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17. We thank A. Berns for a 129/Ola-derived genomic library, D. Mathis and C. Benoist for many helpful suggestions, and A. Livingstone and A. Lanzavecchia for critically reading the manuscript. The Basel Institute for Immunology was founded and is supported by Hoffmann-La Roche Ltd., Basel, Switzerland.

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Regulatory T Cell Clones Induced by Oral Tolerance: Suppression of Autoimmune Encephalomyelitis

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Experimental autoimmune encephalomyelitis (EAE) is a cell-mediated autoimmune disease that serves as an animal model for multiple sclerosis. Oral administration of myelin basic protein (MBP) suppresses EAE by inducing peripheral tolerance. T cell clones were isolated from the mesenteric lymph nodes of SJL mice that had been orally tolerized to MBP. These clones were CD4$^+$ and were structurally identical to T helper cell type 1 (T_H1) encephalitogenic CD4$^+$ clones in T cell receptor usage, major histocompatibility complex restriction, and epitope recognition. However, they produced transforming growth factor–β with various amounts of interleukin-4 and interleukin-10 and suppressed EAE induced with either MBP or proteolipid protein. Thus, mucosally derived T_H2-like clones induced by oral antigen can actively regulate immune responses in vivo and may represent a different subset of T cells.

One of the primary features of the immune system is the discrimination of self from nonself (*1*). Immunologic tolerance provides the immune system with self-nonself discrimination and is crucial to prevent pathogenic reactivities against self antigens. Several mechanisms may contribute to immune tolerance, with the primary one being clonal deletion of autoreactive cells in the thymus (*1–3*). However, not all autoreactive cells are deleted (*3*, *4*), and therefore, mechanisms must exist to regulate autoreactive T cells in the peripheral immune compartment. Mechanisms of peripheral immune tolerance include anergy (*5*) and active suppression (*6*). Active cellular suppression has not been unequivocally demonstrated as a mechanism for peripheral tolerance because of difficulties in cloning and in demonstrating that cloned T cells can actively regulate the responses of other immune cells in vivo.

Oral administration of antigens (oral tolerance) is a classic method of inducing antigen-specific peripheral immune tolerance (*7*). Orally administered antigens can induce active suppression (low antigen dose) or clonal anergy (high antigen dose) (*8*). Oral tolerance is a biologically relevant method of inducing peripheral tolerance: it involves the physiologic interaction of proteins with the gut immune system, a process that has evolved to prevent systemic immune responses to ingested proteins. In addition, the oral administration of autoantigens has become clinically relevant and is being investigated as a treatment for human autoimmune diseases (*9*).

One of the mechanisms of peripheral immune tolerance after orally administered antigens is the generation of regulatory T cells that mediate active suppression (*10*). In the Lewis rat these T cells are CD8$^+$ and act by secreting transforming growth factor–β (TGF-β) after being stimulated by the fed antigen (*11*). These cells do not produce antigen-specific suppressor factors and therefore differ from previously described suppressor T cells that have been difficult to characterize (*6*). Characterization of regulatory T cells that mediate active suppression, including epitope specificity, T cell receptor (TCR) usage, and individual cytokine secretion cannot be established from bulk-cultured cells.

To generate regulatory clones associated with oral tolerance, we fed SJL mice [which are susceptible to experimental autoimmune encephalomyelitis (EAE)] myelin basic protein (MBP) and examined cytokine patterns produced by mesenteric lymph nodes. Mesenteric lymph nodes from animals fed either MBP or hen egg lysozyme (HEL) and cultured in vitro with the relevant antigen secreted TGF-β, interleukin-4 (IL- 4), and IL-10, but minimal interferon (IFN-γ) (Table 1, groups 1 to 4). Cytokine secretion was dependent on antigen-specific stimulation in vitro with the fed antigen. If cells from animals fed MBP and then immunized intraperitoneally with MBP in complete Freund's adjuvant (CFA) were cultured in vitro with MBP, antigen-specific TGF-β secretion was enhanced (Table 1, groups 5 to 7). Thus, in addition to the secretion of T helper cell type 2 (T_H2) cytokines, immune responses in the gut preferentially involve the generation of cells that secrete TGF-β .

