

# The Thymus Plays a Role in Oral Tolerance in Experimental Autoimmune Encephalomyelitis

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The oral administration of myelin proteins has been used for the successful prevention and treatment of experimental autoimmune encephalomyelitis (EAE). We questioned whether the thymus was involved in oral tolerance. In this study, euthymic myelin basic protein (MBP) TCR transgenic mice are protected from EAE when fed MBP but are not protected when thymectomized. Similarly, in a cell transfer system, T cell responses to OVA measured *in vivo* were suppressed significantly only in the OVA-fed euthymic mice but not in the thymectomized mice. We observed that the absence of the thymus dramatically enhanced the Th1 response. We explored three alternatives to determine the role of the thymus in oral tolerance: 1) as a site for the induction of regulatory T cells; 2) a site for deletion of autoreactive T cells; or 3) a site for the dissemination of naive T cells. We found that Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells are increased in the periphery but not in the thymus after Ag feeding. These CD4<sup>+</sup>CD25<sup>+</sup> T cells also express glucocorticoid-induced TNFR and intracellular CTLA4 and suppress Ag-specific proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells *in vitro*. The thymus also plays a role in deletion of autoreactive T cells in the periphery following orally administered MBP. However, thymectomy does not result in homeostatic proliferation and the generation of memory cells in this system. Overall, the oral administration of MBP has a profound effect on systemic immune responses, mediated largely by the generation of regulatory T cells that act to prevent or suppress EAE. *The Journal of Immunology*, 2006, 177: 1500–1509.

**M**ultiple sclerosis (MS)<sup>2</sup> is a chronic disease affecting the CNS of young adults, often first diagnosed between the ages of 20 and 40 years. A small minority (2.7–5%) of MS cases is diagnosed before the age of 15 (1, 2). Recently, it has been found that most of the Ags thought to be involved in MS are expressed in the human thymus (3), suggesting a possible role for the thymus in the development of MS.

Experimental autoimmune encephalomyelitis (EAE), a model for studying MS, is a T cell-mediated autoimmune disease of the CNS. EAE is induced by immunization of susceptible mouse strains with myelin components, including myelin basic protein (MBP), proteolipid protein, or myelin oligodendrocyte glycoprotein or peptides (4). Immunization of H-2<sup>s</sup> (SJL) or H-2<sup>u</sup> (B10.PL or PL/J) mice with MBP or MBP-derived peptides in adjuvant induces a relapsing-remitting chronic form of EAE (5).

The thymus controls organ-specific autoimmune diseases by limiting the development of autoreactive T cells and by generating regulatory T (Treg) cells (6). It has been proposed that the expression of relevant autoantigens in the thymus causes the generation of specific CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (6, 7). MBP is expressed in the thymus at the mRNA and protein levels (8–11). However, a major

encephalitogenic epitope, MBP NAc1–11 peptide, binds weakly to MHC (12) and forms unstable peptide/MHC complexes (13), which cannot mediate efficient negative selection (14). Therefore, it appears that deletion of MBP peptide-reactive T cells in the thymus is incomplete and that autoreactive cells specific for MBP escape to the periphery. Thymic control of autoreactive T cells in the periphery involves deletion of high-affinity T cells as well as the release of high-affinity T cells (Treg cells) (15–17). Therefore, protection from EAE may represent a contribution of both central and peripheral tolerance mechanisms.

Khoury et al. (18) reported that systemic tolerance can be induced in EAE by injection of autoantigen into the thymus of adult animals. Injection of MBP into the thymus induces profound apoptosis of MBP-reactive T cells in the thymus (19) but not all of the Ag-specific T cells die (18). Some surviving MBP-specific T cells are shown to migrate to the periphery, where they remain in an anergic state (18). These findings support the notion that acquired thymic tolerance includes deletion of Ag-specific T cells as well as a peripheral anergy component (19–21).

Oral tolerance refers to the oral administration of protein Ags, which induces a state of systemic immunologic nonresponsiveness specific for the fed Ag. Orally administered Ag first encounters the GALT, which is a well-developed immune network that evolved not only to protect the host from ingested pathogens but also to prevent the host from reacting to dietary proteins. We and others have previously shown that the oral administration of MBP before encephalitogenic challenge results in protection from clinical signs and histopathologic changes of EAE in Lewis rats (22, 23), SJL (H-2<sup>s</sup>) mice (24, 25), B10.PL (H-2<sup>u</sup>) mice (26, 27), C57BL/6 mice (28, 29), and MBP TCR transgenic (Tg) mice (30–32). Feeding any of the three major myelin proteins, MBP, proteolipid protein, or myelin oligodendrocyte glycoprotein is effective in decreasing clinical and histopathologic manifestations of EAE (22–32). Importantly, feeding MBP even after disease is established has proven effective in suppressing EAE clinical signs (26).

