Inactivation of T Cell Receptor Peptide-specific CD4 Regulatory T Cells Induces Chronic Experimental Autoimmune Encephalomyelitis (EAE)

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Summary

T cell receptor (TCR)-recognizing regulatory cells, induced after vaccination with self-reactive T cells or TCR peptides, have been shown to prevent autoimmunity. We have asked whether this regulation is involved in the maintenance of peripheral tolerance to myelin basic protein (MBP) in an autoimmune disease model, experimental autoimmune encephalomyelitis (EAE). Antigen-induced EAE in (SJL × B10.PL)F1 mice is transient in that most animals recover permanently from the disease. Most of the initial encephalitogenic T cells recognize MBP Ac1-9 and predominantly use the TCR Vβ8.2 gene segment. In mice recovering from MBP-induced EAE, regulatory $CD4^+$ T cells (Treg) specific for a single immunodominant TCR peptide B5 (76-101) from framework region 3 of the Vβ8.2 chain, become primed. We have earlier shown that cloned B5-reactive Treg can specifically downregulate responses to Ac1-9 and also protect mice from EAE. These CD4 Treg clones predominantly use the TCR Vβ14 or Vβ3 gene segments. Here we have directly tested whether deletion/blocking of the Treg from the peripheral repertoire affects the spontaneous recovery from EAE. Treatment of F1 mice with appropriate Vβ-specific monoclonal antibodies resulted in an increase in the severity and duration of the disease: even relapses were seen in one-third to one-half of the Treg-deleted mice. Interestingly, chronic disease in treated mice appears to be due to the presence of Ac1-9–specific T cells. Thus, once self-tolerance to MBP is broken by immunization with the antigen in strong adjuvant, TCR peptide–specific CD4 Treg cells participate in reestablishing peripheral tolerance. Thus, a failure to generate Treg may be implicated in chronic autoimmune conditions.

For most of the history of immunology, it had been thought that the modus operandi of the immune system required discrimination between self and nonself, leading to a prevention of response to the former. The presence of self-reactive T cells and the primacy of self-recognition is now considered essential for the normal functioning of the immune system. Indeed, since positive selection is based on recognition of self-peptides in a self-MHC context, to one extent or another, it is clear that responses to foreign antigens depend on cross-reactivity with self (1–3). The ease with which self-reactive T cells can be detected in normal individuals is one indication that negative selection is incomplete and that other mechanisms must operate subsequently to maintain peripheral tolerance to self.

Therefore, self-tolerance to at least some tissue-specific antigens is not entirely a passive process but rather an active dynamic state in which potentially pathogenic self-reactive T cells are prevented from causing disease by other regulatory T cells (4). The differential secretion of lymphokines by T cell subsets offers an explanation for the ability of certain T cells to induce autoimmunity and others to regulate these autoreactive T cells (5). Th1 cytokines have been shown to be involved in cell-mediated autoimmune diseases. For example, IFN-γ, lymphotoxin, or TNF-α secretion correlate with the encephalitogenic capacity of T cell clones reactive to myelin basic protein (MBP)[1] (6). Anti-TNF-α antibodies have been shown to inhibit experimental autoimmune encephalitis (EAE) and collagen-induced arthritis while anti–TGF-β accelerates the disease (reviewed in 4, 5). Both TGF-β and IL-10 can play an important role in regulating autoimmune inflammatory reactions.

Similarly, if immunoregulatory mechanisms malfunction, the resulting deficiency of regulatory T cells could lead to autoimmunity. For example, depletion of a particular subset of T cells results in thyroiditis in mice (7); athymic rats reconstituted with $CD45RB^{high}$ $CD4^+$ T cells alone develop a severe autoimmune condition but when reconstituted with both $CD45RB^{high}$ and regulatory $CD45RB^{low}$

[1] *Abbreviations used in this paper:* EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; PPD, purified protein derivative of mycobacterium tuberculosis; PTx, pertussis toxin; Treg, regulatory $CD4^+$ T cells.

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T cells they were devoid of autoimmune inflammation (8). These and other neonatal depletion and reconstitution experiments suggest that a determining factor in the expression of autoimmunity is the equilibrium between autoreactive and regulatory T cells (9).

EAE is a T cell–mediated autoimmune demyelinating disease of the central nervous system that can be induced after immunization with MBP or its peptide fragments. The TCRs of MBP-reactive T cells in several strains of mice and rats have been shown to be encoded by a limited set of V-region gene segments (10–13). After immunization with MBP, most $CD4^+$ T cells in B10.PL or PL/J mice recognize the immunodominant NH_2 terminal peptide Ac1-9 and a majority of $CD4^+$ T cells use the TCR Vβ8.2 gene segment (10, 11). The limited repertoire of TCR V genes engaged in response to MBPAc1-9 has allowed the use of mAbs to Vβ8 in vivo to successfully prevent or treat EAE (10, 11).