Cells from animals fed MBP and immunized with MBP in CFA were studied in bulk culture to determine which cells secreted the respective cytokines (Fig. 1). Both CD4$^+$ and CD8$^+$ cells secreted TGF-

Table 1. Cytokine production by mesenteric lymph node cells from orally fed SJL mice. Seven groups of SJL/J mice (*27*), four mice each, were fed with MBP or HEL (*24*). Two days after the last feeding, mice in groups 5 to 7 received an intraperitoneal injection of 100 μg of MBP in CFA (*24*). Mesenteric lymph node cells from all groups of mice were prepared 14 days after immunization (or 16 days after the last feeding) and were cultured in serum-free medium containing MBP or HEL (50 μg/ml) as indicated. Culture supernatants were collected at 40 hours for IFN-γ and IL-4 and at 72 hours for TGF-β and IL-10. Cytokine concentrations were determined by ELISA (*28*) and are expressed as picograms per milliliter. Results represent pooled data from three independent experiments.

Group	Antigen used for			TGF-β	IFN-γ	IL-4	IL-10
	Feeding	Immunization	In vitro stimulation				
1	MBP	None	HEL	180 ± 23	<75	<75	<100
2	MBP	None	MBP	429 ± 34	77 ± 36	580 ± 38	350 ± 47
3	HEL	None	HEL	1050 ± 120	88 ± 17	570 ± 108	261 ± 22
4	HEL	None	MBP	230 ± 38	<75	<75	<100
5	MBP	MBP	HEL	320 ± 19	83 ± 41	340 ± 29	246 ± 18
6	MBP	MBP	MBP	6020 ± 233	252 ± 39	965 ± 201	869 ± 31
7	HEL	MBP	MBP	415 ± 49	813 ± 71	520 ± 84	410 ± 50

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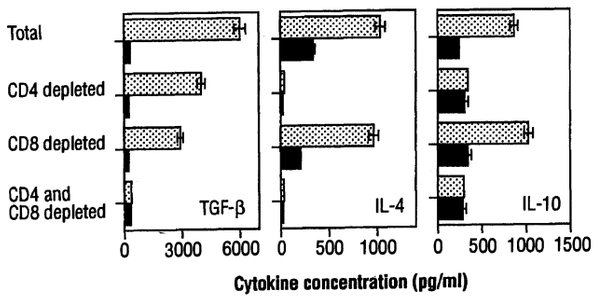

Fig. 1. Cytokine production by CD4$^+$ and CD8$^+$ T cells from orally tolerized animals. Six SJL mice were fed and immunized with mouse MBP (*24*). Mesenteric lymph node cells were prepared 14 days after immunization and were depleted of CD4$^+$ or CD8$^+$ T cells or both by negative selection (*26*). Control CD4$^+$ cells, CD8$^+$ T cells, or both (from mesenteric lymph nodes of naive SJL mice) were then added to the cell preparations to replace the depleted cells from orally tolerized animals. Five million cells were then cultured in 1 ml of serum-free medium with 50 μg of MBP or HEL. Culture supernatants were collected, and cytokine production was determined by ELISA (*28*). Results represent mean ± SD of triplicate cultures and are representative of two independent experiments.

