Immunity, Vol. 19, 317–328, September, 2003, Copyright ©2003 by Cell Press

Epicutaneous Immunization with Autoantigenic Peptides Induces T Suppressor Cells that Prevent Experimental Allergic Encephalomyelitis

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Summary

Information on how suppressor/regulatory T cells can be generated directly in vivo and prevent autoimmunity remains fragmentary. We show here that epicutaneous immunization (ECi) with the immunodominant peptide of myelin basic protein (MBP), Ac1-11, protects mice that are transgenic for an Ac1-11-specific T cell receptor against both the induced and spontaneous forms of experimental allergic encephalomyelitis (EAE). This protection was antigen specific and antigen dose dependent, and was mediated by $CD4^+$/$CD25^-$ T cells whose suppressive activity required cell-cell contact and could transfer protection to naive recipients. These ECi-induced suppressor T cells controlled naive MBP-specific CD4 T cells by inhibiting both their activation and their capacity to secrete IFN-γ. There was no CD4 T cell infiltration in the brain of protected mice. Finally, ECi with autoantigenic peptides protected two nontransgenic models from relapsing-remitting EAE in an antigen-specific and antigen dose-dependent manner.

Introduction

The route and dose of antigen administration have long been known to be key determinants in the experimental induction of immune activation or of immunological tolerance, which might be caused by specific activation of T cells. Recently, epicutaneous (skin) immunization with protein antigens was shown to induce strong Th2 immune responses in mice (Herrick et al., 2000; Wang et al., 1996, 1999). These studies employed an occlusive skin patch in which a protein antigen such as ovalbumin (OVA) was applied to the patch that was then put in direct contact with the shaved skin of mice. In this study, we asked whether the epicutaneous application with tissue-specific autoantigenic peptides prior to disease induction could interfere with the development of Th1 cell-mediated responses directed to the same antigen and thus protect mice from experimental allergic encephalomyelitis (EAE). We applied the myelin basic protein (MBP) peptide, Ac1-11, to the skin of transgenic mice expressing the Ac1-11-specific MBP αβ TCR (Hardardottir et al., 1995). EAE can be induced in these mice, which can also develop spontaneous EAE depending on their genetic makeup (Lafaille et al., 1994). Thus, the MBP-TCR-Tg mouse serves as a model for the human disease multiple sclerosis (Goverman and Brabb, 1996) (MS), an inflammatory attack on the central nervous system (CNS) that results in extensive demyelination and altered neurological functions (Brosnan and Raine, 1996; Steinman, 2001).

Unlike the MBP-TCR-Tg mouse model, which develops acute, monophasic EAE, (SJLxPL/J)F1 and (B10.PLxSJL)F1 mice develop spontaneous remissions and relapses that closely mimic the clinical and pathologic characteristics of MS (Dittel et al., 2000; Kuchroo et al., 2002). Thus, comparison of these different mouse strains treated with their specific CNS antigens will help to determine whether the epicutaneous mode of antigen delivery can be potentially beneficial as a putative therapeutic tool for MS. We therefore epicutaneously immunized (ECi) nontransgenic (B10.PLxSJL)F1 and (SJLxPL/J)F1 mice with their eliciting peptide antigens, Ac1-11 and proteolipid protein (PLP)139-151, respectively. We then attempted to induce disease in order to determine whether they can be protected from an initial attack of EAE and from its spontaneous relapses. In both cases, EAE was prevented.

Results

ECi with Ac1-11 Protects MBP-TCR-Tg Mice from EAE

MBP-TCR-Tg mice underwent ECi with Ac1-11 at varying concentrations (0.1 μg–1 mg) or with PBS. After 2 weeks, we attempted to induce EAE by subcutaneous (sc) immunization with Ac1-11 in CFA and intravenous injection of pertussis toxin (see Experimental Procedures).

All mice ECi with Ac1-11 in a patch prior to EAE induction showed varying degrees of protection compared to PBS ECi controls (Figure 1A). Furthermore, the protection was antigen dose dependent. Mice that were ECi with 10 μg of Ac1-11 showed dramatically greater protection than those that received 0.1 μg, 1 μg, 100 μg, or 1 mg. Not only were the number of mice protected from EAE significantly higher in the group that received 10 μg, but the disease onset occurred at a later time and the disease was less severe (Table 1).

To determine whether protection from EAE was antigen specific or whether any antigen can suppress EAE by some nonspecific mechanism, MBP-TCR-Tg mice were ECi in parallel with varying doses of Ac1-11, OVA, PBS, or other myelin peptides such as myelin oligodendrocyte glycoprotein (MOG)35-55 or proteolipid protein (PLP)139-151, following which we attempted to induce EAE. In contrast to mice receiving 10 μg Ac1-11, mice that received OVA, MOG35-55, or PLP139-155 by the epicutaneous route developed EAE with identical frequency and severity (at all concentrations tested: 10 μg–1 mg; see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/19/3/317/DC1) as the PBS ECi control mice (Figure 1B and Table 1A). This comparison shows that ECi with Ac1-11 is required to confer protection from the Ac1-11 peptide-induced EAE. Therefore, protection cannot be due to some nonspecific mechanism of induction. These data also demon-

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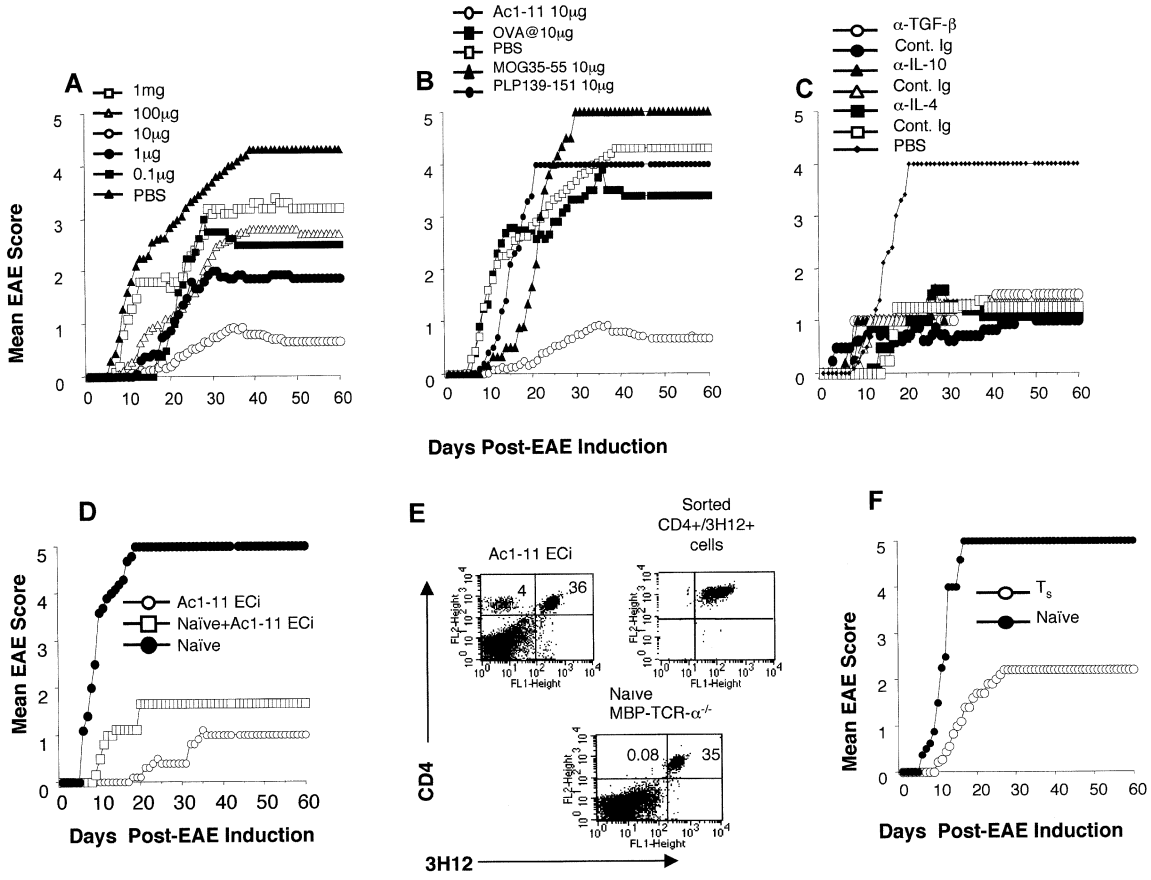

Figure 1. ECi with Ac1-11 Induces Dominant Tolerance in MBP-TCR-Tg Mice

(A) MBP-TCR-Tg mice were ECi with varying doses of Ac1-11: 1 mg, 100 μg, 10 μg, 1 μg, 0.1 μg, or PBS.