Regulatory responses capable of protecting animals from autoimmunity can be raised to the V region of the TCR after vaccination with disease-causing, MBP-reactive $CD4^+$ T cells or more recently, after immunization with TCR peptides from the CDR II (amino acids 39–59) or the framework III (amino acids 76–101) region of the TCR-β or the CDR III region of the TCR-α chain of encephalitogenic T cells (14–17). We have clearly established in the murine model that protection from EAE by TCR peptides can be mediated by regulatory T cells that are physiologically induced after MBP or Ac1-9 immunization (18). We have characterized TCR peptide–reactive T cells at the clonal level and their physiological role during the course of EAE. We have studied the dynamics of the spontaneous physiological induction of anti–TCR-peptide responses during EAE in B10.PL mice. It was shown that T cells directed against the dominant TCR-peptide of Vβ8.2, B5 (76-101), generally were primed during recovery from MBP- induced EAE without the necessity for external challenge by TCR peptides. We have isolated Vβ14-expressing T cell lines, clones, and T cell hybridomas specific for B5, a peptide encompassing framework region 3 of the Vβ8.2 chain. These T cells were $CD4^+$, $CD8^-$, and MHC class II restricted. In adoptive transfer experiments, B5-reactive T cell clones, but not B4-specific T cells, specifically inhibit proliferative responses to Ac1-9 and protect mice from MBP-induced EAE.

Here we have asked whether physiologically activated TCR-peptide B5-specific T cells are directly involved in mediating spontaneous recovery in (SJL × B10.PL)F1 mice. F1 mice were chosen for their consistent acute disease course (<10% of animals develop chronic symptoms) and high disease-incidence to MBP Ac1-9–induced EAE. We show that after treating animals with mAbs against specific Vβ-chains, predominantly used by regulatory $CD4^+$ T cells (Treg) cells, there is delayed recovery and the mice contract chronic EAE. Upon reconstitution with cloned B5-specific T cells these Vβ-depleted mice showed an accelerated recovery. Thus, TCR-peptide specific CD4 T cells that are spontaneously primed in F1 mice are involved in mediating spontaneous recovery from EAE. These findings describe the protective role of Treg cells in restoring peripheral tolerance and preventing outbreaks of EAE. We conclude that chronic and relapsing disease could be due to defective regulation.

Materials and Methods

Mice. SJL and B10.PL mice were purchased from The Jackson Laboratory, Bar Harbor, ME. (SJL × B10.PL)F1 mice were bred under specific pathogen–free conditions in our own colony. Female mice were used at 8–14 wk of age.

Antibodies. The following mAbs were used: anti–CD4-PE (GK1.5 from Becton Dickinson and Co., Mountain View, CA), anti-Vβ14 (14-2, rat IgM, 19), anti-Vβ5 (20), and anti-Vβ3 (KJ-25, 21). Anti-Vβ14 (14-2) antibodies acquired from PharMingen contained 0.1% (wt/vol) sodium azide. Two control experiments were done to rule out any effect of azide on EAE: first, a desalting column was used to purify this IgM molecule; second, an equal amount of azide in saline (50–100 μl of 0.1% solution) was injected in control animals with no effect on EAE. The hybridomas producing anti-Vβ8.2 and anti-Vβ14 antibodies were generously provided by Drs. Michael Bevan (University of Washington, Seattle), and David Raulet (University of California, Berkeley), respectively. Since only a single anti-Vβ14 mAb, 14-2, is available, attempts were made to differentiate simple receptor blocking vs depletion after antibody injection by in vitro culture of peripheral T cells with recombinant IL-2. 3 d after antibody injections, at a time when apparent depletion was detected (see Table 2), nucleated splenic cells were collected from mice given a single injection of the mAb and divided into two fractions. One fraction was immediately stained, whereas the other was cultured for 72–96 h with rIL-2 and then stained with anti-TCR monoclonals. The proportion of cells in each fraction that stained positively for Vβ14 was compared and found to be similar (0.5 vs 0.7), suggesting depletion of T cells.

TCR Peptides. TCR peptides used were same as reported previously (17,18) and were synthesized by S. Horvath (Caltech, Pasadena, CA) using a solid-phase technique on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) and were purified on a reversed-phase column by HPLC (22).

Splenic Proliferation Assay. Spleens of mice were removed 25–35 d after immunization and a single cell suspension was prepared. Splenocytes (8×10^5 cells/well) were cultured in 96-well microtiter plates in 200 μl of serum free medium (HL-1; Ventrex, Portland, ME) supplemented with 2 mM glutamine; peptides were added at concentrations ranging from 0.1 to 7 μM final concentration. Proliferation was assayed by addition of 1 μCi [^{3}H]thymidine (International Chemical and Nuclear, Irvine, CA) for the last 18 h of a 5-d culture, and incorporation of label was measured by liquid scintillation counting.

Induction of EAE. For induction of EAE, mice were immunized subcutaneously with 100 μg Ac1-9 emulsified in CFA and 0.15 μg pertussis toxin (PTx) (List Biological Laboratories, Inc., Campbell, CA) was injected in 200 μl saline intravenously 48 h later. Mice were observed daily for signs of EAE until >60–90 d after MBP Ac1-9 immunization. Mice for some of the initial Treg-deletion experiments were monitored in a double-blind manner by two independent observers. The average disease score for each group was calculated by averaging the maximum severity of all of the affected animals in the group. Disease severity was scored on a five-point scale (18): 1, flaccid tail; 2, hind limb weakness; 3, hind limb paralysis; 4, whole body paralysis; 5, death.

