allergic encephalomyelitis dramatically reduce the severity of their paralysis when treated with adjuvant free aggregated Ig-PLP1. Moreover, IL-10 displays bystander antagonism on unrelated autoreactive T cells, allowing for reversal of disease involving multiple epitopes. Therefore, aggregated Ig-PLP1 likely brings together a peripheral T cell tolerance mechanism emanating from peptide presentation by APCs expressing suboptimal costimulatory molecules and IL-10 bystander suppression to drive a dual-modal T cell modulation system effective for reversal of autoimmunity involving several epitopes and diverse T cell specificities.

Key words: autoimmunity • antigen delivery • bystander downregulation • cytokine antagonism • T cell modulation

Introduction

During development, T cells whose antigen receptors are devoid of self-reactivity exit the thymus and participate in immune surveillance, whereas those bearing receptors endowed with self-reactivity are negatively selected and deleted by programmed cell death (1). This process of T cell screening and selection, known as central tolerance, requires the antigen to be available in the thymus in sufficient quantities and in a form presentable by MHC molecules (2-5). Although central tolerance exerts a tight control on the shaping of the T cell repertoire, some self-reactive T cells still escape the thymus and migrate to the periphery (6-8). If the antigen is available in the periphery, a second round of T cell screening, known as peripheral tolerance, will follow to further minimize autoreactivity (3, 9-13). Presumably, peripheral tolerance develops as a consequence of presentation of autoantigen by nonactivated APCs expressing minimal or no costimulatory molecules (3, 14).

For sequestered autoantigens that are not available for presentation in either the thymus or the periphery, the corresponding T cells will circulate harmlessly. However, events that trigger exposure of those autoantigens, which are usually accompanied by conditions favorable for activation of local APCs, lead to an optimal presentation to and
activation of the circulating T cells (15–18). The results of this T cell activation may be the escalation of inflammatory reactions and injury of specific tissues and organs (19–21).

Supply of antigen in an adjuvant free form might not stimulate the expression of costimulatory molecules on APCs and thereby drive an antigen presentation inadequate for T cell activation (3, 22, 23). Prior studies have in fact indicated that this approach augments autoreactive T cells and promotes recovery from EAE (24–26). However, the usefulness of this approach for modulation of autoimmunity is hampered by the unlimited availability of autoantigen at the injury site and the consequent continuous activation of the self-reactive T cells. In addition, as bystander suppression is unlikely to occur, the approach holds little promise for modulation of T cell-mediated autoimmunity involving multiple antigens. To overcome these issues, an in vitro approach that uses plasmid (27) and viral (28, 29) vector-driven modulatory cytokines was adopted. Indeed, autoreactive T cell clones or hybridomas expressing the cytokine IL-4 or IL-10, as a consequence of transfection or infection, induced recovery from disease when injected into animals with ongoing experimental autoimmune encephalomyelitis (EAE) (27, 28). This is a promising approach and bodes well for the development of practical strategies that could combine both peripheral tolerance and cytokine antagonism to combat autoimmunity.

It has previously been shown that peptide delivery on Igs increases presentation by 100–1,000-fold relative to free peptide (30, 31). It is also known that cross-linking of Fc receptors (FcRs) on target cells by antigen–antibody complexes can trigger the production of cytokines (32–34). Moreover, aggregation of Igs confers the effector functions associated with the Fc fragment without the need for complex formation (35, 36). Here, the encephalitogenic proteolipid protein (PLP1): it was genetically engineered into an Ig molecule (30), and the resulting Ig-PLP1 chimera was aggregated and assayed for modulation of autoreactive T cells and amelioration of active EAE. The results show that aggregated (agg) Ig-PLP1 induced IL-10 secretion by both macrophages and dendritic cells (DCs) but not B cells. In vitro, APCs incubated with agg Ig-PLP1 presented PLP1 to specific T cells. However, because of the IL-10 secreted by the presenting APCs, IFN-γ production by the T cells was impaired. In vivo, when soluble (sol) Ig-PLP1 was injected into mice with ongoing EAE, the severity of disease was slightly reduced. However, when mice were given agg Ig-PLP1, full recovery was achieved. Moreover, agg Ig-PLP1 was able to modulate disease induced by either an encephalitogenic peptide other than PLP1 or by a central nervous system (CNS) homologene. Neutralization of endogenous IL-10 by injection of anti–IL-10 antibody during administration of agg Ig-PLP1 restored disease severity. Therefore, agg Ig-PLP1 triggers IL-10 production by APCs, drives inadequate peripheral presentation of PLP1, and couples both events to modulate autoimmunity involving diverse T cell specificities.

**Materials and Methods**

**Animals.** SJL/J (H-2b) mice were purchased from Harlan, bred, and maintained in our animal care facility for the duration of the experiments.

**Antigens.** Peptides. The peptides used in this study were purchased from Research Genetics, and were HPLC purified to >90% purity. PLP1 peptide (HSLGKWLGHPDKF) encompasses amino acid (aa) residues 139–151 of PLP and is encephalitogenic in SJL/J mice (37). PLP2 peptide (NTWTTTCQSIAPPSK), encompassing aa 178–191 of PLP, is likewise encephalitogenic in SJL/J (38). Myelin basic protein (MBP)-3 peptide (VHFFKNIVTPRP) corresponding to aa residues 87–99 of MBP is also I-Ak restricted, and induces EAE in SJL/J mice (39). Hemagglutinin (HA) peptide, an I-Ek–restricted epitope (31), corresponding to aa residues 110–120 of HA, was used for negative control purposes.

**CNS Homogenate.** 50 frozen undstripped rat brains (Pel-freez Biologicals) were homogenized in PBS using a Waring blender and adjusted to 300 mg/ml with PBS. CNS homogenate was stored at −20°C.

**Ig-PLP Chimeras.** The Ig-PLP1 and Ig-PLP2 chimeras harbored, within the heavy chain CDR3 region, PLP1 and PLP2, respectively, and have been described previously (30, 40, 41). Ig-W is the parental IgG2b antiarsonate antibody, 91A3, not encompassing any PLP peptide, and has been described elsewhere (30). Large-scale cultures of Ig-W, Ig-PLP1, and Ig-PLP2 transfectants were performed in DMEM containing 10% serum supreme (BioWhittaker) and purified on separate rat anti-mouse κ chain sepharose columns to avoid cross-contamination. Subsequently, the Ig chimeras were dialyzed against PBS and concentrated on collagen membranes (Schleicher & Schuell). The chimeras were aggregated by precipitation with 50% saturated (NH4)2SO4 as described (42). In brief, filtered 100% saturated (NH4)2SO4 was added at an equal volume to the sol Ig chimera preparation. The mixture was incubated at 24°C for 1 h with gentle agitation every 10 min. Subsequently, the samples were spun down at 10,000 rpm, and the pellet was resuspended at 1 mg/ml in PBS. Electrophoresis on a 10% acrylamide gel indicated that the sol Ig chimera entered the gel and migrated ~160 kDa. However, the agg Ig chimera did not enter the gel. Knowing that we applied the equivalent of 2 μg of agg Ig chimera, and that the sensitivity of the technology is 0.1 μg, we concluded that at least 95% of the agg Ig chimera preparation is in an aggregate form.