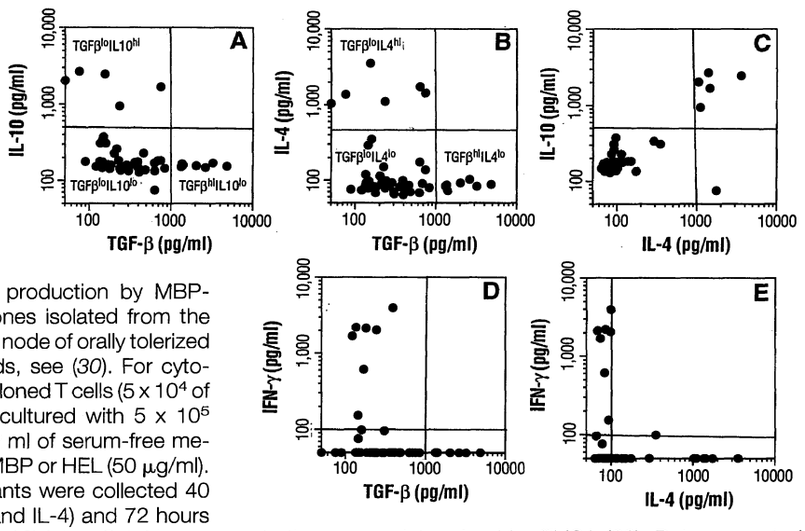

Fig. 2. Cytokine production by MBP-specific T cell clones isolated from the mesenteric lymph node of orally tolerized mice. For methods, see (*30*). For cytokine production, cloned T cells (5 x 10^4 of each type) were cultured with 5 x 10^5 feeder cells in 0.2 ml of serum-free medium containing MBP or HEL (50 μg/ml). Culture supernatants were collected 40 hours (for IFN-γ and IL-4) and 72 hours (for TGF-β and IL-10) later. Cytokine concentrations were determined by ELISA (*28*). Data presented represent the mean of triplicate cultures with MBP minus the mean of cultures with HEL. (**A**) TGF-β versus IL-10; (**B**) TGF-β versus IL-4; (**C**) IL-4 versus IL-10; (**D**) TGF-β versus IFN-γ; (**E**) IL-4 versus IFN-γ. To rule out production of cytokines by feeder cells, cytokine production was also measured after stimulation with a mAb to CD3 (2C11). Comparable amounts of cytokines were detected in all the T cell clones tested.

β, whereas only CD4$^+$ cells secreted IL-4 and IL-10. This is consistent with our previous studies in the Lewis rat in which predominantly CD8$^+$ cells secreted TGF-β (*11*). We thus cloned the CD4$^+$ T cells from these bulk cultures after depletion of CD8$^+$ cells and succeeded in obtaining 48 MBP-specific CD4$^+$ T cell clones. The cytokine profile for each of the 48 clones after activation with MBP or monoclonal antibody (mAb) to CD3 (anti-CD3) was determined by enzyme-linked immunosorbent assay (ELISA) (Fig. 2). Of the 48 clones, 42 produced active TGF-β in addition to various amounts of one or the other T_H2 type cytokines (IL-4 and IL-10). The relative amounts of the three cytokines produced by each clone varied, and the clones could be grouped in three categories depending on the amount of individual cytokines produced. Clones that secreted TGF-β were TGF-β^{hi}, IL-4^{lo}, IL-10^{lo}; TGF-β^{lo}IL-4^{hi}, IL-10^{hi}; or TGF-β^{lo}, IL-4^{lo}, IL-10^{lo} (Fig. 2, A and B). The TGF-β^{hi}-secreting clones produced no IFN-γ or IL-2. It also appeared that the TGF-β secretion was regulated separately from that of IL-4 and IL-10, suggesting that these clones were different than classic T_H2 cells that secrete IL-4 and IL-10. Thus, there was a general correlation between the secretion of IL-4 and IL-10 in an individual clone (Fig. 2C) but this dissociated in clones secreting TGF-β. The cytokine patterns for most T cell clones have been stable for more than 4 months in vitro.