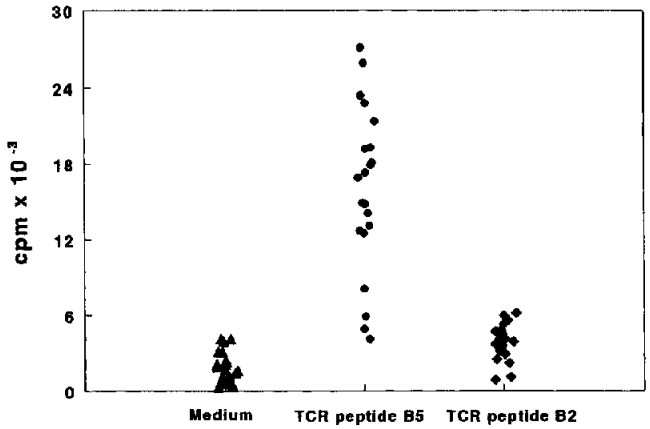

Figure 1. T cells reactive to the immunodominant TCR peptide B5 become primed in mice recovering from EAE. (SJL × B10.PL)F1 mice were immunized with MBP or Ac1-9/CFA and PTx (48 h later). 25–35 d later, the proliferative recall responses to the immunogenic TCR peptides from the Vβ8.2 chain were tested in the spleen. There was no proliferation to TCR peptides in spleen cells from unimmunized F1 mice. By 30 d, at the time of assay, mice had recovered from paralysis. Responses in individual mice (a total of 21 mice) to medium alone (▲), B2 (◆), and B5 (●) at an optimum concentration (3 μM) are shown. The data are expressed as arithmetic means of [^{3}H]thymidine incorporation (cpm × 10^{-3}) in triplicate cultures. These data have been pooled from five individual experiments.

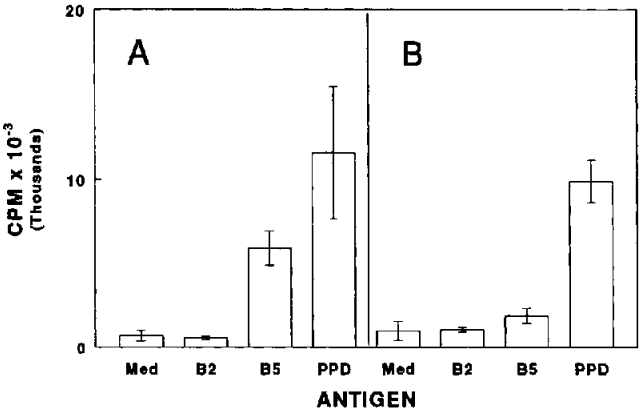

Figure 2. Selection of Vβ14$^+$ CD4$^+$ T cells in the B5 response in vivo. Splenic T cells from F1 mice recovered from MBP-induced EAE were removed, stained, and sorted by FACS® into Vβ14-positive (*A*) and -negative (*B*) CD4 cells using fluorescence-labeled mAbs. Sorted populations were stimulated with the indicated peptides or with PPD and syngeneic, 3,000 rad-irradiated spleen cells. Cultures were pulsed with [^{3}H]thymidine 72 h later and proliferation is shown as the mean values of thymidine uptake in triplicate cultures over the next 24 h.

Flow Cytometry and Cell Sorting Analysis. Antibodies were either purified from hybridoma supernatants by protein A chromatography or from ascites using gel-exclusion chromatography, as recommended by the manufacturers (Middlesex Sciences, Mansfield, MA). Some antibodies were biotinylated with NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL) according to the manufacturer's recommendations. For cell staining, antibodies were used in PBS containing 1% fetal bovine serum. 10^6 cells were stained with 0.5 μg of antibody in a total volume of 50 μl at 4°C for 30 min. Cells were washed twice with PBS and then resuspended in 50 μl of a 1:50 dilution of either FITC-conjugated streptavidin or goat anti-mouse Ig-FITC (Southern Biotechnology Association, Birmingham, AL). After 20 min at 4°C, cells were washed, fixed with 1% paraformaldehyde in PBS, and analyzed using a cytofluorograph (Becton Dickinson and Co., Mountain View, CA).

Tolerance Induction. Tolerance to TCR peptides was induced by two intraperitoneal injections at different doses, 14 or 50 nmol of peptides in IFA (50 μl), the first within 24 h after birth and the second 72 h after birth. 7–8 wk later, tolerized animals were immunized either with TCR peptides/CFA for proliferative T cell responses or with Ac1-9/CFA/PTx for the induction of EAE. In one experiment, tolerized mice (8 wk old) were subsequently vaccinated with TCR peptides to study protection from EAE. Antigen-specific tolerance to MBP peptides was induced by intravenous injection of 400 μg peptide in 0.2 ml PBS, as described previously (23).

Results

Spontaneous Priming of TCR Peptide B5-reactive T Cells in (SJL × B10.PL)F1 Mice after Antigen Injection. To test whether TCR-peptide B5-specific T cells become naturally primed during recovery from antigen-induced EAE in the (SJL × B10.PL)F1 mouse as well as in the B10.PL strain (18), the specific proliferative response to B5 was followed in splenic T cells from mice challenged 25–35 d earlier with MBP/CFA and PTx. A proliferative T cell response to another TCR peptide B2, as control, was also followed in parallel. Clearly, a significant proliferative response to TCR peptide B5 was revealed in the peripheral T cells from mice recovering from EAE (Fig. 1). In contrast, there was no proliferative recall response to another proliferation-inducing or immunogenic TCR peptide, B2. Also, no proliferation was detected in response to B5 in unimmunized mice or in MBP/CFA/PTx-immunized mice before the onset of EAE (data not shown). Thus, during the course of recovery from antigen-induced EAE, T cells reactive to the immunodominant framework region 3 TCR peptide of the Vβ8.2 chain are generated in (SJL × B10.PL)F1 mice, consistent with our earlier observations in the B10.PL strain (18).