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<sup>2</sup> Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; GITR, glucocorticoid-induced TNFR; PT, pertussis toxin; TX, thymectomy; Treg, regulatory T; Tg, transgenic; DTH, delayed-type hypersensitivity; pLN, peripheral lymph node; IRBP, interphotoreceptor retinoid-binding protein.

Two mechanisms have been proposed to explain oral tolerance. High doses of fed Ag induce anergy/deletion of Ag-reactive T cells (23, 32), whereas low doses of Ag induce the generation of regulatory cells (33–36). Recently, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells have received particular attention in association with tolerance. CD4<sup>+</sup>CD25<sup>+</sup> T cells comprise ~5–10% of the peripheral T cell pool and exhibit immunosuppressive capabilities *in vitro* and *in vivo* (37). Papiernik et al. (38) reported that CD4<sup>+</sup>CD25<sup>+</sup> T cells originate in the thymus and are induced to express CD25 at the CD4 single-positive stage. The thymic CD4<sup>+</sup>CD25<sup>+</sup> T cells migrate from the thymus to the periphery (39). In contrast, Thorstenson et al. (39) described a population of naturally occurring polyclonal immunoregulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells induced by specific Ag and that originate in the periphery rather than the thymus. Zhang et al. (33) reported an increase in CD4<sup>+</sup>CD25<sup>+</sup> T cells after oral administration of OVA to OVA TCR Tg mice. Adoptive transfer of these CD4<sup>+</sup>CD25<sup>+</sup> T cells from fed mice suppressed *in vivo* delayed-type hypersensitivity (DTH) responses in recipient mice (33).

To date, the role of the thymus in the induction of mucosal tolerance has not been explored. In the present study, we show that the thymus is necessary for demonstration of oral tolerance following feeding of either MBP or OVA. We explored three alternatives to reveal the role of the thymus in oral tolerance: 1) the thymus serves as a site for the induction of Treg cells; 2) the thymus is a site for deletion of autoreactive T cells; and 3) the thymus is a site for the generation of naive T cells. When the thymus is removed, the possibility exists that peripheral T cells may undergo homeostatic proliferation with the subsequent generation of memory cells. Understanding the possible roles of the thymus in the induction of oral tolerance in EAE might provide new therapeutic approaches for the treatment of MS.

## Materials and Methods

### Animals

V $\alpha$ 4/V $\beta$ 8.2 MBP TCR Tg mice (40) were extensively backcrossed onto the B10.PL background, bred and housed in a clean specific pathogen-free facility at The Ohio State University. Mice were screened by flow cytometry using peripheral blood leukocytes labeled with mAbs directed against the clonotypic TCR (G19) or V $\beta$ 8.2 and CD4. The clonotypic Ab (G19) was a gift from Dr. J. J. Lafaille (New York, NY). Tg animals were used in experiments at 6–8 wk of age.

### Adult thymectomy (TX) and thymocyte transfer

Adult mice (6 wk of age) were given 20  $\mu$ g of gentocin *i.p.* and anesthetized with ketamine (82–110 mg/kg) and xylazine (7.5 mg/kg) *i.p.* During surgery, mice were intubated and maintained on a rodent ventilator. An upper median sternotomy was performed, and both lobes of the thymus were removed (41). The thoracic cage was sutured closed and skin secured. Sham surgery control mice were anesthetized and intubated; their thoracic cage was opened and sutured closed. Mice were allowed to recover and used in experiments 12–14 days after surgery. In some experiments, thymuses were harvested from native MBP TCR Tg mice and processed to form a single-cell suspension. A total of  $10 \times 10^6$  thymocytes in 200  $\mu$ l of PBS was transferred into thymectomized Tg mice by tail vein injection 24 h before feeding.

### Antigens

MBP was extracted from guinea pig spinal cords (Harlan Bioproducts for Science) using the method of Swanborg (42). MBP peptide NAc1–11 ([Ac]-ASQKRPSQRH-[OH]; MW 1293) was obtained from Princeton Biomolecules. OVA was purchased from Sigma-Aldrich.