To further characterize these mucosally derived CD4$^+$ clones that secreted TGF-β, IL-4, and IL-10, three clones were studied in detail for epitope specificity, major his-

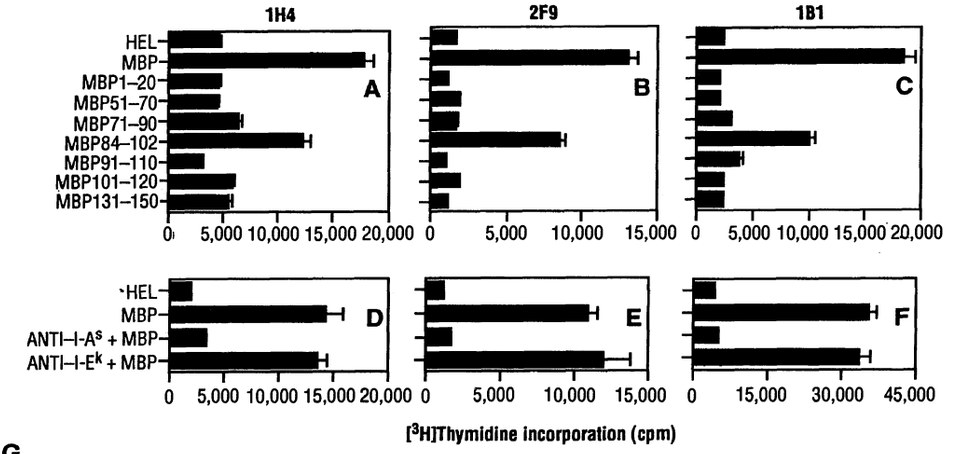

Fig. 3. Peptide specificity and MHC restriction of mucosally derived T cell clones. (**A** to **C**) For peptide specificity, cloned T cells (5 x 10^4 of each type) were cultured with 1 x 10^6 feeder cells in 0.2 ml of medium with 5 μg of HEL, MBP, or MBP peptides. ^{3}H-labeled thymidine (1 μCi) together with 2 U of recombinant IL-2 were added to each culture 72 hours later. Cells were harvested and radioactivity was counted 16 hours later. Data presented represent mean ± SD of triplicate cultures. The experiment was repeated four times for clones 1B1 and 2F9 and two times for clone 1H4. (**D** to **F**) For MHC restriction, cloned T cells (5 x 10^4 of each type) were cultured with normal or pretreated feeder cells (1 x 10^6) in 0.2 ml of medium containing 5 μg of HEL or MBP. Pretreatment of feeder cells was done by incubating cells (10^7 per milliliter) with either mAb 10.2.16 (specific for I-A^s) or mAb 14.4.4.S (specific for I-E^k) ascites (1/80, v/v) (obtained from ATCC) at 37°C for 1 hour. [^{3}H]Thymidine together with 2 U of recombinant IL-2 were added to each culture 72 hours later. Cells were harvested and radioactivity was counted 16 hours later. Data represent mean ± SD of triplicate cultures and are representative of three experiments. (**G**) TCR sequences of regulatory T cell clones and encephalitogenic clone MM4.

G

Clone	V_β	V	D	J	C	Clone	V_α	V	J	C
1H4	V_β17	CASS	TGG	QDTQYFGPGTRLLVL	EDLR	1H4	V_α1	CAVS	KGTNTGKLTFGDGTVLTVKPN	IQ
1B1	V_β17	CASS	LGG	SMNFGPGTRLLVL	EDLR	1B1	V_α3	CAVR	DTGYQNFYFGKGTSLTVIPN	IQ
2F9	V_β4	CASS	QGLG	GAETLYFGSGTRLTVL	EDLR	2F9	V_α1	CAAS	GGTGGYKVVFGSGTRLLVSPD	IQ
MM4	V_β4	CASS	QGLG	GAETLYFGSGTRLTVL	EDLR	MM4	V_α1	CAAS	GGTGGYKVVFGSGTRLLVSPD	IQ