Selection of Vβ14$^+$, CD4$^+$ T Cells In Vivo. Our previous analysis of 29 B5-specific B10.PL-derived T cell clones and hybridomas, showed an oligoclonal TCR Vβ gene usage: a distinct majority (26/29) of T cells used Vβ14; three used Vβ3 gene segments (18). To investigate the in vivo significance of this observation (without introducing a bias as a result of in vitro long-term growth and selection), peripheral T cells from mice recovering from antigen-induced EAE were sorted into Vβ14$^+$ CD4$^+$ and Vβ14$^-$ CD4$^+$ T cell populations. Sorted populations were incubated in the presence of irradiated spleen cells from naive F1 mice with optimum concentrations of different peptides and the proliferative response was determined (Fig. 2). A major proliferative response to B5, but not B2 was found in the Vβ14$^+$ CD4$^+$ T cell population. The diminutive response to B5 in the Vβ14$^-$ fraction could be due to the presence of Vβ3$^+$ T cells in this population. Because of a very low yield of Vβ3$^+$ CD4$^-$ T cells, we could not do a similar analysis.

Table 1. *Neonatal Tolerance Induction to TCR Peptides*

Treatment	T cell proliferative responses			Incidence of EAE (maximum disease score)
	B4	B5	PPD	
		cpm × 1,000		
PBS-tolerized	50.3 ± 1.2	77.8 ± 9.1	112.5 ± 4.1	4/5(5,4,3,3,0)
	41.2 ± 5.2	91.4 ± 1.0	97.8 ± 10.5	
B4-tolerized	2.1 ± 1.7	95.1 ± 1.2	150.5 ± 11.2	6/6(5,4,4,4,4,1)
	4.0 ± 1.2	112.2 ± 5.7	144.2 ± 9.8	
B5-tolerized	61.5 ± 5.1	67.7 ± 10.1	92.5 ± 1.2	6/7(5,5,4,3,1,1,0)
	73.5 ± 11.2	58.8 ± 5.3	118.5 ± 5.6	

Neonatally tolerized mice (two from each group) were challenged (emulsified in CFA) and in vitro recalled with TCR peptides, B4 and B5. LN proliferative responses at optimum concentration of TCR peptides (7 μM) are shown. Remaining mice in each group were immunized with Ac1-9/CFA/PTx to induce EAE.

Both populations showed a comparable response to the purified protein derivative of *Mycobacterium tuberculosis*.

Tolerance Induction in CD4 Treg Populations. To directly test for the functional consequences of the response to B5 in recovery from EAE, attempts were made to induce tolerance in the CD4 Treg population. Groups of mice were neonatally tolerized with TCR peptides, B4, B5, or PBS (Table 1). 7–8 wk later, two mice from each group were challenged with the same peptides in CFA to test for proliferative responses. The remaining mice in each group were challenged with Ac1-9/CFA/PTx to induce EAE. T cell proliferation in the draining LN populations in response to TCR peptides or purified protein derivative of mycobacterium tuberculosis (PPD) are shown in Table 1. Mice neonatally tolerized to B4 and subsequently challenged with B4 did not proliferate. In contrast, B5-tolerized mice still showed a robust proliferative response to a subsequent challenge with B5. Responses to PPD were similar in all groups. In a separate experiment mice tolerized at a higher antigen level, with 50 nmol of B5, still showed a proliferative reponse to B5 (data not shown). The results showing that the incidence and severity of EAE in each group was very similar (Table 1) indicate that B5-specific T cells are not easily tolerized. Likewise, there was no functional tolerance induced and "B5-tolerized" mice could be significantly protected from EAE by subsequent vaccination with B5 as adults. This inability to induce neonatal tolerance to B5 along with the oligoclonality of the TCR V-gene usage led us to use the antibody-depletion approach to test directly the role of B5-reactive T cells in recovery from EAE.

Injection of Vβ-Chain–specific mAbs Leads to a Significant Temporary Depletion of Corresponding Vβ-expressing T Cells. Anti-TCR Vβ region-specific antibodies were used to deplete specific T cells in vivo. We initially used various amounts (25–250 μg) of anti-Vβ14 and/or anti-Vβ3 (0.2 ml vol) to inject (SJL × B10.PL)F1 mice, intraperitoneally. 3 d later, peripheral T cells were analyzed by flow cytometry. Injection of 50–100 μg of anti-Vβ14 or anti-Vβ3 antibody was sufficient to deplete the corresponding T cells considerably (Table 2). A small population of T cells representing ~15% of the original level of CD4 Vβ14-expressing T cells persisted after antibody injections and was not deleted even after injection with 250 μg of antibody. Since mAb 14-2 is IgM and is a rat–mouse hybrid, depletion may be inefficient owing to induction of an anti-rat immune response. Peripheral T cells from some mice were examined for the presence of Vβ14 CD4 T cells at 2, 3, and 6 wk after antibody injection. Although at 2 wk a similar staining pattern to that after 3 d was seen, by 3 wk, normal levels of Vβ14 T cells had almost returned. Thus, depletion of Vβ14-expressing T cells after a single injection with the mAb is incomplete and appears to be relatively short term.