**Induction of EAE**

6–8-wk-old mice were induced for EAE by subcutaneous injection in the footpads, and at the base of the limbs and tail with a 200 μl IFA/PBS (vol/vol) solution containing the autoantigen and 200 μg Mycobacterium tuberculosis H37Rv a 6 h later, the mice were given intravenous 5 × 106 inactivated Bordetella pertussis (Bioport). A second injection of B. pertussis was given after 48 h. Subsequently, the mice were scored daily for clinical signs of EAE as follows: 0, no clinical score; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb paralysis; and 5, moribund or death. In some experiments, purified pertussis toxin

---

**Notes:**

- aAbbreviations used in this paper: agg, aggregated; aa, amino acid; CNS, central nervous system; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; HA, hemagglutinin; MBP, myelin basic protein; PLP, proteolipid protein; sol, soluble.
pertussis organism.

Treatment of EAE with Ig-PLP1

Mice induced for EAE with PLP1, PLP2, a mixture of PLP1 plus PLP2, or CNS homogenate began receiving treatment with Ig-PLP1 after loss of tail tone, which occurs regularly between days 6 and 8 after disease induction. Treatment injections were given intraperitoneally in PBS on days 9, 13, and 17.

Histopathology

Mice treated with agg Ig-PLP1 or agg Ig-W were killed at the peak of the initial phase of disease (day 28 after disease induction), and the brain and spinal cord were removed, fixed with formalin, and embedded in paraffin. Serial cross-sections (6 μm) from the cerebellum, cerebrum, and lumbar cord were cut and stained with hematoxylin and eosin. Perivascular clusters containing at least 20 mononuclear cells were counted as inflammatory foci.

T Cells

TCC-PLP1-1B10. Adult SJL mice were immunized subcutaneously with 100 μg PLP1 peptide in CFA, and 10 d later the draining LN’s were removed and the cells (5 × 10^6 cells/ml) were stimulated with PLP1 (15 μg/ml). After 5 d, the blasts were separated on a Histopaque gradient (Sigma-Aldrich), and then restimulated with peptide and fresh irradiated (3000 rads) syngeneic APCs. 10 d later, the cells were washed, resuspended in media containing 10% T-Stim (Collaborative Research), and rested for 2041

Cytokine Detection

ELISA. ELISA was done according to BD PharMingen’s standard protocol. The capture antibodies were rat anti–mouse IL-2, JES6-1A12; rat anti–mouse IL-4, 11B11; rat anti–mouse IFN-γ, R4-6A2; rat anti–mouse IL-10, JES5-2A5; and rat anti–mouse IL-5, TRFK5. The biotinylated anticytokine antibodies were rat anti–mouse IL-2, JES6-5H4; rat anti–mouse IL-4, BVD6-24G2; rat anti–mouse IFN-γ, XMG1.2; rat anti–mouse IL-10, JES5-16E3; and rat anti–mouse IL-5, TRFK4. ELISA for the detection of active TGF-β was performed using the human TGF-β1 DuoSet kit (Enzyme) according to the manufacturer’s instructions. Bound ligand was revealed using the TMB micro-well peroxidase substrate system (Kirkegaard & Perry Laboratories). Assays were read on a SpectraMax 340 counter (Molecular Devices). Graded amounts of recombinant mouse IL-2, IL-4, IFN-γ, IL-10, IL-5, and TGF-β were included in all experiments for construction of standard curves. The cytokine concentration in culture supernatants was estimated by extrapolation from the linear portion of the standard curve.

Enzyme-linked Immunosorbent Assay (ELISPOT). ELISPOT assays were used to measure the cytokines produced by LN T cells upon stimulation with antigen as described (41). In brief, LN cells (5 × 10^6 cells/100 μl) and the antigen (100 μl/well) were cultured with capture antibody for 24 h. Bound cytokines were revealed with peroxidase and anticytokine antibodies. The anticytokine antibody pairs used here were those described for the ELISA technique. Spots were counted under a dissecting microscope.

Stimulation of Cytokine Production by TCC-PLP1-1B10

Stimulation was performed with both irradiated and fixed APCs. In one case, SJL/J splenocytes were irradiated (3000 rads) and plated (5 × 10^6 cells/well/50 μl) with graded concentrations of antigens (100 μl/well). After 1 h, TCC-PLP1-1B10 cells (0.5 × 10^6 cells/well/50 μl) were added and the culture was incubated for 24 h. For fixed APCs, SJL/J spleen cells (10 × 10^6 cells/well/100 μl) were pulsed with graded amounts of antigen, fixed with 1% paraformaldehyde, and incubated with TCC-PLP1-1B10 cells (0.5 × 10^6 cells/well/100 μl) for 24 h. Detection and quantification of cytokines were then assessed by ELISA from 100 μl of culture supernatant.

Results

Soluble Ig-PLP1 Reduces Paralytic Severity and Suppresses Clinical Relapses in Mice with Ongoing EAE. Prevention and treatment of active autoimmune disease have been achieved by injection of adjuvant-free autoantigens or peptides (24–26, 45–49). However, repetitive injections of the autoantigens are required, and the disease rebounds when the supply of antigen is discontinued (48). One approach that may overcome these setbacks and modulate active disease is the delivery of the self-peptide on Igs. Igs have long half-lives and grant the peptides access to newly synthesized MHC molecules (31, 50), which could lead to efficient peptide loading onto MHC molecules (50) over an extended period of time. To test the Ig delivery system for treatment of active autoimmunity, SJL/J mice were induced for EAE with free PLP1 peptide, and when the clinical signs of disease became apparent the animals were...
given three injections of sol Ig-PLP1 in saline at 4-d intervals and assessed for reduction in disease severity. Control mice were given sol Ig-W, the parental Ig without any PLP1 peptide. The results illustrated in Fig. 1 show that mice treated with the sol Ig-W had an initial severe phase of paralysis with a mean maximal score of 3.7 \pm 0.5 and displayed relapses throughout the 120-d period of examination. The mice treated with sol Ig-PLP1, however, had a reduced severity of paralysis at the initial phase of disease with a mean maximal score of 2.5 \pm 0.3 (P < 0.005) and fully recovered by day 42. Mice treated with 10-fold excess of free PLP1 peptide had a slight reduction in the severity of paralysis at the initial phase of disease (mean maximal clinical score 3.0 \pm 0.2), but never recovered and underwent relapses throughout the entire 120-d observation period (Fig. 1).