### Induction of oral tolerance and EAE

Euthymic and adult thymectomized Tg mice were deprived of food, but not water, for 4 h before oral Ag administration. MBP (100 mg) in 0.5 ml of PBS or PBS alone as vehicle control was administered by gastric intubation to anesthetized mice. To induce EAE, mice were injected *s.c.* with 200  $\mu$ g

of guinea pig MBP combined with CFA containing 200  $\mu$ g of *Mycobacterium tuberculosis*, Jamaica strain, over four sites on the flank. Mice also received two *i.p.* injections of 200 ng of pertussis toxin (PT) (List Biological) at the time of MBP injection and 48 h later. Animals were observed daily for clinical signs and scored as follows: limp tail or waddling gait with tail tonicity, 1+; waddling gait, 2+; partial hind limb paralysis, 3+; full hind limb paralysis, 4+; and death, 5+.

### DTH reaction

BALB/c mice were thymectomized as adults (6–7 wk of age) and allowed to recover for 2 wk after surgery. Lymph node and spleen cells ( $1.5 \times 10^6$  lymphocytes containing  $0.5 \times 10^6$  KJ1–26<sup>+</sup>CD4<sup>+</sup> cells) from euthymic DO11.10 Tg  $\times$  Rag2<sup>-/-</sup> donors were injected into the tail veins of age-matched euthymic and thymectomized BALB/C recipient mice in 0.2 ml of PBS (43). The recipients were fed 100 mg of OVA or PBS 1 day before immunization with 100  $\mu$ g of OVA/CFA at the base of tail. Mice were injected with 20  $\mu$ l of OVA (1 mg/ml) in PBS in the left ear and 20  $\mu$ l of PBS in the right ear 13 days after immunization. Ear thickness was measured 24 h later.  $\Delta$  ear thickness = thickness of left ear – thickness of right ear.

### Flow cytometric analysis

Single-cell suspensions of lymphoid cells derived from the thymus and the peripheral LNs (pLNs) (inguinal, axillary, brachial, cervical, deep cervical, popliteal, periaortic) were stained with anti-V $\beta$ 8.2-FITC (BD Pharmingen), anti-CD4-FITC, anti-CD25-PE (clone PC61), anti-glucocorticoid-induced TNFR (GITR)-PE (eBioscience), anti-CTLA4-PE or a clonotype Ab (G19), and anti-CD4-allophycocyanin or anti-CD25-allophycocyanin using three-color flow cytometry. Isotype control mAbs (BD Pharmingen) were matched for fluorochrome. Cell suspensions containing  $1 \times 10^6$  cells were incubated with labeled Abs diluted in PBS plus azide with 2% rat serum. After a 30-min incubation at 4°C, cells were washed and fixed with 1% paraformaldehyde. A total of 20,000 events/sample was collected and analyzed on a Becton Dickinson FACSCalibur (BD Biosciences) using CellQuest BD Pro software.

Intracellular CTLA4 expression was performed using the Cytofix/Cytoperm kits (BD Pharmingen) with modifications. Briefly,  $2 \times 10^6$  cells were incubated with anti-FITC-CD4 and anti-allophycocyanin-CD25. After washing, cells were incubated with a 3- to 5-fold excess of purified anti-CTLA4 to saturate extracellular CTLA4. Cells were then washed, fixed, and permeabilized according to the manufacturer's instructions. Cells were incubated with anti-CTLA4-PE for intracellular CTLA4 protein detection and then washed, resuspended in 1% paraformaldehyde, and analyzed (at least 20,000 events) by flow cytometry.

Intracellular Foxp3 expression was performed using the PE anti-mouse Foxp3 staining kit (eBioscience). Briefly,  $1 \times 10^6$  cells were incubated with anti-FITC-CD4 and anti-allophycocyanin-CD25. Cells were then washed, fixed, and permeabilized. Cells were incubated with anti-Foxp3-PE for intracellular Foxp3 protein detection.