Anti-Vβ14 and Anti-Vβ3-treated Mice Develop Severe Chronic EAE. We had shown earlier that cloned B5-specific CD4 Vβ14 T cells, when adoptively transferred into

Table 2. *Down-modulation of Vβ14 and Vβ3 T Cells after Treatment of (SJL × B10.PL)F1 Mice with Anti-Vβ14 or Anti-Vβ3 mAbs*

Antibody treatment	Amount	Vβ14 T cells	Vβ3 T cells
	μg	%	%
Ig control	250	2.8	0.9
14-2	250	0.5	1.1
14-2	100	0.6	0.8
14-2	50	0.5	1.1
KJ-25	250	2.8	0.5
KJ-25	100	2.9	0.4

Various concentrations of either 14-2 (anti-Vβ14) or KJ-25 (anti-Vβ3) were injected i.p. into groups of (SJL × B10.PL)F1 mice. 3 d later, nucleated spleen cells were passed over nylon wool and stained with biotinylated 14-2 or KJ-25 antibodies, followed by streptavidin-FITC and CD4-PE. Cells were analyzed by flow cytometry.

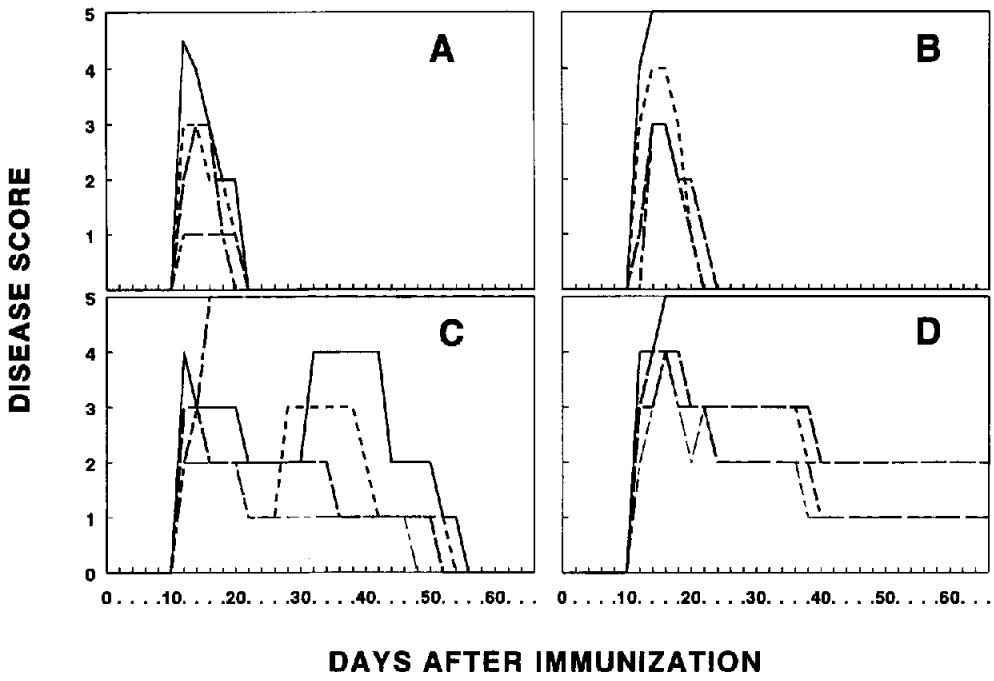

Figure 3. Mice treated with anti-Vβ14 and anti-Vβ3 antibodies display chronic disease and recover poorly from antigen-induced EAE. Four groups (five in each) of F1 mice were immunized with Ac1-9/CFA/PTx for the induction of EAE. 24 h after Ac1-9 injection, 50–100 μg of various mAbs in 200 μl saline were injected intraperitoneally. Mice were monitored daily by two independent observers for disease until day 70. (*A*) control antibody, Rat IgM; (*B*) anti-Vβ3; (*C*) anti-Vβ14; (*D*) anti-Vβ14 and anti-Vβ3.

B10.PL mice, were able to down-regulate encephalitogenic MBP-reactive T cells and prevent MBP-induced EAE (18). Alternatively, mice could be vaccinated with peptide B5 and could also be significantly protected from MBP-induced EAE (17). To directly assess the physiological role of Vβ14$^+$ and/or Vβ3$^+$ CD4$^+$ Treg in spontaneous recovery from EAE, we have asked whether depletion/blocking of these cells affects the duration of disease in F1 mice.

To obtain a reproducible disease course from experiment to experiment, (SJL × B10.PL)F1 mice were immunized subcutaneously with 100 μg of Ac1-9 emulsified with CFA (no additional *M. tuberculosis* was added), followed by a single intravenous injection of 150 ng of PTx in saline, 48 h later. Disease incidence in this protocol was 90–100% and most of the animals (90–95%) showed an acute episode of EAE with spontaneous recovery. For antibody treatment, mice were divided into several groups: mice in one group were left untreated, whereas others were injected with various mAbs on the same day or 24 h after Ac1-9/CFA injection. Results from one experiment are shown in Fig. 3, and the data from three different experiments are summarized in Table 3. Mice treated with anti-Vβ14 and anti-Vβ3 mAb had severe and chronic EAE, with most mice (9/9) recovering very slowly. In fact, some of the mice (30–45%) in the anti-Vβ14-treated group showed relapses and most recovered much later (days 48–70) than mice in the control groups (around day 25–30). Mice treated with anti-Vβ3 only showed no obvious changes in their recovery pattern and were similar to the control Ig-treated group. Mice were also treated with an irrelevant anti-TCR (Vβ5.1) antibody, as a control group, with no significant effect on EAE (Table 3). It is clear that after a single anti-Vβ antibody injection, mice did recover eventually (in different experiments from days 48–70). In one experiment in which we injected mice on days 1, 14, 28, and 42 with both anti-Vβ14 and anti-Vβ3 antibodies, recovery was further delayed in that $^4/_5$ mice showed partial paralysis beyond day 70 (data not shown). Interestingly, mice in the combined anti-Vβ14– and anti-Vβ3–treated group did not stay severely sick (e.g., with a score of 4) but remained partially paralyzed in the tail and hind limbs (score 1 and 2).