Aggregated Ig-PLP1 Displays Higher Efficacy Than Soluble Ig-PLP1 in Reversing Active EAE. Binding of antigen-antibody complexes to FcRs on target cells induces the production of cytokines (34, 51, 52). IL-10, produced by macrophages upon exposure to antigen-antibody complexes, exerts antagonist effects on IL-12 production and reverses proinflammatory responses (34, 52). Similar to complexation of antibodies with antigen, aggregation confers to Igs the effector functions associated with the Fc fragment (35, 36). These include the binding of complement and cross-linking of FcRs. We then reasoned that aggregation of Ig-PLP1 should be able to cross-link FcRs on APCs and drive both cytokine production and efficient loading of PLP1 peptide onto MHC molecules. If this hypothesis proved accurate and IL-10 was among the cytokines produced by APCs, agg Ig-PLP1 should be much more efficient than sol Ig-PLP1 for modulation of active EAE. To investigate this issue, mice were induced for EAE with free PLP1 peptide and when signs of clinical EAE became apparent, they were given three injections of 300 \mu g agg Ig-PLP1 at 4-d intervals and assessed for signs of paralysis. Control mice were treated with agg Ig-W instead of agg Ig-PLP1. As can be seen in Fig. 2 a, the initial phase of paralytic disease severity was reduced from a mean maximum score of 3.3 \pm 0.3 in agg Ig-W-treated animals to 1.1 \pm 0.5 (P < 0.001) in the agg Ig-PLP1 recipient mice. In addition, the animals fully recovered within 9 d of completion of the treatment and never relapsed throughout the entire 120-d observation period, whereas agg Ig-W-treated mice never recovered and showed relapses throughout the entire period of clinical assessment. The effectiveness of agg Ig-PLP1 is also apparent when the paralytic clinical signs of agg Ig-PLP1-treated animals were compared with those of animals injected with sol Ig-PLP1 (P < 0.001) (Fig. 2 b). Indeed, the mean maximum clinical score was much lower and the recovery was faster. Histologic examination of the cerebellum at the peak of disease indicated a lower number of foci.
Table I. Treatment with Agg Ig-PLP1 Ablates Clinical and Histologic EAE

| Treatment     | Clinical EAE: mean maximum severity | Histologic EAE: foci/cross-section |
|---------------|------------------------------------|-----------------------------------|
|               | Cerebrum                           | Lumbar spinal cord                |
| Agg Ig-W      | 3.3 ± 0.3 (P < 0.001)              | 11.7 ± 2.1 (P < 0.001)            |
| Agg Ig-PLP1   | 1.1 ± 0.5                           | 6.5 ± 0.5 (P < 0.001)            |

6-8-wk-old mice were induced for EAE with PLP1 as described in Materials and Methods, and then treated with 300 μg agg Ig-PLP1 or agg Ig-W on days 9, 13, and 17 after disease induction and scored daily for clinical disease. The mean maximum severity was determined by averaging the maximal clinical score obtained from each mouse within a group. To determine histological disease, brains and spinal cords were removed from mice on day 28 after disease induction (peak of disease), fixed in formalin, paraffin embedded, serially cross-sectioned at 6 μm, and then stained with hematoxylin and eosin. Inflammatory foci represent a minimum of 20 mononuclear cells per perivascular cluster.
splenic APCs rather than TCC-PLP1-1B10. In fact, this statement is confirmed by the observation that IL-10 was undetectable when APCs, pulsed with agg Ig-PLP1, were washed and fixed with paraformaldehyde before incubation with TCC-PLP1-1B10 (Fig. 6). The other striking observation from the T cell cytokine assay was that the production of IFN-γ seemed to be decreased as IL-10 production by APCs increased (Fig. 5, c and e). To investigate this issue further, an extended range of Ig-PLP1 concentrations was used for stimulation of bulk and purified APCs, and IL-10 and IFN-γ production was assessed simultaneously from the same tissue culture well. The results presented in Fig. 7 clearly indicate that the IL-10 secreted by the APCs antagonizes the production of IFN-γ by the T cells. Indeed, when the stimulation assay was performed using splenocytes, purified DCs, or enriched peritoneal macrophages as APCs (all of which produce IL-10 upon incubation with agg Ig-PLP1) (Fig. 4), IFN-γ production by the T cells decreased dramatically and became undetectable as the production of IL-10 by APCs increased (Fig. 7, a-c). However, when B cells were used as APCs, which do not produce IL-10 upon incubation with agg Ig-PLP1 (Fig. 4 b), the secretion of IFN-γ by T cells was not affected (Fig. 7 d). Overall, these results indicate that agg Ig-PLP1 triggers IL-10 production by presenting APCs (DC and macrophage), and that IL-10 antagonizes the production of IFN-γ by the T cells.