### Isolation of CD4<sup>+</sup>CD25<sup>+</sup> T cells

CD4<sup>+</sup>CD25<sup>+</sup> cells from vehicle-fed and MBP-fed euthymic Tg mice were purified using the CD4<sup>+</sup>CD25<sup>+</sup> Treg cell isolation kit (Miltenyi Biotec), following the manufacturer's instructions. Deletion of non-CD4<sup>+</sup> T cells from the spleen and pLNs was conducted using indirect labeling of cell populations with biotin-Ab mixture (directed against CD8a, CD11b, CD45R, CD49b, and Ter<sup>119</sup>) and anti-biotin beads via magnetic separation on a depletion column (Miltenyi Biotec). Positive selection of CD4<sup>+</sup>CD25<sup>+</sup> cells was performed by labeling of the flow-through fraction (CD4<sup>+</sup> T cells) with CD25-PE Ab and anti-PE microbeads followed by magnetic separation using two selection columns. To isolate CD4<sup>+</sup>CD25<sup>-</sup> cells, the negative fractions were passed through a depletion column again to deplete any CD4<sup>+</sup>CD25<sup>+</sup> cells. Purity of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells was >95 and >99%, respectively.

### Lymphocyte proliferation

Single-cell suspensions were prepared from pLNs of individual animals. Lymphoid cells were cultured in RPMI 1640 containing 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, and  $5 \times 10^{-5}$  M 2-ME in round-bottom 96-well plates ( $4 \times 10^5$  cells/well). Cells were cultured with OVA (40  $\mu$ g/ml) or NAc1–11 (10  $\mu$ g/ml) in triplicate for 72 h, including a final 18-h pulse with [<sup>3</sup>H]thymidine. In some experiments, CD4<sup>+</sup>CD25<sup>-</sup> responder cells ( $2.5 \times 10^4$ ) were cultured with APCs ( $7.5 \times 10^4$ ) and MBP (40  $\mu$ g/ml), NAc1–11 (3  $\mu$ M), or anti-CD3 (2  $\mu$ g/ml) in the presence or absence of purified CD4<sup>+</sup>CD25<sup>+</sup> cells for 72 h.

Cultures were harvested onto glass-fiber filter mats using a Skatron harvester (Skatron) and were counted by liquid scintillation on a Wallac Beta Plate (LKB).

#### Cytometric bead array analysis

Cell supernatants were harvested and stored at  $-70^{\circ}\text{C}$  until testing. IL-2, IL-4, IL-5, IFN- $\gamma$ , and TNF- $\alpha$  were detected simultaneously using the mouse Th1/Th2 cytokine cytometric bead array kit from BD Biosciences. Briefly, 50  $\mu\text{l}$  of supernatants was mixed with 50  $\mu\text{l}$  of the mixed capture beads and 50  $\mu\text{l}$  of the mouse Th1/Th2 PE detection reagent. The tubes were incubated at room temperature for 2 h in the dark, followed by washing. The samples were analyzed on a FACSCalibur flow cytometer using cytometric bead array software (BD Biosciences). Standard curves were generated for each cytokine using the mixed bead standard, and the concentration of cytokine in the cell supernatants were determined by interpolation from the appropriate standard curve.

#### Apoptosis assay

TUNEL analysis was performed using the In Situ Cell Death Detection kit (Roche Diagnostics). Briefly,  $2 \times 10^6$  cells were stained with PE-anti-V $\beta 8$  and allophycocyanin-anti-CD4, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100/0.1% sodium citrate, and exposed to the TdT/biotin-dUTP mix for 1 h at  $37^{\circ}\text{C}$ . FITC-streptavidin was added for 30 min, followed by washing. Positive control samples were treated with DNase (Genzyme Pharmaceutical) for 10 min before TdT labeling, whereas negative control samples contained no TdT. TUNEL stained samples (at least 20,000 events) were analyzed by flow cytometry.

#### Statistical analysis

For EAE clinical scores, flow cytometry, apoptosis, and proliferation analyses, a nonparametric ANOVA with Tukey's post hoc test was performed to determine differences between vehicle-fed and MBP-fed groups. In some experiments, comparisons were made between  $\text{CD4}^+\text{CD25}^+$  and  $\text{CD4}^+\text{CD25}^-$  cells. Groups were considered significantly different at  $p < 0.05$ .

## Results

### *The thymus plays an essential role in the induction of oral tolerance*

The oral administration of MBP protects rats and mice from acute as well as chronic relapsing EAE (22–23, 27). Immunization of V $\alpha 4/\text{V}\beta 8.2$  MBP TCR Tg mice with MBP or MBP peptide results in the rapid development of EAE because 95% of  $\text{CD4}^+$  T cells in these mice express the V $\alpha 4/\text{V}\beta 8.2$  TCR specific for the immunodominant NAc1–11 epitope of MBP (40). We previously reported that feeding high doses of MBP to MBP TCR Tg mice results in oral tolerance by inducing activation-induced cell death of encephalitogenic T cells (32).