tocompatibility complex (MHC) restriction, and TCR usage. Two clones, 2F9 and 1H4, were chosen that secreted large amounts of TGF-β and one clone, 1B1, that secreted predominantly IL-4 and IL-10. Overlapping 20–amino acid MBP peptides were first tested in bulk culture, and then candidate peptides were used to determine the epitope specificity of individual clones. All three clones reacted with MBP(84–102) (Fig. 3, A to C). Additional clones reacted with other epitopes such as MBP(71–90) (*12*). MHC restriction was studied with clones that were stimulated by antigen-presenting cells treated with mAbs specific for MHC class II alleles (Fig. 3, D to F). Proliferation was inhibited by incubation with mAbs specific for I-A^s but not for I-$E^{k/u}$. (I-A^s is the only type of class II molecule expressed by the SJL strain mice). To study TCR usage, we directly sequenced the TCR α and β chain complementary DNAs (cDNAs) (Fig. 3G), and we further confirmed the V_β usage by staining with V_β chain-specific mAb. For a direct comparison of the TCR used by a MBP-specific T_H1 clone MM4 with that used by the mucosally derived T cell clones, cDNA was isolated from clone MM4 and sequenced across the TCR α and β chains (*13*). We found that MM4 expressed the identical TCR α and β chain nucleotide sequence as that of the TGF-β-producing regulatory clone 2F9 (Fig. 3G). Thus, the mucosally derived regulatory $CD4^+$ clones resemble MBP-specific encephalitogenic $CD4^+$ T cell clones in their epitope specificity, TCR usage, and MHC restriction, but they differ in cytokine profiles.

The major question was whether these mucosally derived $CD4^+$ clones were functional in vivo and could suppress autoimmune encephalomyelitis. Preliminary in vitro experiments showed that clone 2F9 and 1B1 suppressed proteolipid protein (PLP)–specific T_H1 cells and that suppression was abrogated by antibodies to TGF-β and IL-10 (*12*). SJL mice were immunized with MBP to induce EAE and were injected intraperitoneally with 2×10^6 T cells of each clone at the time of immunization (Table 2). Each of the mucosally derived $CD4^+$ clones suppressed EAE, as measured by disease incidence, day of onset, maximum disease score, and fatality. The central nervous system (CNS) inflammatory response was measured in one group of animals protected by the mucosally derived clones. A delayed-type hypersensitivity response was observed in the CNS of control animals with a prominent cellular infiltrate and expression of IL-2 and IFN-γ. In protected animals injected with clone 1B1, there was a reduction in numbers of mononuclear cells, down-regulation of the expression of IL-2 and IFN-γ and up-regulation of IL-4 and IL-10 (*14*). Thus, the mucosally derived $CD4^+$ clones were able to down-regulate a cell-mediated autoimmune disease in vivo.

Bulk T cells from orally tolerized animals can suppress active immune responses to other antigens in the microenvironment, a phenomenon called antigen-driven bystander suppression (*11*, *15*). Such cells suppress by secreting down-regulatory cytokines such as TGF-β after being triggered by specific antigen. We thus investigated whether the $CD4^+$ mucosally derived MBP-specific regulatory T cell clones we generated could affect autoimmune encephalomyelitis induced by another antigen of the CNS, PLP. The PLP-induced disease was also suppressed by MBP-specific regulatory T cell clones (Table 2). However, PLP-specific T cell proliferation was unaffected in the draining lymph nodes of animals injected with MBP-specific regulatory T cells (Δcpm = 69,469) as compared with controls (Δcpm = 75,221), but proliferation was reduced if MBP was added to the culture (Δcpm = 29,275). Thus, bystander suppression only occurs if the antigen recognized by the regulatory clone is present. To demonstrate that clone 2F9 suppressed in vivo by secreting TGF-β, we immunized