(B) To determine whether protection is Ac1-11 specific, mice were ECi with Ac1-11 at 10 μg, with OVA at 10 μg and 100 μg concentrations, with MOG35-55 at 10 μg, with PLP139-151 at 10 μg, or with PBS for comparison. Note that the same group of mice treated with 10 μg of Ac1-11 per patch is shown in both (A) and (B).

(C) MBP-TCR-Tg mice were ECi with 10 μg of Ac1-11 and given anti-IL4, anti-IL-10, anti-TGF-β monoclonal antibodies or their isotype-matched controls, or PBS prior to and during ECi with Ac1-11 as well as after EAE induction.

(D) Irradiated naive B10.PL mice were adoptively transferred with 1×10^7 total splenocytes from Ac1-11 ECi or naive MBP-TCR-Tg mice (control group), or cotransferred with cells from both naive and Ac1-11 ECi mice.

(E) Spleen and lymph node cells from Ac1-11 ECi mice are stained with anti-CD4 and the 3H12 clonotypic antibody (top left). Profiles for sorted $CD4^+/3H12^+$ cells (top right) and naive MBP-TCR-Tg $C\alpha^{-/-}$ splenocytes (bottom) are also shown.

(F) 1×10^6 sorted Ac1-11-specific CD4 T cells from Ac1-11 ECi mice or naive MBP-TCR-Tg mice were adoptively transferred into MBP-TCR-Tg $C\alpha^{-/-}$ recipients.

strate that applying antigen to the skin prior to EAE induction protects MBP-TCR-Tg mice from EAE in a dose-dependent and antigen-specific manner. The abbreviation Ac1-11 ECi will be used to refer to mice that were ECi with the Ac1-11 peptide and were protected from induced EAE.

No Requirement for Th2 Cytokines in ECi-Induced Protection

To determine whether the mechanism of protection against EAE involved the induction of a Th2 immune response in ECi mice, we examined the cytokine profiles both in mice showing protection and in control mice that got EAE. We found no significant production of the cytokines IL-4, IL-10, IL-13, or TGF-β in sera or in antigen-stimulated culture supernatants from antigen-stimulated CD4 T cells from these mice (data not shown). However, control MBP-TCR-Tg mice produced high levels of IL-2 and IFN-γ, while Ac1-11 ECi mice produced low to moderate levels of IL-2 and no IFN-γ in supernatants or sera (discussed later in Figure 4). We next performed cytokine blocking experiments in vivo for IL-4, IL-10, and TGF-β by administering antibodies to these cytokines or isotype-matched controls to mice ECi with 10 μg Ac1-11. Mice that received anti-cytokine antibodies were protected at the same frequency as mice that received the corresponding isotype-matched immunoglobulin (Figure 1C and Table 2C). This suggests that IL-4, IL-10, and TGF-β may not play a significant role in mediating protection from EAE in this system.

Disease Protection Can Be Transferred to Naive MBP-TCR-Tg Recipients

We next investigated whether peripheral lymphoid cells from Ac1-11 ECi mice can suppress EAE induction in naive MBP-TCR-Tg mice. Splenocytes from Ac1-11 ECi

Table 1. EAE Incidence in Ac1-11 ECi and Control MBP-TCR-Tg Mice

A. Antigen/Dose	n	# Mice with EAE	# Recovered	Day of Disease Onset	Mean EAE Score
Ac1-11 1 mg	11	6/11	1/6	8	3.2
Ac1-11 100 μg	30	16/30	1/16	8	2.8
Ac1-11 10 μg	30	13/30	9/13	15	0.7
Ac1-11 1 μg	13	8/13	3/5	14	1.9
Ac1-11 0.1 μg	6	5/6	2/5	18	2.5
OVA 100 μg	21	17/21	1/17	7	4.3
PBS	18	15/18	0/15	6	4.3
MOG35-55 10 μg	11	11	0/11	7	5
PLP139-151 10 μg	6	4	0/4	8	4
B. Total Splenocytes (Spcs) or CD4 T Cells from Ac1-11 ECi Mice Can Transfer Dominant Suppression					
Spcs Ac1-11 ECi	5	2/5	1/2	18	1.2
Spcs naive Tg	5	5/5	0/5	5	5
Ac1-11 ECi:naive MBP Tg	5	3/5	1/3	10	1.9
CD4 Ac1-11 ECi	9	4/9	1/4	8	2.4
CD4 naive MBP-TCR $Ca^{-/-}$	4	4/4	0/4	5	5

In (A) n refers to number of mice for two to four experiments; in (B) n refers to number of mice for one experiment. Experiments in (B) were done a minimum of six times with very similar outcomes.

mice or from control naive MBP-TCR-Tg mice were transferred into sublethally irradiated, naive B10.PL recipients. Five days later, we attempted to induce EAE. Less than 1 week after EAE induction, the control mice developed flagrant EAE and all such mice died before 2 weeks. By contrast, EAE was significantly suppressed in recipients that were cotransferred with both naive MBP-TCR-Tg and Ac1-11 ECi cells, and more so in recipients that received splenocytes only from Ac1-11 ECi mice (Figure 1D and Table 1B). Thus, splenocytes from Ac1-11-ECi mice can protect recipient mice carrying naive MBP-TCR-Tg T cells from EAE.

Ac1-11-Specific CD4 T Cells Mediate Protection

There are examples where CD4 T cells are involved in resistance to EAE (Olivares-Villagomez et al., 1998; Van de Keere and Tonegawa, 1998). We therefore hypothesized that CD4 T cells were involved in the suppression of EAE in Ac1-11 ECi mice and that these cells were Ac1-11 specific (see Figure 1B). CD4 T cells from Ac1-11 ECi (Figure 1E) or naive MBP-TCR-Tg mice, after being stained and sorted with a clonotypic antibody (Figure 1E), were transferred into 4-week-old MBP-TCR-$C\alpha^{-/-}$ recipients. The MBP-TCR-$C\alpha^{-/-}$ mice are devoid of most endogenous CD4 and CD8 $\alpha\beta$ T cells, so they are comprised almost entirely of MBP-TCR-Tg CD4 T cells (Figure 1E). The use of these mice allowed us to evaluate the effect of CD4 T cells on a quasi-pure population of naive CD4 MBP-TCR-Tg T cells. EAE was induced 5 days after transfer. Mice receiving CD4 T cells from Ac1-11 ECi mice show significant protection from EAE: five out of nine mice survived, while four out of nine mice

Table 2. $CD4^+CD25^-$ T Cells Confer Protection from Induced EAE

A. Treatment	n	Mice with EAE	Maximum or Mean EAE Score	# Mice Recovered	Disease Onset
0.25×10^6 CD4/$CD25^+$	3	3/3	5	0	1 week
0.5×10^6 CD4/$CD25^+$	3	3/3	5	0	2 weeks
1.0×10^6 CD4/$CD25^+$	3	2/3	1 and 1.5	2/2	2.5 weeks
0.25×10^6 CD4/$CD25^-$	3	3/3	5	0/3	2 weeks
0.5×10^6 CD4/$CD25^-$	3	2/3	2.5 and 5	1/2	2.5 weeks
$1. \times 10^6$ CD4/$CD25^-$	3	1/3	1	1/3	2.5 weeks
B. Cells from Ac1-11 EC-sp Mice but Not Controls Protect MBP-TCR-$C\alpha^{-/-}$ Mice from Spontaneous EAE					
Ac1-11 10 μg	5	3/5	4	1/3	3.5 months
PBS	5	5/5	5	0/5	7 weeks
CD4 T cells from Ac1-11 ECi mice	5	0/5	0	N/A	None up to 7 months
CD4 T cells from PBS ECi mice	5	4/5	4	0/4	3 months
C. In Vivo Anti-Cytokine Treatment in Mice ECi with Ac1-11					
Anti-IL-4	5	2	1.6	1	9 days
Anti-IL-4 control (rat Ig)	4	1	1.25	0	12 days
Anti-IL-10	5	3	1.3	2	8 days
Anti-IL-10 control (mouse Ig)	5	4	1.6	3	8 days
Anti-TGF-β (1,2,3)	5	1	1	0	8 days
Anti-TGF-β control (mouse IgG1)	5	1	0.8	1	6 days

In (A)–(C), n refers to the number of mice per experimental group for one experiment. Mean EAE score refers to (C). Experiments in (B) were done two to four times and in (A) three times with similar outcomes.

developed EAE and died. By contrast, all control mice died from EAE within a few days of disease induction (Figure 1F and Table 1B). These data show a role for antigen-specific CD4 T cells in protection against EAE and demonstrate that tolerance induced by ECi with Ac1-11 is dominant, as protection can be transferred to naive MBP-TCR-Tg recipients. We therefore refer to CD4 T cells isolated from Ac1-11 ECi mice as suppressor T cells (T_s).