It is reported that oral tolerance is not inducible in older mice ( $>4$  mo of age) (44). Involution of the thymus has been implicated as a potential cause for the lack of tolerance in older animals. Therefore, we directly examined the role of the thymus in MBP-induced oral tolerance. MBP TCR Tg mice were thymectomized as adults and allowed to recover from thymectomy surgery. Euthymic or thymectomized Tg mice were then fed 100 mg of MBP and immunized 1 day later for EAE. The oral administration of MBP to euthymic MBP TCR Tg mice is observed to significantly protect the MBP-fed mice from EAE (Fig. 1A). In contrast, adult-thymectomized Tg mice fed MBP showed clinical signs of EAE equivalent to vehicle-fed controls (Fig. 1B). We also observed that sham surgery control mice (vehicle-fed and MBP-fed) showed similar results as euthymic mice (Table I). These data strongly suggest that the thymus plays a key role in the induction of oral tolerance in EAE.

We also assessed the role of the thymus in oral tolerance induced by feeding a non-self Ag, OVA, in a non-Tg system. We used the T cell transfer protocol described by Kearney and Jenkins

**FIGURE 1.** The thymus plays a role in oral tolerance. MBP TCR Tg mice were thymectomized as adults (6–7 wk of age), and allowed to recover for 2 wk after surgery. Age-matched euthymic (A) and thymectomized (B) Tg mice were fed 100 mg of MBP or PBS 1 day before immunization with MBP/CFA/PT. Mice were scored daily for disease, and the mean score of each group  $\pm$  SEM is shown ( $n = 14$ – $22$ /group). C, BALB/c mice were thymectomized similarly as adults and allowed to recover after surgery. A total of  $1.5 \times 10^6$  lymphocytes (containing at least  $0.5 \times 10^6$  KJ1–26 $^+$ CD4 $^+$  cells) from DO11.10 Tg  $\times$  Rag2 $^{-/-}$  donors was injected i.v. into age-matched euthymic and thymectomized BALB/c mice ( $n = 5$ – $6$ /group). The recipients were fed 100 mg of OVA or PBS 1 day before immunization with 100  $\mu\text{g}$  of OVA/CFA. Mice were challenged with 20  $\mu\text{l}$  of OVA (1 mg/ml) in PBS in the left ear or 20  $\mu\text{l}$  of PBS in the right ear 13 days after immunization. Ear thickness was measured 24 h later (C).  $\Delta$  ear thickness = thickness of left ear – thickness of right ear. \*, Values are statistically different from vehicle-fed mice at  $p \leq 0.05$ .