Synergy between Endogenous IL-10 and Peripheral Tolerance for In Vivo Modulation of Aggressive T Cells. Systemic antigen given to animals without adjuvant usually drives tolerance operating through antigen presentation by peripheral APCs expressing minimal or no costimulatory molecules (3, 22, 23). Incubation of purified macrophages or DCs with sol or agg Ig-PLP1, which allows for efficient loading of peptide onto MHC class II molecules, does not lead to upregulation of B7-1, B7-2, or CD40 (data not shown). Furthermore, as agg Ig-PLP1 causes the production of IL-10 by APCs (Fig. 4), it is likely that IL-10, as has previously been shown (53, 54), inhibits upregulation of costimulatory molecules on APCs.
As IL-10 has been defined to antagonize Th1 cytokines (55) and possibly interfere with inflammatory functions, we postulated that the effectiveness of agg Ig-PLP1 in T cell modulation and reversal of disease lies on inadequate peptide presentation by APCs expressing minimal costimulatory molecules and the inhibitory function of IL-10 produced by such APCs. To test this hypothesis, mice were induced for EAE with PLP1 peptide, and when the signs of paralysis became apparent, the mice were given agg Ig-PLP1 together with anti–IL-10 antibody and assessed for reduction in disease severity. The results presented in Fig. 8 indicate that the severity of paralysis was restored when in vivo IL-10 was neutralized by the anti–IL-10 antibody. In fact, mice treated with agg Ig-PLP1 alone had a mean maximal clinical score of 1.1 ± 0.5, whereas the mice injected with both agg Ig-PLP1 and anti–IL-10 antibody had a score of 3.0 ± 0.3, which is comparable to the 3.3 ± 0.3 (P > 0.23) score seen in mice treated with agg Ig-W. Furthermore, control mice given agg Ig-PLP1 together with rat IgG, instead of anti–IL-10 antibody, did not restore disease severity and had a mean maximal score of 1.6 ± 0.2. Injection of anti–IL-10 antibody together with agg Ig-W neither reduced nor exacerbated the severity of disease. These results indicate that agg Ig-PLP1–induced IL-10 plays a significant role in controlling disease severity, and that for the effects of IL-10 to occur, a specific interaction between APCs and the target T cells is required. In support of this statement is the observation that treatment with sol Ig-PLP1 plus exogenous IL-10 reduces the severity of paralysis to the same extent as agg Ig-PLP1 (Fig. 8 b). Sol Ig-PLP1, which does not induce detectable levels of IL-10, ameliorates the disease slightly with a mean maximal score of 2.5 ± 0.3, whereas sol Ig-PLP1 together with exogenous IL-10 further reduces the disease to a mean maximal clinical score of 1.1 ± 0.3, which is comparable to the 1.1 ± 0.5 score obtained with mice treated with agg Ig-PLP1. Furthermore, for endogenous IL-10 to modulate the disease, a physical bridging of the APCs to the T cells seems to be required. This conclusion is drawn from the observation that treatment of diseased mice with a mixture of agg Ig-W and free PLP1 peptide, instead of agg Ig-PLP1, did not reduce the severity of disease (Fig. 9). Overall, effective T cell downregulation requires physical interaction between IL-10–producing APCs and the target pathogenic T cell. The likely explanation for this requirement is that IL-10 as a paracrine cytokine needs to be in close proximity to T cells in order to achieve antagonism.

Treatment with Aggregated Ig-PLP1 Decreases the Clinical Severity of a Drive EAE Induced by Multiple Epitopes. IL-10 produced by APCs as a result of agg Ig-PLP1–mediated FcR cross-linking may antagonize specific T cells engaged to the PLP1–MHC ligand on the APCs as well as neighboring T cells with unrelated specificity. This phenomenon, known as bystander suppression, has proven effective in IL-4 (56) and IL-10 (57) settings. One way to find out if bystander suppression could be ascribed to the IL-10 in our experimental system is to induce EAE with a mixture of epitopes and test if treatment with agg Ig-PLP1 could...
modulate unrelated autoreactive T cells and ameliorate the disease. This experiment was carried out, and the results presented in Fig. 10a show that mice with ongoing EAE induced by a mixture of PLP1 and PLP2 peptides manifested reduced severity of paralysis and fully recovered by day 33 after disease induction upon treatment with agg Ig-PLP1, whereas animals treated with agg Ig-W had severe paralysis and did not recover from the disease during the 50-d period of clinical assessment. Therefore, endogenous IL-10 may have displayed downregulatory effects on PLP2-specific T cells. Induction of disease with PLP2 peptide should expose whole PLP and drive spreading and activation of PLP1-specific T cells (17, 58). In this case, injection of sol Ig-PLP1 to reduce the severity of EAE. Groups of mice (eight per group) were induced for EAE with 100 μg PLP1 and on days 9, 13, and 17 were given intraperitoneally in PBS 300 μg agg Ig-PLP1 (agg Ig-PLP1); 300 μg agg Ig-PLP1 plus 500 μg rat anti-mouse IL-10 antibody, 2A5 (agg Ig-PLP1 + anti-IL-10); 300 μg agg Ig-PLP1 plus 500 μg rat IgG (agg Ig-PLP1 + rat IgG); 300 μg agg Ig-W (agg Ig-W); or 300 μg agg Ig-W plus 500 μg rat anti-mouse IL-10 antibody, 2A5 (agg Ig-W + anti-IL-10). All the injections were done intraperitoneally in PBS. (b) Exogenous IL-10 synergizes with sol Ig-PLP1 to reduce the severity of EAE. Groups of mice (eight per group) were induced for EAE with 100 μg PLP1 and on days 9, 13, and 17 were given intraperitoneally in PBS 300 μg sol Ig-PLP1 (sol Ig-PLP1); 300 μg agg Ig-PLP1 (agg Ig-PLP1); 300 μg sol Ig-PLP1 plus 400 U recombinant IL-10 (sol Ig-PLP1 + IL-10); or 300 μg agg Ig-W (agg Ig-W).

modulation of unrelated autoreactive T cells and ameliorate the disease. This experiment was carried out, and the results presented in Fig. 10a show that mice with ongoing EAE induced by a mixture of PLP1 and PLP2 peptides manifested reduced severity of paralysis and fully recovered by day 33 after disease induction upon treatment with agg Ig-PLP1, whereas animals treated with agg Ig-W had severe paralysis and did not recover from the disease during the 50-d period of clinical assessment. Therefore, endogenous IL-10 may have displayed downregulatory effects on PLP2-specific T cells. Induction of disease with PLP2 peptide should expose whole PLP and drive spreading and activation of PLP1-specific T cells (17, 58). In this case, injection of agg Ig-PLP1 should bridge IL-10-producing APCs to PLP1-specific T cells and promote bystander suppression of these cells as well as neighboring PLP2-specific T cells. To address this issue, mice were induced for EAE with PLP2 peptide, and when signs of paralysis became apparent, they were treated with agg Ig-PLP1. Fig. 10b shows that although the initial phase of paralysis in these mice is only slightly milder than untreated mice, the animals quickly recovered by day 26, and unlike the untreated mice, did not relapse for the remaining period of clinical assessment. These results strengthen the notion of bystander suppression and suggest that epitope spreading offers an opportunity to modulate disease at a later stage of paralysis.

To further explore the breadth of this approach in T cell downregulation, we tested agg Ig-PLP1 for modulation of unrelated autoreactive T cells and ameliorate the disease. This experiment was carried out, and the results presented in Fig. 10a show that mice with ongoing EAE induced by a mixture of PLP1 and PLP2 peptides manifested reduced severity of paralysis and fully recovered by day 33 after disease induction upon treatment with agg Ig-PLP1, whereas animals treated with agg Ig-W had severe paralysis and did not recover from the disease during the 50-d period of clinical assessment. Therefore, endogenous IL-10 may have displayed downregulatory effects on PLP2-specific T cells. Induction of disease with PLP2 peptide should expose whole PLP and drive spreading and activation of PLP1-specific T cells (17, 58). In this case, injection of agg Ig-PLP1 should bridge IL-10-producing APCs to PLP1-specific T cells and promote bystander suppression of these cells as well as neighboring PLP2-specific T cells. To address this issue, mice were induced for EAE with PLP2 peptide, and when signs of paralysis became apparent, they were treated with agg Ig-PLP1. Fig. 10b shows that although the initial phase of paralysis in these mice is only slightly milder than untreated mice, the animals quickly recovered by day 26, and unlike the untreated mice, did not relapse for the remaining period of clinical assessment. These results strengthen the notion of bystander suppression and suggest that epitope spreading offers an opportunity to modulate disease at a later stage of paralysis.