Protection against EAE Does Not Depend on $CD4^+CD25^+$ T Cells

There is strong evidence that the $CD4^+CD25^+$ T cell subset confers protection in a variety of organ-specific autoimmune disease models (Sakaguchi et al., 1995; Salomon et al., 2000; Shevach, 2001). To examine whether $CD4^+CD25^+$ T cells are involved in protection against EAE in our model, we sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from pooled spleens and lymph nodes of Ac1-11 ECi mice. These subsets were adoptively transferred into young naive MBP-TCR-C$\alpha^{-/-}$ recipients. We then attempted to induce EAE 5 days later. All recipient mice that received less than 1×10^6 $CD4^+CD25^+$ T cells developed EAE and died. Recipients that received 0.5×10^6 and 1×10^6 $CD4^+CD25^-$ T cells developed less severe EAE (Figure 2A and Table 2A). Therefore, $CD4^+CD25^-$ T cells are sufficient to confer protection from EAE. Furthermore, $CD4^+CD25^-$ T cells show a somewhat greater degree of suppression than $CD4^+CD25^+$ T cells, as protection was observed at lower cell numbers than that observed by transferring $CD4^+CD25^+$ cells. It is worth noting that the $CD4^+CD25^+$ subset never represented more than 5% of the CD4 T cell population in the Ac1-11 ECi mice (data not shown).

CD4 T Cells from ECi Mice Are Unresponsive and Inhibit Proliferation of Effector Cells

We next assessed the proliferative capacity of CD4 T cells from Ac1-11 ECi mice, along with control mice with a clinical score of 3.5. These were compared with cells from naive MBP-TCR-Tg mice to determine their ability to proliferate. Cells from Ac1-11 ECi mice proliferated less efficiently and produced little to no IL-2 as compared to both control and naive mice, which indicates varying degrees of unresponsiveness (Lechler et al., 2001) (Figure 2B). To determine whether the unresponsiveness could be restored by IL-2, we treated CD4 T cells from Ac1-11 ECi mice with exogenous IL-2 and antigen. There was a 2-fold increase in proliferation in the presence of IL-2 and antigen (Figure 2C). IL-2 without antigen did not induce proliferation. We then asked whether these T_s cells can suppress the proliferation of naive CD4 T cells in vitro, as previously reported (Chai et al., 1999). Naive MBP-TCR-Tg CD4 T cells were cocultured with CD4 T_s cells at various cell ratios. T_s cells inhibited the proliferative response of naive MBP-TCR-Tg T cells in a cell dose-dependent manner. To determine whether this was indeed suppression and not simply inhibition of proliferation due to competition for space or access to APCs, we cocultured naive CD4 T cells from MBP-TCR-Tg mice with CD4 T cells from naive nontransgenic mice at the same cell ratios as above. Naive MBP-TCR-Tg cells proliferated similarly with or without the addition of naive nontransgenic CD4 T cells (Figure 2D). We conclude that ECi induced antigen-specific CD4 T cells able to suppress the activation of Ac1-11 specific naive CD4 T cells in response to cognate stimulation.

Suppression by T_s Cells Is Contact Dependent, but Independent of IL-4, IL-10, and TGF-β

We next performed in vitro Th2 cytokine blocking experiments to determine whether antibodies to IL-4, IL-10, or TGF-β can inhibit the suppression of naive MBP-TCR-Tg CD4 T cell proliferation. As shown in Figure 2E, suppression of naive MBP-TCR-Tg T cell proliferation in vitro was not blocked by anti-cytokine mAbs treatment. This result is fully consistent with the in vivo data and indicates that IL-4, IL-10, or TGF-β cytokines did not play any significant role in disease suppression. To determine whether contact between effector T cells and T_s cells is necessary for suppression to occur, we performed transwell-based assays in which T_s cells were separated or not separated from effector cells in the presence of APCs and antigen. The results show definitively that T_s cells need to be in contact with effectors and/or APCs for suppression to occur (Figure 2F).

ECi with Ac1-11 Can Suppress Spontaneous EAE

We asked whether ECi with Ac1-11 could protect MBP-TCR-C$\alpha^{-/-}$ mice from spontaneous EAE. As explained above, T cells in these mice are comprised almost entirely of MBP-TCR-Tg CD4 T cells (Figure 1E). Therefore, similar to RAG$^{-/-}$ mice, they develop spontaneous EAE by 6 weeks of age (Lafaille et al., 1994; Olivares-Villagomez et al., 1998). Naive MBP-TCR-C$\alpha^{-/-}$ mice were ECi with 10 μg Ac1-11. Disease onset occurred at 7 weeks in control mice, and by 4.5 months, all such mice had developed EAE and died (Figure 3A and Table 2B). In contrast, the first symptoms of EAE developed in Ac1-11 ECi mice at 3.5 months and some mice did not develop EAE (Table 2B). Hence, ECi with Ac1-11 can protect MBP-TCR-C$\alpha^{-/-}$ mice from spontaneous EAE. These mice will be referred to as Ac1-11 ECi-sp.

We next asked whether CD4 T cells from Ac1-11 ECi-sp mice could protect against induced EAE. CD4 T cells from Ac1-11 ECi-sp or control mice were transferred to MBP-TCR-C$\alpha^{-/-}$ recipients prior to EAE induction. The onset of EAE was the same in both groups, and the control mice died in less than 1 week. Recipients with cells from Ac1-11-ECi-sp mice showed a significant lag in disease of 30 days, and by 35 days all mice had died (Figure 3B). This is in contrast with the above data in Figure 1E where CD4 T cells from Ac1-11 ECi mice almost totally inhibited the development of induced EAE. These data imply that immunization with Ac1-11 in CFA, after ECi with Ac1-11, is required to generate dominant suppression (or T_s).

Earlier, we showed that CD4 T cells from Ac1-11 ECi mice suppressed induced EAE development (Figure 1F). We also investigated whether these same CD4 T cells can protect against spontaneous EAE. Sorted CD4 T cells from pooled spleen and lymph nodes from Ac1-11 ECi mice or naive MBP-TCR-C$\alpha^{-/-}$ mice were adoptively transferred into naive MBP-TCR-C$\alpha^{-/-}$ recipients. Dis-