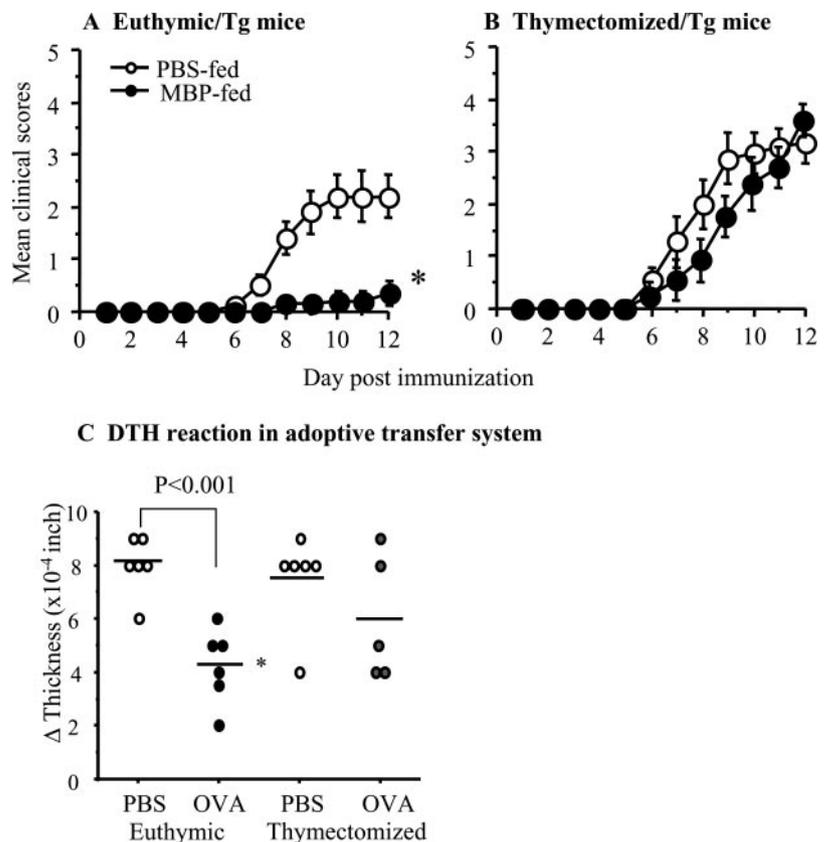


Table I. Orally administered MBP protects adult euthymic but not thymectomized (TX) MBP TCR Tg mice from EAF<sup>a</sup>

Treatment	Incidence of EAE (%)	Mean Day of Onset <sup>b</sup>	Mean Cumulative Clinical Score <sup>c</sup>	Mean Maximum Severity <sup>d</sup>	Mortality
PBS fed	20/22 (91%)	9.00 ± 3.84	12.25 ± 9.21	2.59 ± 1.77	4/22 (18%)
MBP fed	10/16 (63%)	15.71 ± 8.94*	4.63 ± 6.30*	1.50 ± 1.76*	1/16 (6%)
Sham/PBS fed	10/10 (100%)	7.70 ± 0.95	14.75 ± 3.23	5.00 ± 0.00	10/10 (100%)
Sham/MBP fed	10/11 (91%)	9.45 ± 3.75*	10.41 ± 5.29*	3.73 ± 2.17*	8/11 (73%)
TX/PBS fed	16/16 (100%)	7.69 ± 1.35	16.22 ± 8.64	4.28 ± 1.22	8/16 (50%)
TX/MBP fed	22/22 (100%)	8.64 ± 1.59	15.39 ± 6.31	3.26 ± 0.74	8/22 (36%)
TX/thymocyte transfer/PBS fed	3/3 (100%)	9.7 ± 3.2	37.0 ± 2.8	4.7 ± 0.3	1/3 (33%)
TX/thymocyte transfer/MBP fed	2/3 (67%)	11.0 ± 3.5	16.0 ± 12.3*	3.0 ± 2.6	1/3 (33%)

<sup>a</sup> MBP TCR Tg mice were thymectomized or sham-treated as adults and allowed to recover for 2 wk after surgery. Age-matched euthymic, thymectomized, and sham-treated Tg mice were fed 100 mg of MBP or PBS 1 day before immunization with MBP/CFA/PT. Mice were scored daily for disease, and the mean score of each group ± SD is shown.

<sup>b</sup> The two groups of Tg mice were housed in specific pathogen-free conditions (2 wk).

<sup>c</sup> Mean of the sum of scores for each animal over the entire observation period. Animals who died during the observation period received only a single score of 5 on the day of death (5 = death).

<sup>d</sup> Mean of the highest clinical score exhibited by individual animals within a group during the entire course of EAE.

\*,  $p < 0.05$  by  $t$  test.

(43), in which D011.10 Tg T cells recognizing an epitope of OVA were transferred to BALB/c recipients. BALB/c mice were thymectomized as adults and allowed to recover from surgery. Lymphocytes from DO11.10 Tg × Rag2<sup>-/-</sup> donors were transferred i.v. to age-matched euthymic and thymectomized mice. The recipients were fed OVA or PBS 1 day before immunization with OVA/CFA. Mice were challenged with OVA in PBS in the left ear or PBS alone in the right ear 13 days after immunization for assessment of DTH responsiveness. Fig. 1C shows that the DTH reaction is suppressed significantly in the OVA-fed euthymic group but not in the OVA-fed thymectomized group. Thus, the data in this OVA transfer model agree with the findings in EAE using direct feeding of Tg mice, supporting the conclusion that the thymus plays an essential role in oral tolerance.