Table 2. MBP-specific $CD4^+$ T cell clones generated by oral tolerance suppress EAE. Cloned mucosal T_H2 cells (clones 1B1, 2F9, or 1H4) or concanavalin A–activated $CD4^+$ splenic T cells from SJL mice (T cell blast) were stimulated for 2 days with mouse MBP and feeder cells. T cell blasts were then expanded in medium containing 10% mouse TCGF, washed, and separated from feeder cells by centrifugation through a ficoll isopaque gradient. On day 0, each SJL mouse received (i) an intraperitoneal injection of 0.2 ml of phosphate-buffered saline (PBS) or PBS containing 2×10^6 T cells; (ii) subcutaneous immunization with 400 μg of mouse MBP or 100 μg of mouse PLP139–151 peptide (HSLGKWLGHPDKF) in 0.15 ml of PBS emulsified in an equal volume of CFA containing mycobacterium tuberculosis H37 RA (4 mg/ml); and (iii) an intravenous injection of 200 ng of pertusiss toxin in 0.2 ml of PBS. Mice received another injection of pertusiss toxin (200 ng/mouse) (List Biological Laboratories, Campbell, California) on day 2 and were monitored for symptoms of EAE as described (*29*). Statistical analysis was performed by χ^2 analysis for disease incidence and fatality and by Student's *t* test for maximum score and onset day. For 1B1, 2F9, and 1H4 groups, $P < 0.001$ compared with T cell blast or PBS control for all EAE parameters listed. Cytokine concentrations (picograms per milliliter) were determined as described (*28*). Dashes indicate not applicable or not tested. Single letter abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Parameter	2F9	1H4	1B1	T cell blast	PBS
		Cytokine profile			
TGF-β	1102	1261	154	-	-
IFN-γ	<50	<50	<50	-	-
IL-2	<20	<20	<20	-	-
IL-4	223	<75	3566	-	-
IL-10	729	513	2471	-	-
		MBP-induced EAE			
Disease incidence	2/6	2/6	2/9	9/12	8/10
Onset day ± SD	19.5 ± 0.5	18.5 ± 0.5	18.0 ± 1.0	14.4 ± 0.7	13.9 ± 1.2
Maximum score ± SD	0.5 ± 0.8	0.5 ± 1.2	1.3 ± 0.9	3.0 ± 0.9	2.9 ± 0.5
Fatality	0/6	0/6	0/9	3/12	2/10
		PLP-induced EAE			
Disease incidence	4/10	-	6/12	10/10	23/24
Onset day ± SD	16.8 ± 2.0	-	15.3 ± 1.7	12.8 ± 1.1	13.4 ± 1.2
Maximum score ± SD	1.9 ± 1.3	-	1.5 ± 1.4	4.1 ± 0.8	3.8 ± 0.9
Fatality	0/10	-	0/12	4/10	8/24

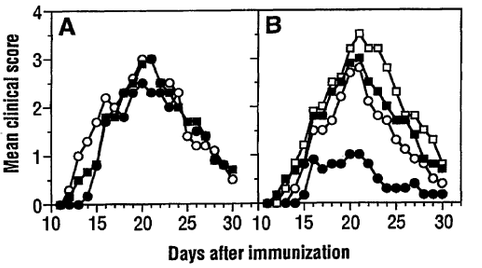

Fig. 4. TGF-β mAb neutralizes the in vivo suppressive activity of regulatory T cell clone 2F9. SJL/J mice, six animals per group, were immunized for EAE with PLP peptide (139–151) as described in Table 2. Nine days after immunization, animals were injected intraperitoneally as follows: 100 μg of control mouse Ig every other day for a total of four injections [(**A** and **B**), filled squares]; 2×10^6 MBP-specific T_H1 cells (clone MM4) [(A), open circles]; 2×10^6 HEL-specific T_H2 cells [(A), filled circles]; 100 μg of mouse TGF-β mAb every other day for a total of four injections [(B), open squares]; 2×10^6 regulatory cells (clone 2F9) together with 100 μg of mouse TGF-β mAb and then 100 μg of mouse TGF-β mAb every other day for a total of three injections [(B), open circles]; and 2×10^6 regulatory cells (clone 2F9) together with 100 μg of control mouse Ig and then 100 μg of control mouse Ig every other day for three injections [(B), closed circles]. Maximal disease severity of animals injected with 2F9 = 1.0 ± 0.8 and animals injected with 2F9 + TGF-β mAb = 2.8 ± 0.9 ($P < 0.01$).