Table 3. *Chronic EAE in Mice Treated with anti-TCR Antibodies*

Treatment	Incidence of acute EAE	Disease onset	Average score	Incidence of chronic EAE*
	day 15	*d*		*day 35*
None	9/11	10–13	2.8	0/11
Ig control	8/10	9–14	2.7	1/10
Anti-Vβ3	8/11	10–13	2.9	1/11
Anti-Vβ5	5/5	9–12	3.0	0/5
Anti-Vβ14	16/17	9–13	3.4	11/17
Anti-Vβ14 + Anti-Vβ3	9/9	9–14	3.6	9/9

Age-matched female (SJL × B10.PL)F1 mice were treated intraperitoneally with various mAbs (50–100 μg/mouse) either on the same day of Ac1-9/CFA injection, or 24 h later in 200 μl saline. PTx (150 ng) was given i.v. 48 h after ac1-9 injection in 200 μl saline.

*P values between the control group (monophasic disease) and anti-Vβ14/anti-Vβ3-treated group (chronic disease with delayed recovery) was $P < 0.001$, and was calculated using the chi-square test with Yates correction.

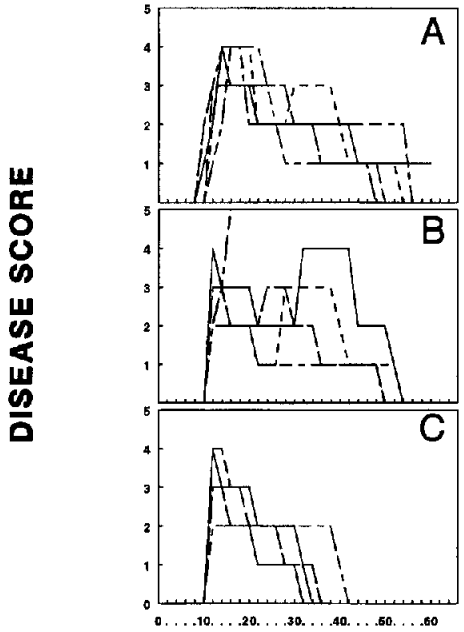

Figure 4. Adoptively transferred Treg clone B5.2 accelerates recovery in Treg-depleted mice. Three groups of F1 mice were injected with Ac1-9/CFA/PTx for EAE and were treated with anti-Vβ14 antibody as in Fig. 3 C. On day 12, at the time of onset of EAE, mice were injected intraperitoneally with either saline only (*A*), or with 10^6 B4.1 T cells (*B*), or with 10^6 B5.2 T cells (*C*). The disease was monitored as in Fig. 3.

Adoptive Transfer of the B5-specific, Vβ14-expressing Regulatory T Cell Clone B5.2 Accelerates Recovery in Anti-Vβ–treated F1 Mice. We next wanted to test whether adoptively transferred B5-specific Treg cells could reestablish a regulatory balance and normalize the disease course in Treg-depleted mice. First, groups of mice (SJL × B10.PL)F1 were treated with anti-Vβ14 antibody, intraperitoneally, as in Fig. 3. Mice in one of the anti-Vβ14-treated groups were injected (i.p.) with 10^6 cloned B5.2 T cells at the time (day 12) of onset of EAE. Mice in the other two groups were injected with either saline or with a B4-specific T cell clone, B4.1 (10^6). As shown in Fig. 4, mice injected with the B5.2 clone recovered much earlier (4/5 mice recovered by day 36) than mice in the control anti-Vβ14 treated group (0/5 by day 36). Mice in the B4.1-injected group failed to recover in an accelerated fashion. It should be pointed out that at ~2 wk after injection, the anti-Vβ14 apparently had reduced to an ineffective level, so that the transferred B5.2 cells manage to perform their regulatory functions.

Chronic Disease and Relapses in Anti-Vβ–treated Mice Are Due to the Presence of Dominant Peptide Ac1-9–specific T Cells. We have shown earlier that T cells specific for other subdominant determinants appear late in F1 mice after immunization with Ac1-9 (24). Recently, we have shown that when peptide MBP121-140, containing a subdominant determinant, was injected in CFA with PTx, it was capable of causing EAE in B10.PL mice as well as in F1 mice (25, and Kumar, V., unpublished observation). It is not yet clear whether in vivo–primed T cells specific for these later determinants are encephalitogenic and/or participate in chronic EAE in this system.