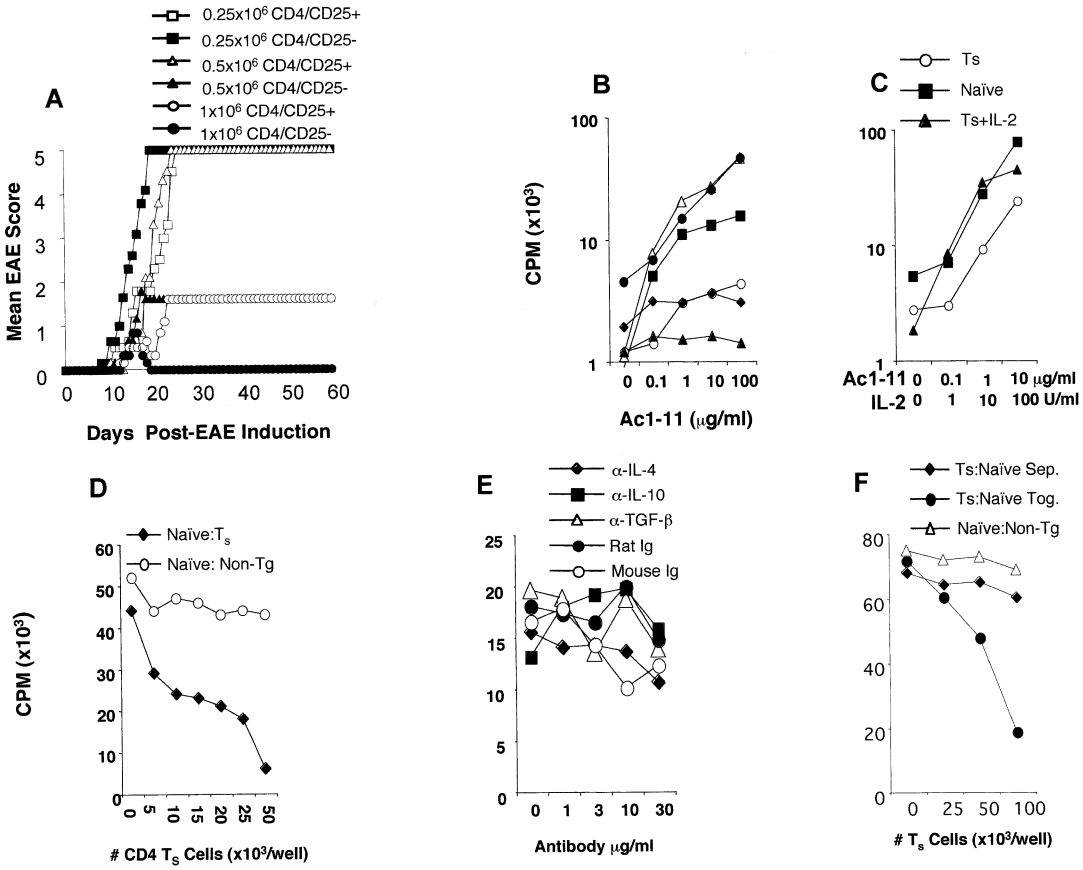

Figure 2. T_s Cells Suppress Effector Cell Proliferation by Cell-to-Cell Contact and Are Functionally Altered

(A) MBP-TCR-Tg $C\alpha^{-/-}$ mice were adoptively transferred with varying doses of sorted $CD4^+CD25^+$ T cells (0.25×10^6 [open square], 0.5×10^6 [open triangle], 1×10^6 [open circle]) or $CD4^+CD25^-$ T cells (0.25×10^6 [closed square], 0.5×10^6 [closed triangle], 1×10^6 [closed circle]) from Ac1-11 ECi mice and monitored for EAE.

(B) Sorted CD4 T cells from various Ac1-11 ECi mice (closed triangle, closed diamond, closed square, open circle), naive MBP-TCR-Tg mice (open triangle), or mice with end-stage EAE (closed circle) were stimulated in vitro with Ac1-11 to determine their proliferation status.

(C) Sorted CD4 T cells from Ac1-11 ECi (open circle) or naive MBP-TCR-Tg mice (closed square) were cultured with APCs and varying concentrations of Ac1-11 alone; CD4 T cells from Ac1-11 ECi mice were also cultured with APCs, and fixed Ac1-11 (10 μg/ml), and varying concentrations of IL-2 (closed triangle).

(D) Sorted CD4 T cells from Ac1-11 ECi mice, when cocultured with naive MBP-TCR-Tg T cells in the presence of peptide and APCs, could suppress naive MBP-TCR-Tg CD4 T cell proliferation in vitro in a dose-dependent manner.

(E) Varying concentrations of anti-IL-4, anti-IL-10, anti-TGF-β mAbs, and their corresponding isotype-matched controls did not block the T_s cell-mediated suppression of naive CD4 T cell proliferation in the presence of fixed numbers of APCs, T_s cells, and antigen in vitro.

(F) In a transwell assay, various numbers of T_s cells were cultured directly with fixed numbers of APCs, effector cells, and antigen (closed circle), or separated from effector cells by a membrane (closed diamond). No inhibition was observed when T_s cells were separated from effector cells, and inhibition was specific to T_s cells as CD4 T cells from nontransgenic littermates (open triangle) showed no inhibition of proliferation.

ease onset occurred at 3 months in control mice and by 4 months, four of the five mice died while one mouse remained free of EAE symptoms. All recipients that received cells from Ac1-11 ECi mice were without any clinical signs of EAE at 7 months (Figure 3C and Table 2A). Thus, CD4 T cells from Ac1-11 ECi mice can suppress spontaneous disease.

ECi with Autoantigen Can Protect Mice from Relapsing-Remitting EAE

In (SJLxPL/J)F1 mice, relapsing-remitting EAE (RR-EAE) can be induced by immunization with the immunodominant peptide of proteolipid protein (PLP)139-151 in CFA (Karandikar et al., 1998). The (B10.PLxSJ/L)F1 mice can also develop RR-EAE by immunization with Ac1-11 in CFA (Dittel et al., 2000). We ECi (SJLxPL/J)F1 mice with varying doses of PLP139-151 or with OVA, PBS, or MOG35-55 as controls, after which we induced disease with PLP139-151 in CFA. (B10.PLxSJL/J)F1 mice were ECi with 10 or 100 μg Ac1-11 or with MOG35-55 as control. Complete protection was observed in mice that were ECi with 1 mg/ml of PLP139-151; mice that were ECi with 100 or 10 μg PLP139-151 had the same time of disease onset as control mice. However, disease was much less severe in mice that were ECi with 100 μg PLP139-151 when compared to the 10 μg group; both of these groups developed significantly less severe disease as compared to the controls. This same pattern

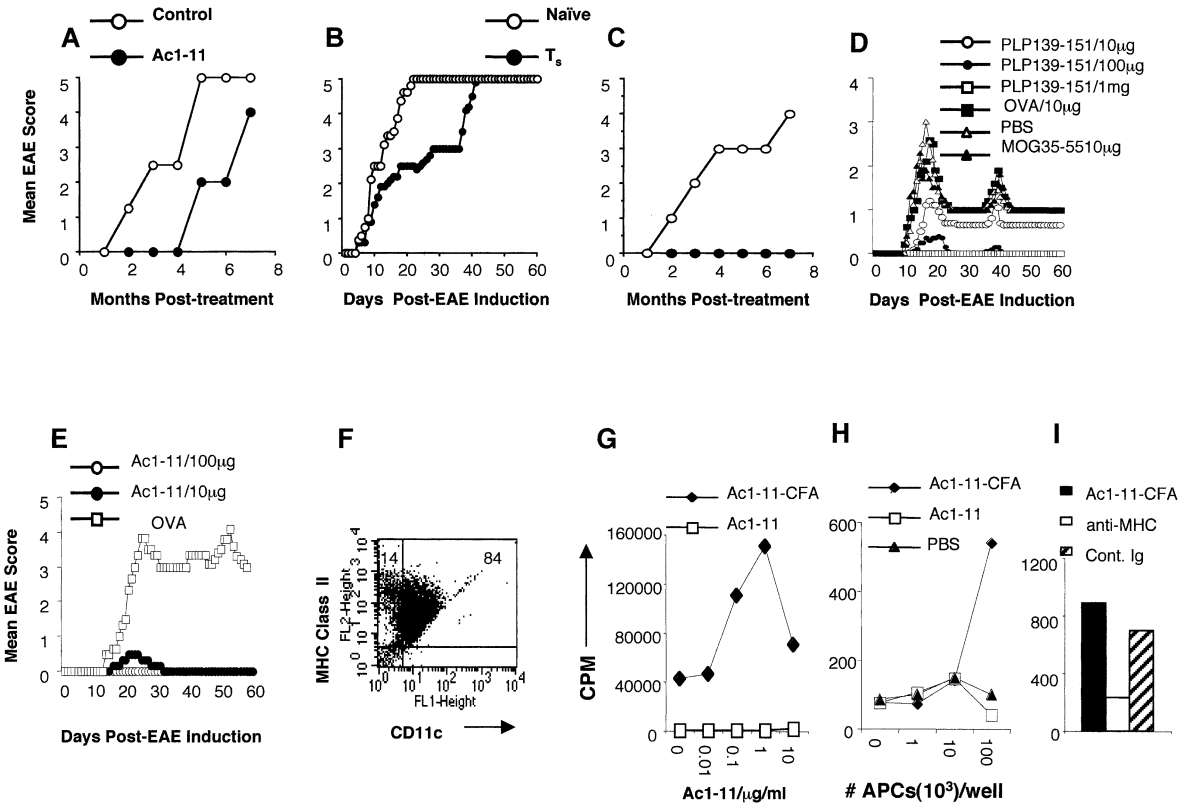

Figure 3. ECi with CNS Antigens (Ac1-11, PLP139-151) Suppress Spontaneous and R-R-EAE

(A) Four- to six-week-old MBP-TCR-Tg $C\alpha^{-/-}$ mice when ECi with Ac1-11 show substantial protection from spontaneous EAE when compared to PBS controls.