To further explore the breadth of this approach in T cell downregulation, we tested agg Ig-PLP1 for modulation of unrelated autoreactive T cells and ameliorate the disease. This experiment was carried out, and the results presented in Fig. 10a show that mice with ongoing EAE induced by a mixture of PLP1 and PLP2 peptides manifested reduced severity of paralysis and fully recovered by day 33 after disease induction upon treatment with agg Ig-PLP1, whereas animals treated with agg Ig-W had severe paralysis and did not recover from the disease during the 50-d period of clinical assessment. Therefore, endogenous IL-10 may have displayed downregulatory effects on PLP2-specific T cells. Induction of disease with PLP2 peptide should expose whole PLP and drive spreading and activation of PLP1-specific T cells (17, 58). In this case, injection of agg Ig-PLP1 should bridge IL-10-producing APCs to PLP1-specific T cells and promote bystander suppression of these cells as well as neighboring PLP2-specific T cells. To address this issue, mice were induced for EAE with PLP2 peptide, and when signs of paralysis became apparent, they were treated with agg Ig-PLP1.
tion of disease induced with CNS homogenate, which incorporates a full range of myelin autoantigens. The results presented in Fig. 11 clearly indicate that mice injected with agg Ig-PLP1 had mild signs of paralysis in the initial phase of paralysis and fully recovered by day 24 after disease induction without any relapses for the 60-d period of clinical assessment. Control mice treated with agg Ig-W instead of agg Ig-PLP1 had a disease pattern similar to that of untreated animals (Fig. 11). These results indicate that the downregulatory function of agg Ig-PLP1 extends to both intra- and intermolecular epitopes and suppresses diverse T cell specificities.

Exposure to IL-10 seems to be the likely mechanism underlying downregulation and suppression of pathogenic myelin-specific T cells. The source of IL-10, as demonstrated in Figs. 4 and 6, is APCs such as DCs and macrophages. However, the broadened effectiveness and the endurance of T cell modulation in this setting raise the question of whether the bystander suppression was due to antagonism of the pathogenic T cells by APCs' IL-10, or to downregulation by regulatory T cells generated under the effect of such IL-10 (59). The rationale for this statement derives from previous studies suggesting that IL-10 enables naive T cells to develop into regulatory cells (59) that could produce IL-10, IL-5, or TGF-β and inhibit the function of pathogenic T cells thereby sustaining suppression (46, 60-63). To address this issue, the LN T cells from mice that were recovering from CNS-induced paralysis subsequent to treatment with agg Ig-PLP1 were stimulated with antigen and tested for proliferation and production of cytokines markers of regulatory T cells. The results presented in Fig. 12 show that 2 d after the final injection of agg Ig chimeras, proliferation to myelin peptides was significant in the mice treated with the control Ig-W but at background levels for those recipient of agg Ig-PLP1. Similarly, while the mice injected with agg Ig-W had significant amounts of IL-2 and IFN-γ, those treated with agg Ig-PLP1 had neither Th1- nor Th2-type cytokines and did not produce IL-10, IL-5, or TGF-β. Similar results were obtained when the mice were tested at day 9 after completion of the treatment regimen (data not shown). Furthermore, splenic T cells and cells harvested from the peritoneum showed a similar pattern of responses (data not shown). Overall, these results suggest that the typical proliferative and cytokine responses trademark of regulatory T cells are undetectable in this particular setting of systemic treatment of active autoimmunity.
Discussion

Previous studies have demonstrated that delivery of peptides on Igs increases presentation to T cells in vitro by 100–1,000-fold relative to free peptide (30, 31) and induces stronger T cell responses in vivo (31, 64). Internalization of Ig-peptide chimeras into APCs via FcRs and access of the attached peptide to newly synthesized MHC class II molecules are most likely responsible for such effective presentation (31, 50). The efficacy of peptide delivery by Igs seems to extend to peripheral APCs expressing minimal or no costimulatory molecules, as injection of the Ig-PLP1 chimera without adjuvant into diseased mice modulates PLP1-specific pathogenic T cells and ameliorates EAE (Fig. 1). This statement is supported by the finding that 200 nmol of PLP1 in the form of free peptide reduced the severity of disease only slightly and the animals never recovered, but 20 nmol of peptide in the form of sol Ig-PLP1 reduced the severity of the initial phase of disease and most of the animals fully recovered by day 42 (Fig. 1). The effectiveness of Ig-PLP1 in disease reversal was more dramatic when the chimera was given to mice in an aggregated form (Figs. 2 and 3, and Table I). The explanation we wish to put forth for this observation is that agg Ig-PLP1 displays an antagonistic function against T cells at two levels. On the one hand, as Ig-PLP1 was injected without any adjuvant it is likely that presentation was carried out mostly by peripheral APCs not expressing optimal costimulatory molecules, thereby leading to inactivation of the T cells. In fact, incubation of purified DCs and macrophages with sol or agg Ig-PLP1 did not induce upregulation of B7 or CD40 costimulatory molecules. On the other hand, the presenting APCs produce IL-10, which further antagonizes the T cells engaged to them through PLP1 peptide. The evidence for this dual-modal mechanism for T cell turn off derives from two essential observations. First, soluble adjuvant-free Ig-PLP1, which does not induce detectable levels of IL-10 (Fig. 4a), modulates the disease with significantly fewer copies of peptide than free PLP1 peptide (Fig. 1). Second, agg Ig-PLP1, which induces IL-10 production by macrophages and DCs (Fig. 4b), enhances T cell antagonism, as the mice display a significant reduction in the frequency and size of inflammatory foci both in the brain and spinal cord (Fig. 3, and Table I), manifest very mild clinical signs of EAE, and fully recover from paralysis by day 25 after disease induction (Fig. 2). The contribution of IL-10 to T cell modulation and disease amelioration is supported by both in vitro and in vivo data. Indeed, the TCC-PLP1-B10 T cell clone, which proliferates and produces both type I and II cytokines upon incubation with free PLP1 (Fig. 5), significantly reduced IFN-γ production when agg Ig-PLP1 was used for stimulation (Fig. 5). This is well illustrated in Fig. 7, which shows that the production of IFN-γ by T cells is inversely proportional to the IL-10 production by DCs and macrophages within the same cell culture. In vivo, neutralization of APCs' IL-10 by anti-IL-10 antibodies during treatment with agg Ig-PLP1 restores the severity of disease to scores comparable with Ig-W treatment (Fig. 8a). Furthermore, the combination of recombinant IL-10 and sol Ig-PLP1 reduces disease severity to the same extent as agg Ig-PLP1 (Fig. 8b). All together, these results suggest that effective control of EAE requires synergy of antagonistic functions against T cells, and points out the need for a multimodal strategy to contain autoimmunity. In fact, systemic injection of IL-10 has proven inefficient for modulation of autoimmunity (65), and only overexpression of IL-10 by means of transgenic manipulation (66), targeted delivery of the cytokine by means of engineered T cells (27), or viral gene transfer (29) has proven effective for control of T cell–mediated autoimmunity. The agg Ig-PLP1 approach brings together a peripheral tolerance-like mechanism and the antagonism activity of IL-10 to strengthen the modulatory functions against aggressive T cells. Furthermore, as IL-10 exerts its downregulatory function in a paracrine fashion, it may counteract neighboring Th1 cells regardless of antigen specificity. This behavior, known as bystander suppression (56, 57), seems to contribute to disease modulation by agg Ig-PLP1. Indeed, mice induced for EAE with a mixture of PLP1 and PLP2 peptides recovered from paralysis when they were treated with agg Ig-PLP1 (Fig. 10). The likely explanation for these results is that IL-10 produced by the APCs exerts an antagonist effect on neighboring T cells specific for PLP2 peptide, thereby modulating disease involving two epitopes. Moreover, when the disease was initiated with PLP2 peptide, the treatment with agg Ig-PLP1 allowed paralysis to peak to a significant clinical score but the recovery was expeditious and relapses did not occur. This pattern could suggest that IL-10-mediated bystander suppression had to await exposure of PLP and generation of PLP1-specific T cells to focus the presenting APCs and their IL-10 on the pathogenic T cells. This has twofold importance for the usefulness of this approach for treatment of autoimmunity. First, using this strategy for intervention at the initial phase of disease will likely modulate paralysis, as the target T cells should become available to focus the APCs and their IL-10 on neighboring pathogenic T cells regardless of their specificity. Second, as epitope spreading seems to follow a sequential order and sustain specific relapses (58), one could devise a regimen to target T cell modulation at later phases in the relapses. The data presented in Fig. 11, showing that agg Ig-PLP1 modulates disease induced with CNS homologue, argue for the broad effectiveness of the approach and support the notion that bystander suppression offers a strategy for intervention at various stages of the disease.