We wanted to test whether chronic disease or even relapses in some cases (30–45%) in the anti-Vβ–treated mice were due to the presence of T cells with specificities for determinants other than Ac1-9, namely subdominant determinants, such as MBP81-100/A^s or MBP121-140/A^u. T cells specific for these determinants do not predominantly use Vβ8.2 or Vβ13 gene segments, which are prevalent among Ac1-9–specific T cells. Therefore, anti-Vβ14/anti-Vβ3-treated mice were depleted of Ac1-9–specific T cells during the course of chronic EAE and their disease and recovery were followed. As shown in Table 4, mice in the control group, with Ac1-9–specific T cells intact, showed chronic EAE in that 7/7 mice still had clinical signs of the disease by day 35. In contrast, mice devoid of Ac1-9–reactive T cells (anti-Vβ8.2– and anti-Vβ13–treated) showed accelerated recovery, despite the loss of Treg, and despite the presence of T cells directed to the subdominant determinant of MBP. Only 1 out of 9 mice in this group showed clinical EAE by day 35.

Since non–Ac1-9-reactive T cells, including T cells specific for other myelin antigens, could use $Vβ8^+$ or $Vβ3^+$ TCR, we induced antigen-specific tolerance and studied its effect on the chronicity of EAE in Treg-depleted mice.

Table 4. *A Chronic Disease Course in Anti-Vβ–treated Mice Can Be Attributed to the Continued Presence of Ac1-9–specific T Cells*

Treatment	Incidence of acute EAE	Average clinical score	Incidence of chronic EAE
	day 14		*day 35*
Experiment 1			
Ig control	7	3.1	7/7
Anti-Vβ8.2 + Anti-Vβ13	9	3.4	1/9*
Experiment 2			
MBP 41-58	5	2.9	5/5
MBP Ac1-9	5	3.0	0/5*

Groups of (SJL × B10.PL)F1 mice treated with both anti-Vβ14 and anti-Vβ3 mAbs and matched for the severity of EAE symptoms were treated at day 14 after disease onset with, in experiment 1, i.p., either an IgG control Ab or a combination of anti-Vβ8.2 (F23.2) and anti-Vβ13 (MR 12-4) mAbs (100 μg of each antibody per mouse), and in experiment 2, i.v., with 400 μg of the control peptide MBP 41-58 or Ac1-9 to deplete Ac1-9–specific T cells. Mice were scored daily for symptoms of EAE until days 60 and 55, respectively.

*P values between the control and Ac1-9–depleted group was $P < 0.001$, and was calculated using the chi-square test with Yates collection.

During the course of EAE (on day 14), mice were injected intravenously with 400 μg of Ac1-9 or MBP 41-58 peptide in saline (Table 4). Mice tolerized with Ac1-9 recovered relatively quickly (days 23–25), whereas five out of five mice in the control group tolerized with MBP 41-58 contracted chronic disease and had tail paralysis beyond day 35. Thus, chronic EAE in Treg-depleted mice still seems to be driven by the presence of Ac1-9–specific T cells.

Discussion

We have attempted to determine the importance of the activation of Vβ14 and Vβ3 regulatory CD4 T cells in the development and course of EAE in the (SJL × B10.PL)F1 mice. In this strain, TCR-peptide–specific T cells specific for the immunodominant TCR β-chain peptide B5 are physiologically induced via antigenic stimulation, without any exogenous challenge with the TCR peptide. Importantly, here we have shown that the deletion/blocking of CD4 $V\beta14^+/V\beta3^+$ Treg cells from the peripheral T cell repertoire leads to an increase in severity of EAE and poor recovery from disease in F1 mice.

TCR Peptide B5-specific T Cells Predominantly Expressing the Vβ14 Gene Segment Are Naturally Revealed in (SJL × B10.PL)F1 Mice Recovering from Antigen-induced EAE. Demonstration of the induction of TCR peptide B5-reactive CD4 T cells during the course of antigen-induced EAE suggests that a determinant within the B5 peptide (amino acids 76–101) from the Vβ8.2 chain of the T cell receptor is physiologically processed and presented in a class II ($I\text{-}A^u$) context. There are two other peptides (B2 and B4) from the same Vβ chain that are capable of inducing T cell proliferative responses when the peptide form is used for immunization (17). These latter peptides seemingly are not naturally processed and presented, at least in the presence of B5, because spontaneous T cell reactivity to them is not detected during the course of EAE.

Determinant(s) within the B5 peptide appear to be dominant: first, LN cells from mice challenged with recombinant single-chain TCR molecules containing the entire Vβ8.2 chain, respond to B5 in in vitro proliferative recall assays and not to other TCR peptides (Kumar, V. et al., manuscript submitted for publication); second, spleen cells from naive animals are capable of stimulating B5-reactive T cell clones in vitro in the absence of exogenous peptide (18). Thus, APCs appear to constitutively process and potentially present B5 in vivo. In view of the prevalence of the TCR Vβ8 family in peripheral T cells (15–30%), it is not surprising that TCR Vβ8 peptides are generated which are capable of binding to MHC molecules in vivo. Among these, B5 seems to possess the appropriate combination of availability and affinity of binding to A^u to render it dominant. Another possible interpretation is that some other molecule(s) on the surface of splenic APC could be cross-reactive (26) and mimic the B5 TCR determinant. We are currently examining these issues.

Typically, dominant determinants on self-antigens induce thymic tolerance (23). Thus, why is the T cell repertoire directed against the dominant TCR peptide B5 still intact? Are these TCR-peptide–MHC complexes not present in the thymus or do they fail to mediate negative selection? It is likely that if determinants in the B5 region were able to be processed and presented by the thymic APCs, T cells expressing only high affinity TCR would be deleted sparing the low affinity TCR-bearing T cells to be positively selected. Our data demonstrate an inability to induce neonatal tolerance to B5 and is consistent with the idea that most B5-specific T cells are not deleted, but rather may be primed, as has recently been reported (27). These results are similar to a recent report in the rat model where neonatal tolerance to the CDR2 peptide does not lead to a change in the course of EAE (28). However, rats tolerized as adults to this peptide contract severe disease (28).