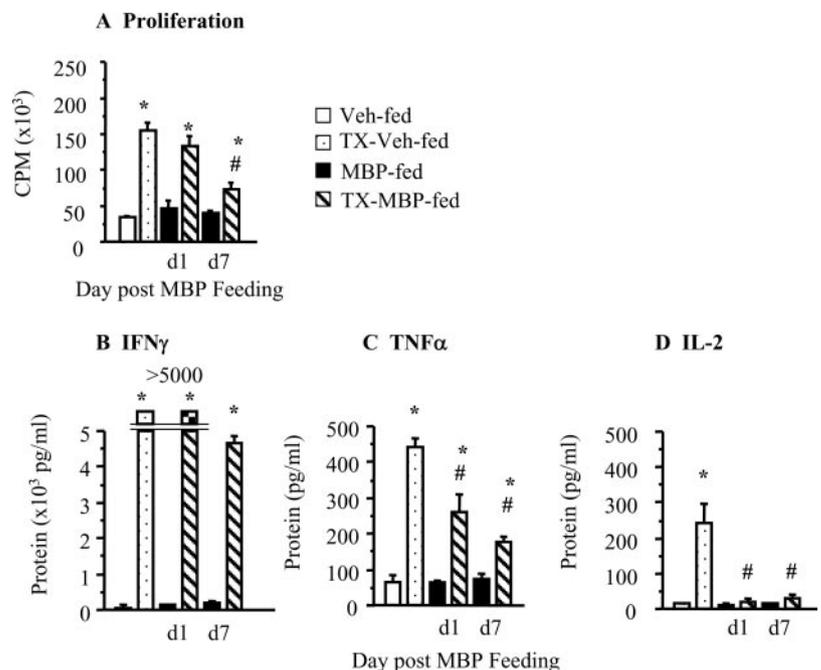
We next determined whether reconstitution of thymectomized Tg mice with thymocytes would be sufficient to restore oral tolerance and thus protect the mice from EAE. Briefly, thymocytes from naive MBP TCR Tg mice were dissociated from an intact thymus and transferred into a thymectomized MBP TCR Tg mouse that was then fed MBP and immunized for EAE (Table I).

Thymectomized mice reconstituted with thymocytes showed a delay in onset and significantly less severe clinical signs of EAE following MBP feeding compared with reconstituted vehicle-fed mice (Table I) as well as nonreconstituted MBP Tg mice (Fig. 1B and Table I). The data suggest that 1) returning thymocytes to a thymectomized mouse restores the capacity for oral tolerance; and 2) supplying thymocytes cannot restore tolerance and protection without oral Ag. It is possible that a specific type of thymocytes (CD4<sup>+</sup>CD8<sup>-</sup>), as well as the environment/structure of the thymus (thymic stroma), plays an important role in induction of oral tolerance.

#### The absence of the thymus dramatically enhances the Th1 response

In Fig. 1B and Table I, we noted that the severity of disease in thymectomized mice is higher than in the euthymic group (Fig. 1A). Therefore, we analyzed T cell function in thymectomized vs nonthymectomized mice. We observed that thymectomy significantly elevates the proliferative response to anti-CD3 (Fig. 2A), as well as MBP (data not shown). The increased proliferation was

**FIGURE 2.** Thymectomy enhances Th1 cell activation. The splenocytes were cultured with anti-CD3 for 48 h and pulsed with [<sup>3</sup>H]thymidine for 18 h (A). Culture supernatants were collected and analyzed by the cytometric bead assay for Th1/2 cytokine production (B–D). Two mice per group were analyzed at each time point. \*, Values are statistically different between euthymic and thymectomized mice, and #, values are statistically different from vehicle-fed mice in the euthymic or thymectomy group at  $p < 0.05$ .



accompanied by significant Th1 cytokine production, including IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 (Fig. 2, B–D). In contrast, Th2 cytokine production (IL-4 and IL-5) was not different between euthymic and thymectomized groups (data not shown). Fig. 2 shows that thymectomy significantly enhances the proliferative response and Th1 cytokine production (IFN- $\gamma$ , TNF- $\alpha$ , and IL-2) in vehicle-fed compared with euthymic mice. Although TNF- $\alpha$  and IL-2 levels in thymectomized mice are reduced by MBP feeding, TNF- $\alpha$  levels are still significantly higher than in MBP-fed euthymic mice (except IL-2). Taken together, this strong enhancement of the Th1 response is thymus dependent.