animals with PLP peptide (139–151) and injected clone 2F9 on day 9 after immunization. As shown in Fig. 4, treatment with antibodies to TGF-β reversed the protection of clone 2F9 (Fig. 4B). An MBP-specific T_H1 clone (MM4) or an HEL-specific T_H2 clone used as controls did not suppress PLP-induced EAE (Fig. 4A). No disease was observed when $CD4^+$ regulatory clones alone were given at a dose of 5×10^6 cells per injection.

An unusual aspect of the T_H2-like regulatory clones derived from orally tolerized animals is their secretion of TGF-β. We derived a number of T_H2 clones after subcutaneous immunization with PLP and found that only an occasional T_H2 clone produced TGF-β, unlike the T cell clones derived after oral administration of PLP (*16*). TGF-β appears to participate in the local function of the gut and serves as a helper or switch factor for immunoglobulin A (IgA) production in the mucosa (*17*). TGF-β may also be involved in the homing mechanisms of cells to high endothelial venules (*18*). Thus, mucosally derived $CD4^+$ cells producing mainly TGF-β may be considered another T helper subset (T_H3?) with both mucosal T helper function and down-regulatory properties for T_H1 cells.

Our studies suggest that cells capable of regulating immune responses are not a special type of suppressor cell but are conventional T cells whose major down-regulatory activity is mediated by their cytokines. The cytokine profile appears stable and is dependent on the microenvironment in which they are induced. During oral tolerance in the Lewis rat, $CD8^+$ cells are generated that suppress immune responses both in vitro and in vivo through the secretion of TGF-β (*11*). The epitopes responsible for triggering these $CD8^+$ cells to produce TGF-β in the Lewis rat are different from the epitopes in the Lewis rat that trigger $CD4^+$ encephalitogenic cells (*19*), and this most probably relates to the fact that they are class I restricted. The cloning of $CD8^+$ cells from orally tolerized SJL mice will allow the characterization of $CD8^+$ mucosally derived regulatory cells. The $CD8^+$ regulatory T cell clones in lepromatous leprosy mediate immune suppression through IL-4 production (*20*). Other investigators have described anti-idiotypic T cells specific for encephalitogenic clones that down-regulate EAE (*21*). The mechanism by which these cells suppress have not been described, although in some instances it may relate to cytotoxicity against encephalitogenic T cells. Anti-idiotypic $CD4^+$ regulatory T cells in rats recovering from EAE mediate suppression through IL-4 and TGF-β (*22*). During the natural recovery from EAE in the Lewis rat, TGF-β and IL-4 are expressed in the brain, and in mice recovering from relapsing EAE, IL-10 is expressed (*23*).

It thus appears that regulatory T cells are normally generated during the course of immune responses, and they function by the production of suppressive cytokines. Furthermore, induction of these cells relates to the immune microenvironment in which they are induced, and they are preferentially induced by the mucosal immune system. Therefore these cells may have an important biologic function in maintaining peripheral tolerance in the host.