Vβ14 and/or Vβ3 CD4 Treg Cells Are Crucial for Spontaneous Recovery from Antigen-induced EAE. Delayed recovery from Ac1-9–induced EAE in F1 mice after treatment with Treg-reactive anti-Vβ mAbs indicates that the presence of TCR-peptide–specific CD4 T cells is crucial for a quick spontaneous recovery in these mice. It is interesting that rats tolerized as adults to the Vβ 8 CDR2 peptide contract severe EAE but this tolerance did not result in chronic disease or prolonged recovery (28). Oligoclonality in TCR Vβ-gene usage by spontaneously primed CD4 Treg cells was further confirmed by the demonstration that the most significant delay in recovery occurred in animals treated with both anti-Vβ14 and anti-Vβ3 mAbs. Interestingly, most of the animals, despite treatment with both mAbs, were able to partially recover. There are at least four explanations for this finding; (*a*) incomplete depletion of Treg expressing Vβ14 or Vβ3; (*b*) emergence of CD4 Treg using alternate Vβ gene segments; (*c*) emergence of T cells specific for other subdominant or cryptic MBP determinants which induce Th2 cells, leading to immune deviation in a Th2 direction, accompanied by down-regulation of Th1; (*d*) recruitment of CD8 T cells, which become independent of CD4 Treg in the down-modulation of encephalitogenic potential. These hypotheses are not mutually exclusive and are currently being examined.

Finally, relapses in some of the Treg-depleted animals, as well as the occurrence of chronic EAE in a mouse strain that normally gets an acute episode of EAE, suggest that the spontaneous chronic autoimmune disease course found in some experimental models or in humans could reflect a crucial defect in tight regulation. In monozygotic twins, frequently only one suffers from chronic autoimmune disease: in such cases defective regulation could be decisive in the manifestation of the disease. Conversely, stronger and more efficient regulatory effectors may exist in EAE-resistant mouse strains, protecting them from antigen-induced autoimmunity.

Chronicity of EAE in Treg-depleted Mice Seems To Be Dependent on the Presence of T Cells Specific for the Immunodominant Determinant Ac1-9. In (SJL × B10.PL)F1 mice, the initial response to MBP focuses on the immunodominant

determinant Ac1-9 within the NH_2-terminal fragment. However, owing to determinant spreading (24), T cells reactive to other subdominant determinants, for example, 81-100/A^s, and 121-140/A^u are revealed in splenic T cell proliferation assays. It has been shown that challenge with certain subdominant peptides in strong adjuvants (CFA and PTx), or adoptive transfer of in vitro–activated T cells into naive mice result in EAE (Kumar, V., unpublished observation; 29). It is not yet clear whether in vivo–primed T cells reactive to these latter determinants are critical for the chronic course of EAE. Our findings in Ac1-9–specific T cell–depleted mice suggest that the continued presence of the initially dominant peptide–reactive T cells may be required for sustaining chronic EAE in this model. However, it is likely that in the chronic-relapsing model of EAE, recruited T cells reactive to newly revealed determinants could participate in the disease, as suggested recently in the SJL mouse (29).

How Do the CD4 Treg Regulate Responses to MBP? It is likely that activated $V\beta14^+$ regulatory T cells traffic to inflammatory sites in the central nervous system and locally down-regulate Ac1-9–specific responses through secretion of modulatory cytokines, for example, TGF-β, IL-4, or IL-10 (reviewed in references 4 and 5). Consistent with this notion, the presence of Vβ14 mRNA in central nervous system tissue during recovery from EAE, around day 12, has recently been demonstrated in (PL/J × SJL) mice (30). Preliminary analysis of the cytokine secretion profile of CD4 Treg reveals that these cells are Th0-like in their ability to secrete IL-2 and IFN-γ, as well as IL-4 and IL-5 (Kumar, V., unpublished observations).

CD8 deletion experiments suggest that once activated, the regulatory B5-reactive $CD4^+$ T cells recruit $CD8^+$ T cells which ultimately down-regulate encephalitogenic T cells (18). The CD8 cells can recognize distinct TCR determinants in an MHC class I context, displayed on the surface of MBP-specific effector cells (31). Recognition of the activated, encephalitogenic $V\beta8.2^+$ effector cells by TCR-peptide–specific $CD8^+$ T cells may thus be one pathway of specific regulation. Indeed, we have recently characterized a potential class I determinant on the Vβ8.2 chain distinct from B5. Mice vaccinated with this 10-mer peptide are completely protected from MBP/Ac1-9-induced EAE in an unusual dose-related fashion, the protective dose being very low (Kumar, V., unpublished observations). Other experiments in mice devoid of $CD8^+$ T cells, either by anti-CD8 treatment or CD8 gene targeting, have also shown that CD8 cells play an important role in regulating further episodes of EAE (32, 33).

In conclusion, it is likely that TCR $V\beta14^+$ CD4 regulatory T cells are an essential part of a physiological T cell regulatory circuit that establishes tolerance to self MBP in the (SJL × B10.PL)F1 mouse (34). It appears that disruption of this cellular circuitry can result in an anomalous persistence and chronicity of autoimmune disease.

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