(B) Pooled spleen and lymph node cells collected 5.5 months after ECi from Ac1-11 ECi-sp mice (closed symbol) or from control mice (open symbol) were adoptively transferred into naive MBP-TCR-Tg $C\alpha^{-/-}$ recipients, and EAE was induced.

(C) CD4 T cells from Ac1-11 ECi mice (closed symbol) or from naive MBP-TCR-Tg $C\alpha^{-/-}$ mice (open symbol) were transferred into naive MBP-TCR-Tg $C\alpha^{-/-}$ recipients that were monitored for spontaneous disease.

(D) (SJL x PLJ)F1 mice were ECi with varying doses of PLP139-151 peptide. As controls, mice were ECi with OVA, PBS, or MOG135-155 peptide, another CNS antigen.

(E) (B10.PL x SJL)F1 mice were ECi with Ac1-11 at 100 μg, 10 μg, or OVA as control.

(F) Representative MHC class II/CD11c distribution for epidermal APCs prepared after epicutaneous immunization.

(G) Epidermal APCs from mice ECi with Ac1-11 or Ac1-11-CFA were incubated with naive antigen-specific CD4 T cells. CD4 T cells were reisolated prior to stimulation with Ac1-11 and fresh APCs.

(H) APCs from skin-draining lymph nodes of mice that were ECi with Ac1-11, Ac1-11-CFA, or PBS were cultured with naive antigen-specific CD4 T cells without exogenous antigen.

(I) APCs from skin-draining lymph nodes of Ac1-11-CFA ECi mice were cultured with naive antigen-specific CD4 T cells and Ac1-11 plus anti-MHC class II (Y3JP) mAb or isotype control.

was observed in the spontaneous relapse phase of the disease (Figure 3D and Table 3).

In the (B10.PLxSJL/J)F1 mice, significant protection was seen at both peptide (Ac1-11) concentrations (10 and 100 μg). Mice that were ECi with 100 μg of Ac1-11 never developed disease; one mouse in the 10 μg group

Table 3. ECi with Their Cognate Antigens Protected (SJLxPLJ)F1 and (B10.PLxSJL)F1 Mice from R-R-EAE

Mouse Strain	Antigen/Dose	n	# Mice with EAE	# Mice Recovered	Days of EAE Onset	Mean Initial EAE Score	# Mice Relapsed 1st/2nd
(SJLxPLJ)F1	PLP139-151/10 μg	15	7	5	11	1.2	2/2
(SJLxPLJ)F1	PLP139-151/100 μg	10	3	3	13	0.4	1/1
(SJLxPLJ)F1	PLP139-151/1 mg	5	0			0	
(SJLxPLJ)F1	MOG35-55/10 μg	5	5	4	9	2.3	2/3
(SJLxPLJ)F1	OVA/100 μg	5	5	4	10	2.6	3/3
(SJLxPLJ)F1	PBS	10	10	8	10	3	5/3
(B10.PLxSJL)F1	Ac1-11/10 μg	4	1	1	16	0.5	0/0
(B10.PLxSJL)F1	Ac1-11/100 μg	3	0			0	
(B10.PLxSJL)F1	MOG35-55/10 μg	5	5	2	11	3.8	2/2

developed mild disease, fully recovered, and did not relapse over the 60 days of disease monitoring. Control mice developed fulminant EAE: three out of five mice died and the other two relapsed (Figure 3E and Table 3). These data confirmed that ECi with autoantigenic peptide can protect different strains of mice both from the primary disease and the relapses that follow in an antigen dose-dependent and antigen-specific manner.

Additional studies indicated that CD4 T cells from protected (B10.PI x SJL)F1 mice displayed characteristics similar to those of T_s cells from the MBP-TCR-Tg mouse model. They were unresponsive to stimulation with their cognate peptide (Ac1-11) in vitro (see Supplemental Figure S2 at http://www.immunity.com/cgi/content/full/19/3/317/DC1), they did not produce IL-4, IL-10, IL-13, or TGF-β cytokines upon stimulation (data not shown), and they could suppress responder cell proliferation in vitro (Supplemental Figure S3). There was also no change in the frequency of $CD4^+CD25^+$ T cells upon ECi and during long-term protection in both (B10.PL x SJ/L)F1 and (SJL x PL/J)F1 mice. Thus, ECi-induced protection could operate in relapsing-remitting models of EAE and is therefore not restricted to the MBP-TCR-Tg model.

Epidermal Antigen-Presenting Cells Influence the Priming of Naive CD4 T Cells

We explored the possibility that, in ECi mice, epidermal dendritic cells may play a role in the induction of T_s cells. Subsequent to epicutaneous application, the antigen may be gradually and continuously presented to antigen-specific CD4 T cells in skin-draining lymph nodes. Due to the absence of adjuvant, such presentation could impair the functionality of naive T cells. In experiments where MBP-TCR-Tg mice were ECi with Ac1-11 in CFA, we found that mice were clearly not protected from EAE (see Supplemental Table S1). We then isolated antigen-presenting cells from the epidermis of mice that were ECi with Ac1-11 alone or Ac1-11 in CFA for 30 min to an hour. As shown by FACS analysis, the vast majority of such cells expressed MHC class II and CD11c on their surface (Figure 3F). We incubated these APCs in the presence of naive MBP-TCR-Tg CD4 T cells without exogenous antigen. We could detect a weak proliferation only in the presence of epidermal APCs from Ac1-11 + CFA ECi mice (data not shown). We also analyzed CD4 T cells after reseparating them from the epidermal APCs. The reisolated CD4 T cells were incubated with fresh irradiated APCs and varying concentrations of Ac1-11. As shown in Figure 3G, CD4 T cells that were preincubated with epidermal APCs from Ac1-11 ECi mice failed to proliferate upon stimulation. By contrast, CD4 T cells that were preincubated with epidermal APCs from Ac1-11-CFA ECi mice proliferated robustly. We also analyzed APCs collected from skin draining (superficial inguinal and/or axillary) lymph nodes from freshly ECi mice for their ability to stimulate naive antigen-specific CD4 T cells without the addition of exogenous antigen. Remarkably, naive antigen-specific CD4 T cells incubated with lymph node-derived APCs from mice ECi with Ac1-11-CFA showed a detectable proliferation while antigen-specific CD4 T cells incubated with lymph node-derived APCs from Ac1-11 ECi or PBS-treated mice displayed a background response. This showed that Ac1-11 was effectively captured by skin dendritic (Langerhans) cells in ECi mice and carried to the draining lymph nodes (Figure 3H). This proliferation was specific as it could be neutralized by addition of anti-MHC class II (Y3JP) antibody (Figure 3I). From these data, and from the data showing that mice ECi with peptide + CFA are not protected from EAE, we infer that the epicutaneous delivery of antigen in the absence of adjuvant (i.e., absence of costimulatory signal induction) affects the priming of naive antigen-specific T cells and alters their functionality.

T_s Cells Suppress the Secretion of IFN-γ by Effector Cells Both In Vitro and In Vivo

We have consistently found IFN-γ present in the sera of MBP-TCR-Tg mice with active EAE followed by a concomitant rise in IFN-γ titer as disease progressed. This is in contrast to healthy or naive MBP-TCR-Tg mice that do not produce any substantial level of IFN-γ in their sera. We examined pooled sera of Ac1-11 ECi mice or controls for the presence of IFN-γ prior to (week 0) and during ECi (weeks 1 and 2) and post-EAE induction (weeks 3–6). Only mice with progressive EAE produced any substantial amount of IFN-γ in their sera that increased with disease severity. There was no significant level of IFN-γ present in sera of Ac1-11 ECi mice (Figure 4A). Similar observations were made in tissue culture supernatants from antigen-stimulated CD4 T cells from Ac1-11 ECi mice. This is compared to high IFN-γ levels produced by naive CD4 T cells or CD4 T cells from mice with active disease after being stimulated with antigen in vitro (Figure 4B). To further test the effect of Ac1-11 ECi on IFN-γ production in MBP-TCR-Tg mice, we tested IFN-γ levels in supernatants from cultures in which T_s cells suppressed the proliferation of naive antigen-stimulated CD4 T cells. T_s cells suppressed IFN-γ secretion by effector cells at least 2-fold (Figure 4C). This is strong evidence that ECi-induced T_s cells directly impair the Ac1-11-inducible secretion of IFN-γ by naive Ac1-11-specific CD4 T cells.