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24. Oral tolerance was induced by a multiple low-dose feeding regimen. Each SJL mouse was fed with 0.5 mg of either HEL (Sigma, St. Louis, MO) or mouse MBP dissolved in 0.5 ml of phosphate-buffered saline (PBS) by gastric intubation with an 18-gauge stainless steel feeding needle (Thomas Scientific, Swedesboro, NJ). Mouse MBP was prepared from the brain tissue by a modified method of Deibler *et al.* (*11*); the purity of the MBP preparation was confirmed by gel electrophoresis and amino acid analysis. Mice were fed every other day for a total of five times. Two days after the last feeding, each mouse received an intraperitoneal injection of 100 μg of mouse MBP in 0.25 ml of PBS emulsified in an equal volume of CFA containing mycobacterium tuberculosis H37 RA (400 μg/ml, Difco, Detroit, MI).
25. The culture medium used in this study was Dulbecco's modified essential medium (DMEM) supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), 10% heat-inactivated fetal bovine serum (Biowhittacker, Walkersville, MD), and 5×10^{-5} M of 2-mercaptoethanol (Sigma, St. Louis, MO). The mouse T cell growth factor (TCGF) was prepared as described [Y. Chen *et al.*, *J. Immunol.* **152**, 3 (1994)].
26. The $CD4^+$ and $CD8^+$ T cells were purified by positive selection and depleted by negative selection with magnetic beads per manufacturer's instruction (Advanced Magnetics, Cambridge, MA). The mAbs specific for CD4 [clone GK1.5, American Type Culture Collection (ATCC)] and CD8 (clone 53–6.72, ATCC) were purified from the culture supernatants through a protein G column and added to the cell suspension at a ratio of 1 to 5 μg per 10^6 cells per milliliter. The treatment was repeated two times, and separated cell populations were more than 99% $CD4^+CD8^-$, $CD4^-CD8^+$, or $CD4^-CD8^-$ as determined by direct flow cytometry.
27. Female SJL/J mice, 6 to 8 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in Harvard Medical School Animal Care Facilities under virus-free conditions.
28. For the ELISA for cytokines the following reagents were purchased from Pharmingen (San Diego, CA): purified rat mAbs to mouse IL-2 (clone JES-1A12), IL-4 (clone BVD4–1D11), IL-10 (clone JES5–2A5), and IFN-γ (clone R4–6A2); biotinylated rat mAbs to mouse IL-2 (clone JES6–5H4), IL4 (clone BVD6–24G2), IL-10 (clone SXC-1), and IFN-γ (clone XMG1.2); and recombinant mouse IL-2, IL-4, IL-10, and IFN-γ. Polyclonal chicken antibody to TGF-β1 was purchased from R&D Systems (Minneapolis, MN); purified bovine TGF-β1 and mAb to mouse TGF-β (clone 1D11.16) were from Celtrix Pharmaceuticals (Santa Clara, CA). Quantitative ELISA for IL-2, IL-4, IL-10, and IFN-γ were performed with paired mAbs specific for corresponding cytokines per manufacturer's recommendations. TGF-β1 was determined without acid activation as described (*19*).
29. Scoring of EAE: 0, no disease; 1, tail paralysis; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb plus forelimb paralysis; and 5, moribund.
30. To generate MBP-specific T cell clones, we fed and immunized six SJL mice with mouse MBP as described (*24*). Fourteen days after immunization, MLNs were harvested and a single-cell suspension was prepared. Cells were then cultured in medium containing mouse MBP (25 μg/ml) for 4 days (*25*). Mouse T cell growth factor (TCGF) (10%, v/v) was then added to the culture, and cells were further stimulated for a week before cloning (*25*). For T cell cloning, $CD4^+$ T cells were first purified by positive selection as described (*26*) and diluted to 1 cell per milliliter in medium containing mouse MBP (25 μg/ml) and 10% mouse TCGF, together with syngeneic irradiated spleen cells as feeder cells (5×10^6/ml). Aliquots (0.2 ml per well) were dispensed into 96-well flat bottom plates (Corning, Corning, NY). Wells exhibiting cell growth 10 to 14 days later were scored and expanded in medium containing 10% mouse TCGF. Subcloning of MBP-specific T cells were conducted in a similar fashion. For maintaining T cell clones, cells (2×10^5 per milliliter) were first rested for 7 days with feeder cells (5×10^6/ml) and then stimulated with mouse MBP (25 μg/ml), mouse TCGF (10%), and feeder cells for 4 days. This cycle of resting and stimulation was necessary for expanding MBP-specific T cells producing TGF-β.

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