At the mechanistic level, the in vitro data demonstrate that while IL-10 downregulates the Th1 type cytokine IFN-γ, produced by the TCC-PLP1-B10 Th0 clone, production of the Th2 cytokine IL-4 was not affected (Fig. 5). This suggests that IL-10 antagonism partially affects the specific functions of the T cell rather than driving full inactivation or death of the cells. Whether IL-10 exerts a similar activity on differentiated Th1 cells in vivo remains to be investigated. On a speculative basis, if IL-10 affects IL-12...
synthesis by the APCs, a complex regulatory mechanism could be triggered around T cell–APC interactions possibly via CD40–CD40L, resulting in downregulation of Th1 function and suppression of IFN-γ production (67–69).

On the other hand, one could speculate that APCs' IL-10 may promote the development of regulatory T cells (46, 59–63), which in turn would help in downregulation of the pathogenic T cells. In fact, this would provide a good argument for the broad and sustainable bystander suppression, as regulatory T cells could produce suppressive cytokines such as IL-10 and TGF-β (61, 63). However, the results presented in Fig. 12 provided no support for this speculation and neither proliferation nor IL-10, IL-5, or TGF-β were obtained subsequent to treatment with agg Ig-PLP1. Although high concentrations of peptide were used for in vitro stimulation and cells from various tissues have been assayed for production of IL-10, IL-5, and TGF-β, this does not provide a definitive exclusion of regulatory cells. A few decades ago, Jerne (70) postulated that the immune system keeps itself in check by sustaining an equilibrium of interaction among its components. Pathogenic T cells could be kept harmless by a tight control from regulatory T cells. A perturbation of these interactions would lead to oscillations in the levels of each component. At the time of recovery, the regulatory T cells may have returned to minimal levels below the sensitivity of our assays.

Overall, agg Ig-PLP1 promotes efficient peptide loading onto MHC molecules and couples a peripheral tolerance-like mechanism and directed IL-10 bystander suppression to counteract aggressive T cells and modulate complex autoimmune involving multiple epitopes. One should emphasize that the effectiveness of the approach and its bystander suppression likely lies on the physical link of the Ig vehicle to the peptide, which serves to bridge the APCs to the T cell and focus IL-10 on the neighboring pathogenic lymphocytes. This dual-modal approach, which proves to be efficient in this autoimmune setting where a high precursor frequency probably entertains the severe paralysis (71), may prove feasible for treatment of human multiple sclerosis, which likely involves several myelin autoantigens (19, 72).

This work was supported by grant RG2967A2/1 from the National Multiple Sclerosis Society, grant RO1-N037406 from the National Institutes of Health, and grant R01-101564 from Astral, Inc. (a subsidiary of Alliance Pharmaceutical Corp., San Diego, CA).