Lack of CD4 T Cell Infiltration in the Brain Parenchyma of Ac1-11 ECi-Protected Mice

EAE is characterized by the infiltration of autoreactive CD4 T cells into the CNS (Brosnan and Raine, 1996; Goverman, 1999). To ascertain whether CD4 T cells from Ac1-11 ECi mice can infiltrate the CNS, we stained serial brain sections from perfused mice using anti-CD4 monoclonal antibody (Baron et al., 1993). Brains from young naive and diseased immunized mice (clinical EAE score = 1.5 and 3) were stained as controls. Infiltration by CD4 T cells was seen only in the brain of the diseased mice, whereas no infiltration was seen in the perivascular regions or in the parenchyma of the brains of Ac1-11 ECi, Ac1-11 ECi-sp, or naive mice (Figures 4Da, 4Db, and 4Dc, respectively, and summarized in Figure 4E). Therefore, the lack of disease symptoms observed in Ac1-11 ECi mice correlates with the lack of CD4 T cell infiltration in the brain. This is well in line with the data showing that T_s cells preempt the activation of naive Ac1-11-specific CD4 T cells. Altogether the data indicate

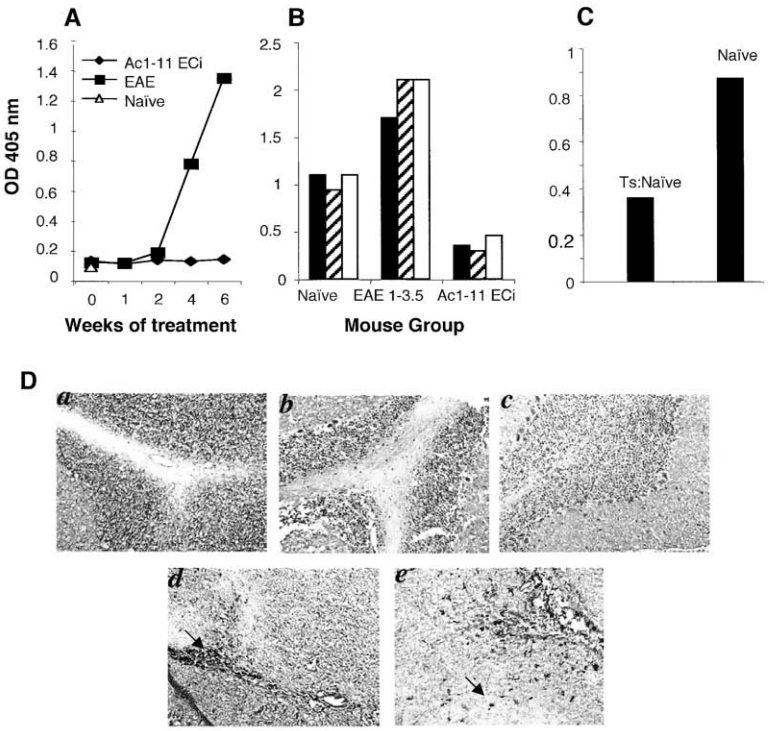

Figure 4. T_s Cell-Mediated Suppression of IFN-γ Secretion by Naive CD4 T Cells and Lack of CD4 T Cell Infiltration in the Brain Parenchyma of Disease-Resistant Mice

(A) Sera from disease resistant (Ac1-11 ECi), diseased (EAE), and unmanipulated (naive) mice were tested for the presence of IFN-γ by ELISA prior to (week 0) and during ECi (weeks 1–2) as well as after EAE induction (weeks 3–6).

(B) CD4 T cells from protected (Ac1-11 ECi) mice, normal MBP-TCR-Tg (naive) mice, or mice with active (score 1–3.5) EAE were stimulated in vitro with Ac1-11, and the supernatants were tested for IFN-γ production by ELISA. Three mice were analyzed in each group.

(C) ELISA measurement of IFN-γ production in supernatants obtained from cultures in which T_s suppressed the proliferative response of naive MBP-TCR-Tg T cells to cognate stimulation.

(D) No CD4 T cell infiltration is detectable in the brain parenchyma of protected mice by immunohistochemistry. Sections of perfusion-fixed brain tissue from Ac1-11 ECi mice protected from spontaneous EAE (a), induced EAE (b), naive mice (c), and mice with clinical signs of EAE (score of 1.5 [d] and 3 [e]).

(E) Detailed summary of histological analysis.

E

	Mice	*n*	Type of EAE	ECi	EAE Score	Total # Fields Scored	Avg # Fields With infiltration
a	MBP-TCR-Cα$^{-/-}$	2	Spont	+	0	60	0
b	MBP-TCR-Tg	3	Ind	+	0	90	0
c	MBP-TCR-Tg(naïve)	3	None	-	0	90	0
d	MBP-TCR-Cα$^{-/-}$	3	Spont	-	1.5	60	18
e	MBP-TCR-Tg	3	Ind	-	3	90	58

Ind (induced); Spont (spontaneous)

that T_s cells act on the induction phase of the disease and not by controlling autoreactive T cells in the CNS.

Discussion

Epicutaneous immunization with protein or peptide antigens in the absence of adjuvant can generate Th2 immune responses (Herrick et al., 2000; Wang et al., 1996, 1999). We reasoned that the delivery of autoantigenic peptide through the skin could interfere with the development of inflammatory autoimmune diseases where Th1 cells play a central role (King and Sarvetnick, 1997). We observed that the simple epicutaneous application of the Ac1-11 peptide effectively protects MBP-TCR-Tg mice and MBP-TCR-Cα$^{-/-}$ mice from developing induced and spontaneous EAE, respectively. Disease suppression was mediated by CD4 T cells because the adoptive transfer of sorted CD4 T cells from protected mice was sufficient to protect naive syngeneic recipients from developing disease.

Relative to naive MBP-TCR-Tg CD4 T cells, CD4 T cells able to confer protection were poorly responsive or unresponsive to cognate stimulation and therefore appeared functionally inactivated. Such a state of unresponsiveness is consistent with the assumption that, under the conditions used, Ac1-11 is gradually and continuously captured by skin resident dendritic (Langerhans) cells (Cumberbatch et al., 2000), which in the absence of adjuvant do not acquire their high stimulatory potential (Linsley and Ledbetter, 1993; Ragazzo et al., 2001; Wulfing and Davis, 1998). Ac1-11 applied to the epidermis could be captured and transported to draining lymph nodes because APCs derived from skin-draining lymph nodes of ECi mice could induce a detectable and specific proliferation of naive MBP specific CD4 T cells. APCs derived from epidermal cell preparations differed in their capacity to stimulate naive CD4$^+$ MBP-TCR-Tg T cells depending on whether they were isolated from mice epicutaneously exposed to autoantigen alone or to autoantigen plus adjuvant. Further, exposure to epidermal CD11c$^+$ MHC class II$^+$ cells from Ac1-11 ECi mice clearly converted naive MBP-TCR-Tg CD4 T cells into T cells unresponsive to cognate stimulation. Not surprisingly, Ac1-11-CFA-ECi MBP-TCR-Tg mice were

not protected from EAE induced by immunization. Instead, the onset of the clinical signs of disease was significantly accelerated in these mice (see Supplemental Table S1).

CD4 T cells from Ac1-11 ECi mice were capable of inhibiting both the proliferation of, and the IFN-γ secretion by, naive $CD4^+$ MBP-TCR-Tg T cells in response to Ac1-11 stimulation in vitro. This inhibition required direct contact with effector cells and did not rely on killing as similar numbers of living cells were recovered in the presence of CD4 T cells from protected mice or of control CD4 T cells (data not shown). Together with the protective effect observed in transfer experiments, these results indicate that the epicutaneous delivery of Ac1-11 to Tg mice expressing the Ac1-11-specific MBP-TCR generates CD4 T cells with inhibitory activity. We believe that the induced inhibitory cells are best described by the term "suppressor T cells" (T_s), which was first coined by Gershon and coworkers to emphasize the negative side of the immunoregulatory potential of T lymphocytes (Gershon and Kondo, 1971; Hu et al., 1983; Ptak et al., 1983).