Submitted: 6 Octorber 1999
Revised: 19 April 2000
Accepted: 27 April 2000

References

1. Goldrath, A.W., and M.J. Bevan. 1999. Selecting and maintaining a diverse T-cell repertoire. Nat. Rev. 402:255–262.
2. Cibotti, R., J.M. Kanellopoulos, J.P. Cabaniol, O. Halle-panenko, K. Kosmatopoulos, E. Sercarz, and P. Kourilsky. 1992. Tolerance to self-protein involves its immunodominant but does not involve its subdominant determinants. Proc. Natl. Acad. Sci. USA. 89:416–420.
3. Fowlkes, B.J., and F. Ramsdell. 1993. T-cell tolerance. Curr. Opin. Immunol. 5:873–879.
4. Herold, K.C., A.G. Montag, and F. Buckingham. 1992. Induction of tolerance to autoimmune diabetes with islet antigens. J. Exp. Med. 176:1107–1114.
5. Amula, M.J. 1993. The inability to process a self-peptide allows autoreactive T cells to escape tolerance. J. Exp. Med. 177:567–571.
6. Burns, J., A. Rosenzweig, B. Zweiman, and R.P. Lisak. 1983. Isolation of myelin basic protein-reactive T cell lines from normal human blood. Cell. Immunol. 81:435–440.
7. Liu, G.Y., P.J. Fairchild, R.M. Smith, J.R. Prowle, D. Kioussis, and D.C. Wraith. 1995. Low avidity recognition of self-antigen by T cells permits escape from central tolerance. Immunity. 3:407–415.
8. Yan, T., H. Burkhardt, T. Ritter, B. Broker, K.H. Mann, W.M. Berling, K. von der Mark, and F. Emmerich. 1992. Specificity and TCR β chain usage of a human collagen type II-reactive T cell clone derived from a healthy individual. Eur. J. Immunol. 22:51–56.
9. Arnold, B., G. Schonrich, and G. Hammerling. 1993. Multiple levels of peripheral tolerance. Immunol. Today. 14:12–14.
10. Kosaka, H., and J. Sprent. 1993. Tolerance of CD8+ T cells developing in parent F1 chimeras prepared with supralethal irradiation: step-wise induction of tolerance in the intrathymic and extrathymic environments. J. Exp. Med. 177:367–378.
11. McCormack, J.E., J.E. Callahan, J. Kappler, and P.C. Marrack. 1993. Profound deletion of mature T cells in vivo by chronic exposure to exogenous superantigen. J. Immunol. 150:3785–3792.
12. Ochsa, B., and H. von Boehmer. 1991. Peripheral selection of the T cell repertoire. Science. 251:1225–1228.
13. Webb, S., C. Morris, and J. Sprent. 1990. Extrathymic tolerance of mature T cells clonal elimination as a consequence of immunity. Curr. Opin. 63:1249–1256.
14. Jenkins, M.K., and J.G. Johnson. 1993. Molecules involved in T-cell costimulation. Curr. Opin. Immunol. 5:361–367.
15. Brocke, S., A. Gaur, C. Piercy, A. Gautam, K. Gijbels, C.G. Fathman, and L. Steinman. 1993. Functional evidence for epitope spreading in experimental autoimmune encephalomyelitis by bacterial superantigen. Nature. 365:642–644.
16. Wucherpfennig, K.W., and J.L. Strominger. 1995. Molecules mimicry in T-cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Curr. Opin. Immunol. 7:695–705.
17. Crée, B.L., C.L. Vanderlugt, M.C. Dal Canto, and S.D. Miller. 1995. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. J. Exp. Med. 182:75–85.
18. Sercarz, E.E., P.V. Lehmann, A. Metani, G. Benichou, A. Miller, and K. Moudgil. 1993. Dominance and crypticity of T cell antigenic determinants. Annu. Rev. Immunol. 11:729–766.
19. Steinman, L. 1996. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. Curr. Opin. 85:299–302.
20. Tisch, R., and H. MeDevitt. 1996. Insulin-dependent diabetes mellitus. Curr. Opin. 85:291–297.
21. Feldmann, M., F.M. Brennan, and R.N. Maini. 1996. Rheumatoid arthritis. Curr. Opin. 85:307–310.
22. Jacobs, M.J., A.E. van den Hock, L.B. van de Putte, and S. Mackay. 1996. Rheumatoid arthritis. Curr. Opin. 85:307–310.
23. Mueller, D.L., and M.K. Jenkins. 1995. Molecular mechanisms underlying functional T-cell unresponsiveness. Curr. Opin. Immunol. 7:375–381.

24. Elliott, E.A., H.I. McFarland, S.H. Nye, R. Coffey, T.M. Wilson, J.A. Wilkins, S.P. Squinto, L.A. Matis, and J.P. Mueller. 1996. Treatment of experimental encephalomyelitis with a novel chimeric fusion protein of myelin basic protein and proteolipid protein. J. Clin. Invest. 98:1602–1612.

25. Gaur, A., B. Wiers, A. Liu, J. Rothbard, and C.G. Fathman. 1992. Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. Science. 258:1491–1494.

26. Liblau, R., R. Tisch, N. Bercovici, and H.O. McDavitt. 1997. Systemic antigen in the treatment of T-cell-mediated autoimmune diseases. Immunol. Today. 18:599–604.

27. Mathisen, P.M., M. Yu, J.M. Johnson, J.A. Drazba, and V.K. Tuohy. 1997. Treatment of experimental autoimmune encephalomyelitis with genetically modified memory T cells. J. Exp. Med. 186:159–164.

28. Shaw, M.K., J.B. Lorenz, A. Dhawan, R. Dalcanto, H.Y. Tse, A.B. Tran, C. Bompaine, S.L. Eswaran, S. Brocke, N. Sarvetnick, et al. 1997. Local delivery of interleukin 4 by retrovirus-transduced T lymphocytes ameliorates experimental autoimmune encephalomyelitis. J. Exp. Med. 185:1711–1714.

29. Ma, Y., S. Thornton, L.E. Duwel, G.P. Boivin, E.H. Gianini, J.M. Leiden, J.A. Bluestone, and R. Hirsch. 1998. Inhibition of collagen-induced arthritis in mice by viral IL-10 gene transfer. J. Immunol. 161:1516–1524.

30. Legge, K.L., B. Min, N.T. Potter, and H. Zaghouani. 1997. Presentation of a T cell receptor antagonist peptide by immunoglobulins ablates activation of T cells by a synthetic peptide or proteins requiring endocytic processing. J. Exp. Med. 185:1043–1053.

31. Zaghouani, H., R. Steinman, R. Nonacs, H. Shah, W. Gerhard, and C. Bona. 1993. Presentation of a viral T cell epitope expressed in the CDR3 region of a self immunoglobulin molecule. Science. 259:224–227.

32. deo, Y.M., R.F. Graziano, R. Repp, and J.G. van de Winkel. 1997. Clinical significance of IgG Fc receptors and FcγR-directed immunotherapies. Immunol. Today. 18:127–135.

33. Polat, G.L., J. Lauffer, I. Fabian, and J.H. Passwell. 1993. Cross-linking of monocyte plasma membrane Fc alpha, Fc gamma or mannose receptors induces TNF production. Immunology. 80:287–292.

34. Sutterwala, F.S., G.J. Noell, P. Saltzman, and D.M. Mosser. 1998. Reversal of proinflammatory responses by ligation of the macrophage Fcγ receptor type I. J. Exp. Med. 188:217–222.

35. Christian, C.L. 1960. Studies on aggregated gamma-globulin I & II. J. Immunol. 84:112–121.

36. Rosqvist, E., T. Jossang, and J. Feder. 1987. Thermal properties of human IgG. Mol. Immunol. 24:495–501.

37. Tuohy, V.K., Z. Lu, R.A. Sobel, R.A. Laursen, and M.B. Lees. 1989. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. J. Immunol. 142:1523–1527.

38. Greer, J.M., V.K. Kuchroo, R.A. Sobel, and M.B. Lees. 1992. Identification and characterization of a second encephalitogenic determinant of myelin proteolipid protein (residues 178–191) for SJL mice. J. Immunol. 149:783–788.

39. Brocke, S., K. Gijbels, M. Allegraft, I. Ferber, C. Peiry, T. Blankenstein, R. Martin, U. Utz, N. Karin, D. Mitchel, et al. 1996. Treatment of experimental encephalomyelitis with a peptide analog of myelin basic protein. Nature. 379:343–346.

40. Legge, K.L., B. Min, C. Pack, J. Caprio, and H. Zaghouani. 1999. Differential presentation of an altered peptide within fetal central and peripheral organs supports an avidity model for thymic cell development and implies a peripheral readjustment for activation. J. Immunol. 162:5738–5746.

41. Min, B., K.L. Legge, C. Pack, and H. Zaghouani. 1998. Neonatal exposure to a self-peptide-immunoglobulin chimera circumvents the use of adjuvant and confers resistance to autoimmune disease by a novel mechanism involving interleukin 4 lymph node deviation and interleukin γ- mediated splenic anergy. J. Exp. Med. 188:2007–2017.

42. Chase, M.W., L. Gidez, L. Levine, B. Mach, W.T. Murakami, C.A. Williams. 1968. Chemical analyses. In Methods in Immunology and Immunchemistry. Vol. 2. C.A. Williams and M.W. Chase, editors. Academic Press, New York. 249–341.

43. Doyle, A.G., and I.P. Fraser. 1996. Murine macrophages isolation, cultivation, and characterization. In Weir’s Handbook of Experimental Immunology, L.A. Herzenberg, D. Weir, L.A. Herzenberg, and C. Blackwell, editors. Blackwell Science, Cambridge, MA. 154.1–154.8.

44. Romani, N., N. Bhardwaj, M. Pope, F. Koch, W.J. Swiggard, U.O. Doherty, M.D. Witmer-Pack, L. Hoffman, G. Schuler, K. Inaba, et al. 1996. Dendritic cells. In Weir’s Handbook of Experimental Immunology, L.A. Herzenberg, D. Weir, L.A. Herzenberg, and C. Blackwell, editors. Blackwell Science, Cambridge, MA. 156.1–156.14.

45. Critchfield, J.M., M.K. Racke, J.C. Zuniga-Pflucker, B. Cannella, C.S. Raine, J. Goverman, and M.J. Lenardo. 1994. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. Science. 263:1139–1143.

46. Chen, Y., J.I. Inobe, V.K. Kuchroo, J.L. Baron, C.A. Janeway, Jr., and H.L. Weiner. 1996. Oral tolerance in myelin basic protein T-cell receptor transgenic mice: suppression of autoimmune encephalomyelitis and dose-dependent induction of regulatory cells. Proc. Natl. Acad. Sci. USA. 93:388–391.

47. Devaux, B., F. Enderlin, B. Wallin, and D.E. Smilk. 1997. Induction of EAE in mice with recombinant human M O G, and treatment of EAE with a M O G peptide. J. Neuroimmunol. 75:169–173.

48. Leadbetter, E.A., C.R. Bourque, B. Devaux, C.D. Olson, G.H. Sunshine, S. Hirani, B.P. Wallner, D.E. Smilk, and M.P. Hopp. 1998. Experimental autoimmune encephalomyelitis induced with a combination of myelin basic protein and myelin oligodendrocyte glycoprotein is ameliorated by administration of a single myelin basic protein peptide. J. Immunol. 161:504–512.

49. Staykova, M.A., R.D. Simmons, and D.O. Willenborg. 1997. Inhibition of soluble myelin basic protein protects long-term against induction of experimental autoimmune encephalomyelitis. Immunol. Cell Biol. 75:54–64.

50. Brumaneau, T.D., W.J. Swiggard, R.M. Steinman, C.A. Bona, and H. Zaghouani. 1993. Efficient loading of identical viral peptide onto class II molecules by antigenized immunoglobulin and influenza virus. J. Exp. Med. 178:1795–1799.

51. Berger, S., H. Bällo, and H.J. Stutte. 1996. Immune complex-induced interleukin-6, interleukin-10 and prostaglandin
secretion by human monocytes: a network of pro- and anti-inflammatory cytokines dependent on the antigen:antibody ratio. Eur. J. Immunol. 26:1297–1301.

52. Berger, S., R. Chandra, H. Ballo, R. Hildenbrand, and H.J. Stutte. 1997. Immune complexes are potent inhibitors of interleukin-12 secretion by human monocytes. Eur. J. Immunol. 27:2994–3000.

53. Steinbrink, K., M. Wolff, H. Jonuleit, J. Knop, and A.H. Enk. 1997. Induction of tolerance by IL-10-treated dendritic cells. J. Immunol. 159:4772–4780.

54. Ding, L., P.S. Linsley, L.-Y. Huang, R.N. Germain, and E.M. Shevach. 1993. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. J. Immunol. 151:1224–1234.

55. Fiorentino, D.F., A. Zlotnik, P. Vieira, T.R. Mosmann, M. Howard, K.W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J. Immunol. 146:3444–3451.

56. Falcone, M., and B.R. Bloom. 1997. A T helper cell 2 (Th2) immune response against non-self antigens modifies the cytokine profile of autoimmune T cells and protects against experimental allergic encephalomyelitis. J. Exp. Med. 185:901–907.

57. Stohlman, S.A., L. Pei, D.J. Cua, Z. Li, and D. Hinton. 1999. Activation of regulatory cells suppresses experimental allergic encephalomyelitis via secretion of IL-10. J. Immunol. 163:6338–6344.

58. Tuohy, V.K., M.Y. Yu, J.A. Kawczak, J.M. Johnson, P.M. Mathisen, B. Weinstock-Guttman, and R.P. Kinkel. 1998. The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. Immunol. Rev. 164:93–100.

59. Groux, H., A. O’Garra, M. Bigler, M. Rouleau, S. Antonenko, J.E. de Vries, and M.G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nat. Med. 3:737–742.

60. Asseman, C., S. Mauze, M.W. Leach, R.L. Coffman, and F. Powrie. 1999. An essential role for regulatory T cells that inhibit intestinal inflammation. J. Exp. Med. 190:995–1003.

61. Groux, H., and F. Powrie. 1999. Regulatory T cells and inflammatory bowel disease. Immunol. Today. 20:442–445.

62. Seddon, B., and D. Mason. 1999. Block the CD40L–CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion. J. Exp. Med. 189:693–698.

63. Jerne, N.K. 1974. Towards a network theory of the immune system. Ann. Immunol. (Paris). 125C:373–389.