At least two major mechanisms might account for the absence of clinical symptoms in Ac1-11 ECi MBP-TCR-Tg mice. First, MBP-TCR-Tg T cells could acquire a pathogenic potential upon immunization and traffic to the CNS but not cause lesions due to in situ control by cells with inhibitory activity (Brabb et al., 2000). Alternatively, suppression could occur early in the periphery and preempt the differentiation of pathogenic T cells. Our histological results definitively ruled out the first hypothesis; there were neither mild tissue lesions nor CD4 T cell infiltration in the CNS of disease-resistant mice. In addition, there was no deficiency in the expression of the VLA-4 homing molecules known to be crucial for the migratory capacity of pathogenic MBP-TCR-Tg T cells (Baron et al., 1993) in these mice (data not shown). These observations imply that under our experimental conditions T_s cells generated by ECi protect mice from developing disease by interfering with the priming of naive MBP-specific T cells in lymphoid organs. The experimental support for this conclusion is 2-fold. First, as mentioned above, purified CD4 T cells from disease-resistant mice had the capacity to block the specific activation (proliferative response and IFN-γ secretion) of purified naive MBP-TCR-Tg CD4 T cells in vitro. Second, in sharp contrast with control mice developing clinical signs of EAE, we could not detect the Th1 cytokine IFN-γ in the serum or in culture supernatant of cells from disease-resistant mice. The in vivo CD4 T cell-mediated suppression we observed is therefore fundamentally distinct from regulatory mechanisms able to control CD4 T cells in situ after they have crossed the blood-brain barrier (Brabb et al., 2000).

We repeatedly failed to detect significant secretion of the anti-inflammatory cytokines IL-4, IL-10, IL-13, or TGF-β by T_s cells by ELISA upon specific stimulation or in suppression assays in vitro. Molecular analysis such as RPA also failed to show significant increase in cytokine transcript levels in these cells (data not shown). Consistent with this was the fact that the infusion of monoclonal antibodies able to neutralize IL-4, IL-10, and TGF-β in vivo appeared to have no significant impact on the ECi-induced protection from disease. In vitro suppression was unaffected as well.

It has been shown that delivery of antigen through the intestinal mucosa could either recruit (Zhang et al., 2001) or generate (Thorstenson and Khoruts, 2001) $CD4^+CD25^+$ T cells with immunosuppressive properties. However, we observed that ECi had no positive impact on the representation of the $CD4^+CD25^+$ T cell pool in mice that were protected against EAE by ECi. In addition, sorted $CD4^+CD25^-$ T cells alone could suppress induced EAE upon adoptive transfer and, on a per cell basis, were more potent in doing so than $CD4^+CD25^+$ T cells. We conclude that there was no greater dependence on $CD4^+CD25^+$ T cells than on $CD4^+CD25^-$ cells in the disease resistance conferred by the T_s cells generated by ECi with Ac1-11. Suppressive activity among $CD4^+CD25^-$ T cells has indeed been reported both in mice and rat (Annacker et al., 2000; Apostolou et al., 2002; Stephens and Mason, 2000). Most interestingly, the naturally occurring $CD4^+$ T cell population able to protect MBP-TCR-Tg mice from spontaneous EAE is itself characterized by the $CD25^-$ phenotype (Olivares-Villagomez et al., 2000).

During the course of transfer experiments, we noticed that CD4 T cells from Ac1-11 ECi mice could fully protect naive recipients from both spontaneous and induced EAE. Although CD4 T cells from Ac1-11 ECi-sp mice also fully protected against spontaneous disease, they only transiently reduced disease severity upon active induction. Possibly, these results could be described by a model where ECi with Ac1-11 induces naive CD4 T cells to adopt a presuppressor state that is converted into a dominant suppressor state upon immunization with antigen, adjuvant, and pertussis toxin.

Although EAE developing in the MBP-TCR-Tg mouse shares many characteristics with MS in humans, the absence of spontaneous relapses limits their utility as a model for elucidating the full scope of MS pathogenesis. Another important feature is the lack of a normal or physiologic T cell repertoire; greater than 95% of CD4 T cells in this mouse model bears the same TCR specificity. In this paper, we also demonstrate that ECi with autoantigen is not limited to the MBP-TCR-Tg mouse model, as we can protect against EAE in mouse models with a diverse T cell repertoire. For instance, the epicutaneous application of the immunodominant peptide of PLP (139-151) protected unmanipulated (SJL x PL/J)F1 mice from relapsing-remitting EAE. This protection, like that observed in the MBP-TCR-Tg mouse, was antigen specific and antigen dose dependent. In contrast with the MBP-TCR-Tg mouse model, maximum protection was observed at higher peptide concentrations in both the (SJL x PL/J)F1 and (B10.PL x SJL/J)F1 hybrids. Protection was observed both against the initial disease phase and in spontaneous relapses. Thus, ECi with autoantigen prior to disease induction protects genetically unmanipulated mice from both primary and recurrent disease.

The influence of antigen dose and route of delivery has been shown to be a key variable in inducing immune tolerance (Weiner, 2001). Ac1-11 at 10 μg on a skin patch resulted in the highest level of protection, while higher (100–1 μg) and lower doses (0.1–1 mg) of peptide on the skin patch resulted in more limited protection.

The converse is true in the (SJL x PL/J)F1 and (B10.PL x SJL/J)F1 mice. It is possible that in the MBP-TCR-Tg mouse, high antigen concentrations could potentially induce a number of MBP-TCR-specific CD4 T cells to differentiate into effector cells. Antigen doses lower than 10 μg may be too small for efficient induction of T_s to occur, perhaps because of the vast excess of MBP-TCR-Tg CD4 T cells. Therefore, the majority of MBP-TCR-Tg CD4 T cells could remain naive, become pathogenic upon immunization, and cause EAE. Conversely, the number of potentially autoreactive pathogenic T cells is rather small in nontransgenic models relative to the MBP-TCR-Tg mouse model, and induction of suppressor cells may require a higher concentration of antigen.

In summary, we have demonstrated that ECi with a tissue-specific self-peptide can fully protect mice from developing an autoimmune response driven by CD4 T cells specific for the same determinant. Disease resistance was mediated by induced CD4 suppressor T cells. The induced protection was not dependent on engagement/recruitment of $CD4^+CD25^+$ T cells, and it could operate against both spontaneous and induced disease. We also demonstrated that the epicutaneous application of CNS antigens to nontransgenic mice can fully protect them from primary EAE and from spontaneous relapses. Collectively, these findings may help in the design of new, noninvasive therapies to prevent or to treat chronic inflammatory autoimmune disorders such as multiple sclerosis, type 1 diabetes, and rheumatoid arthritis.

Experimental Procedures

Mice

MBP-$TCR^{+/-}$ transgenic mice (MBP-TCR-Tg) express the rearranged genes encoding the α (Vα4) and β (Vβ8.2) chains of an autoreactive, I-A^u-restricted TCR specific for the N-terminal acetylated peptide (Ac1-11) of MBP (Hardardottir et al., 1995). MBP-TCR-Tg mice were mated to TCR-$C\alpha^{-/-}$ mice to generate MBP-TCR-$C\alpha^{-/-}$ mice. (SJLxPLJ)F1 female mice 4–6 weeks old, and B10.PL (H2u) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). (B10.PLxSJL)F1 mice were generated by crossing SJL with B10.PL. All mice are housed in the pathogen-free facility at Yale University, New Haven, CT.

Epicutaneous (Skin) Patch Immunization and EAE Induction

The backs of mice were shaved with an electric razor after being lightly anaesthetized with methoxyflurane (Schering-Plough, Union, NJ). One day later, Ac1-11 or PLP139-151 (0.1 μg/ml to 1 mg/ml) in 100 μl PBS was applied to the gauze on an occlusive patch (DuoDERM Extra Thin; Convatec, Princeton, NJ) and applied to the shaved area (Herrick et al., 2000). As controls, MOG35-55, PLP139-151, or OVA (Grade V, Sigma Chemical Co., St. Louis, MO) (100 μg/ml or 10 μg/ml) in PBS, or PBS were used. The patch was left in place for 1 week. This was repeated for a second week. At the end of week 2 the patch was removed and EAE was induced by immunizing mice subcutaneously in the flanks with Ac1-11 or PLP139-151 in complete Freund's adjuvant (CFA), followed by intravenous pertussis toxin injection on the day of immunization and 2 days later.

EAE Scoring

A numerical score was assigned to mice based on the severity of the disease symptom. 0 = no disease; 1 = limp tail; 2 = weak tail and partial hind limb paralysis; 3 = total hind limb paralysis; 4 = both hind limb and fore limb paralysis; 5 = dead. Mice with a score of 4 were euthanized.

T Cell Proliferation, Suppression, Anti-IL-2 Treatment, and Transwell Experiments

Sorted CD4 T cells from spleen and lymph nodes of naive MBP-TCR-Tg mice were plated at 5×10^4cells/well in the presence of 2×10^5 irradiated B10.PL spleen cells/well as antigen-presenting cells in Click's EHAA medium (Life Technologies) supplemented with 5% fetal calf serum. Ac1-11 (1–10 μg/ml) alone or with IL-2 (1–100 U/ml) with fixed concentration of Ac1-11 (10 μg/ml) was added to the cultures. After 48 hr at 37°C, cells were pulsed with 1 μCi of [^{3}H]thymidine, harvested 18–24 hr later, and counted in a β plate scintillation counter. To determine whether CD4 T cells from Ac1-11 ECi mice can suppress the proliferation of naive CD4 T cells from MBP-TCR-Tg mice, various numbers of CD4 T cells from Ac1-11 ECi mice or CD4 T cells from nontransgenic mice (controls) were cocultured with a fixed number of naive MBP-TCR-Tg CD4 T cells (5×10^4). 2×10^5 irradiated APCs were added, and a fixed concentration of Ac1-11 (5 μg/ml) was used for stimulation. Culture conditions were as described above. Transwell assays were done in 24-well plates (Costar, Cambridge, MA) with fixed numbers of effector cells, APCs, and Ac1-11 (5 μg/ml) in the presence or absence of varying numbers of T_s or nontransgenic CD4 T cells as control.

Adoptive Transfer

Total splenocytes (pooled spleen and lymph node cells) from naive (control) or Ac1-11 ECi mice were washed in sterile PBS two to three times, and 1×10^7 cells were resuspended in a final volume of 200 μl PBS and administered intravenously to irradiated (600 rads), naive B10.PL mice 1 day after irradiation. EAE was induced 5 days after transfer. CD4 T cells were stained with the clonotypic antibody 3H12 and sorted. 1×10^6 Ac1-11-specific CD4 T cells were also resuspended in 200 μl of PBS and administered intravenously to MBP-TCR-$C\alpha^{-/-}$ recipients. EAE was induced 5 days after transfer.

Tissue Preparation and Immunohistochemistry

Mice were perfused and their fixed brains and spinal cords removed and prepared exactly as described (Baron et al., 1993). Immunostaining was performed on 8 μm sections with biotinylated anti-CD4 monoclonal antibody as primary antibody.

Antibodies and FACS Sorting

Cells were stained with anti-CD4-FITC conjugate or anti-CD4-PE (Caltag, Burlington, CA), anti-mouseVβ8.1/2-FITC or PE (Pharmingen, San Diego, California, USA), and/or anti-CD25-PE or with anti-clonotypic antibody, 3H12 (a kind gift from Dr. Juan Lafille). Anti-MHC class II antibody (Y3JP) was prepared by our laboratory; anti-CD11c-FITC mAb was purchased from Pharmingen. The cells were incubated at 4°C for 30 min before being washed and were then sorted on a FACstar plus. Sorted cells were analyzed on a FACSscan analyzer (Beckton Dickinson). IL-2, anti-IL-4 (11B11), anti-IL10 (SXC1), and anti-I-A^u (Y3JP) were all produced by our laboratory. Anti-TGF-β (β1, β2, β3; clone # 1D11) was purchased from R&D systems (Minneapolis, MN). Biotinylated anti-CD4 was purchased from Caltag. CD4 T cells were isolated from skin-draining lymph node preparation using goat anti-mouse IgM, goat anti-Rat IgG, and goat anti-mouse IgG magnetic beads (Qiagen, Hilden, CA) as per manufacturer's protocol.

Anti-Cytokine Treatment In Vivo and In Vitro

Mice were treated intraperitoneally with 1 mg of anti-IL-4 or anti-IL-10, or 0.5 mg of anti-TGF-β or isotype-matched controls (in 300 μl PBS) at 1 week intervals prior to (week 0) and during ECi (weeks 1 and 2) and for 2 weeks after disease induction (weeks 3 and 4). Sorted naive CD4 T cells (5×10^5) were cocultured with APCs (2×10^5) and Ac1-11 at 10 μg/ml in the presence of 5×10^5 T_s cells and neutralizing monoclonal antibodies to IL-4, IL-10, TGF-β, or their isotype-matched Ig as controls (rat IgG1, mouse IgG1).

IFN-γ ELISA

Sera were obtained from mice at day 0 (week 0, prior to skin patching), week 1 (1 week after skin patching), week 2 (end of 2 weeks of skin patching), and weeks 3 to 6 (after EAE induction), pooled from three different experiments, and frozen at −70°C until use. Supernatants were collected (at 24 and 48 hr, and day 5 and 14

days) from in vitro-stimulated sorted CD4 T cells from pooled spleen and lymph node from Ac1-11 ECi, naive, or diseased mice. ELISAs were performed on supernatants or 10-fold serial dilutions of sera in PBS. 96-well microtiter plates (Dynex Technologies Inc., Chantilly, VA) were coated with 2 mg/ml of purified anti-mouse IFN-γ in 0.1 M sodium carbonate buffer and incubated at 4°C overnight. Plates were washed three times with PBS-0.05% Tween-20 (American Bioanalytical, Natick, MA), then blocked with 1% BSA in borate-buffered saline, and incubated at 37°C for 1 hr. Sera or supernatant samples and IFN-γ standard (recombinant murine IFN-γ, Pharmingen) were incubated for 2 hr at room temperature and then washed three times in PBS-0.5% Tween. Biotinylated anti-mouse INF-γ (2 μg/ml, XMG1.2, Pharmingen) was added, and plates were incubated at 37°C for 1 hr. After three washings, streptavidin-alkaline-phosphatase conjugate (Pharmingen) was added, and plates were incubated for 1 hr at room temperature and then washed three times. Plates were developed with the enzyme substrate, *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets; Sigma) and read at optical density, 405 nm.

ECi and Epidermal Cell Preparation and Isolation of APCs from Skin-Draining Lymph Nodes

B10.PI mice were ECi with 100 μg of Ac1-11, Ac1-11 in CFA, or PBS in a patch for 30 min to an hour after which the mice were sacrificed, shaved from the neck to the base of the tail, and bathed in ethanol. The fatty tissue was removed, and the skin was then cut into 1 cm strips and placed in a petri dish containing trypsin (Sigma) with the dermal side facing down. The petri dishes with the skin samples were incubated at 37°C for 1.5 hr. The epidermis, which was held facing up, was scraped off with smooth forceps while carefully avoiding dermal contamination by lifting up after scraping along the strip of skin so as to take only the epidermal sheet. The epidermis was put in 50 ml tubes containing trypsin and DNase-1 (Boehringer-Mannheim) and kept on ice until all the strips were done. The epidermal preparation was next incubated at 37°C in a shaking water bath for 10 min and then filtered to remove debris. The cells were centrifuged for 10 min at 1000 rpm at 4°C. The crude epidermal preparation was resuspended in media containing pen/strep (100×; Gibco) and 0.1% of DNase-1. Using 4 ml of media per dish of skin, each dish was put over a Histopaque (Sigma) gradient and centrifuged for 20 min at 1200 rpm at room temperature. The interface cells (called interface epidermal cells) were harvested, washed in media, and counted. Langerhans cells can be expected to be between 5%–20%. Epidermal cells were plated in 24-well plates (Falcon, Becton Dickinson) at 1×10^5 at a 1:5 ratio with naive antigen-specific CD4 T cells and incubated for 48 hr. CD4 T cells were separated from epidermal APCs by magnetic bead fractionation (as described above) and cultured (5×10^4/well) with fresh irradiated APCs (2×10^5) and varying concentrations of antigen. B10.PI mice were ECi with 10 μg of Ac1-11 or Ac1-11 in CFA, or PBS for 18 hr, and their lymph nodes were harvested for APCs. Antibodies to remove CD4 (11b11), CD8 (TIB150), and NK1.1 (HB191) and complement (Cedarlane Distributors, Ontario, Canada) were added to cell suspension and incubated for 1 hr at 37°C to obtain APCs. Cells were plated in varying numbers in the presence of 5×10^4 naive antigen-specific CD4 T cells without antigen and with or without the presence of anti-I-A^u antibody (0–30 μg/ml) or a control Ig and incubated for 48 hr.

Acknowledgments

This work is dedicated to the memory of Dr. Charles A. Janeway, Jr. We thank C. Annicelli and Joanne Apicelli for help with mice, A. Etgen for reading the manuscript, Elise Tran for technical assistance, and C. Herrick and H. MacLeod for help with the skin patch. This work was supported by the Howard Hughes Medical Institute and by NIH grants AI14579 and Po1A136529 to C.A.J.

Received: April 10, 2003
Revised: May 13, 2003
Accepted: June 4, 2003
Published: September 16, 2003

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