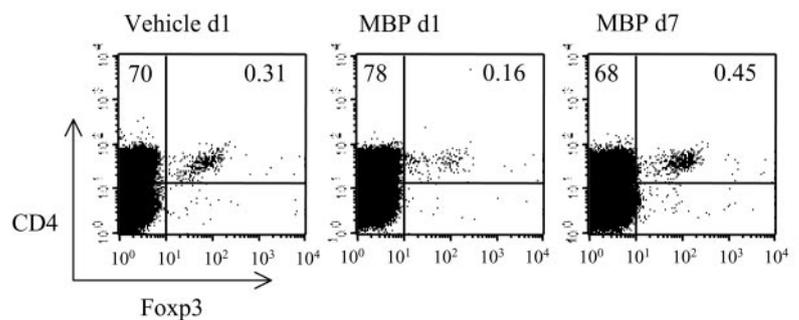
*The thymus plays a role in the generation of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells in the periphery after MBP feeding*

To determine the role played by the thymus in oral tolerance, we explored three alternatives: 1) the thymus serves as a site for generation of Treg cells; 2) it is a site for deletion of autoreactive T cells; and/or 3) the thymus serves as a site from which naive T cells are disseminated. In the absence of the thymus, T cells may undergo homeostatic proliferation with the subsequent generation of memory cells (45–48). To investigate the first alternative, we examined Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells in the thymus and periphery after feeding MBP in euthymic and thymectomized mice. As

shown in Fig. 3A, Foxp3<sup>+</sup>CD4<sup>+</sup> thymocytes were observed in the thymus of both vehicle-fed and MBP-fed euthymic mice. Interestingly, Foxp3<sup>+</sup>CD4<sup>+</sup> thymocytes were reduced 1 day after MBP feeding (Fig. 3A, middle panel), with a greater number of such cells observed at 7 days after feeding (Fig. 3A, right panel). These results imply that 1) the Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> thymocytes may traffic into the periphery or be deleted 1 day after MBP feeding, and 2) the emergence of new lymphocyte precursors on day 7 in the thymus most likely represents new, natural/induced Treg cells.

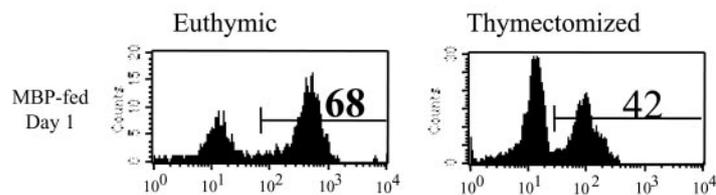
In Fig. 3, B and C, we examined Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells in the pLNs of both euthymic and thymectomized mice fed vehicle or MBP. We found that the percentage of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells is reduced in thymectomized MBP-fed mice compared with vehicle-fed thymectomized mice or the MBP-fed euthymic group (Fig. 3B). Interestingly, the level of Foxp3 expression was lower in thymectomized mice compared with euthymic mice on day 1 after MBP feeding (Fig. 3B). Moreover, the absolute number of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells is increased significantly in the LN of euthymic MBP-fed vs thymectomized mice 1 day after MBP feeding, which was not observed at the day 7 time point (Fig. 3C). In addition to Foxp3, we also examined other Treg markers, including intracellular CTLA4 and GITR on CD4<sup>+</sup>CD25<sup>+</sup> T cells. The expression of intracellular CTLA4 (71%; data not shown) and

### A Foxp3 in the thymus

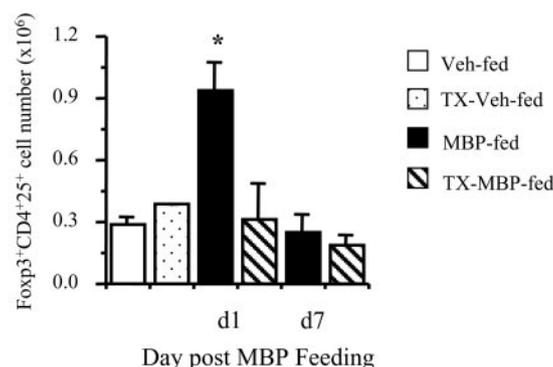


**FIGURE 3.** Increased Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells in MBP-fed euthymic mice compared with thymectomized control mice. MBP TCR Tg mice were thymectomized as adults and allowed to recover for 2 wk after surgery. Age-matched euthymic and thymectomized mice were fed 100 mg of MBP or PBS. On the indicated day after feeding, the expression of intracellular Foxp3 and CD4 on thymocytes in euthymic mice (A) and intracellular Foxp3 expression on gated CD4<sup>+</sup>CD25<sup>+</sup> T cells in the pLNs in both euthymic and thymectomized mice (B) were analyzed by flow cytometry. Total Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cell number = total pLN cell number  $\times$  percentage of CD25<sup>+</sup>  $\times$  percentage of Foxp3<sup>+</sup>CD4<sup>+</sup>. C, Two mice per group; \*, values are statistically different from vehicle-fed and thymectomized MBP-fed mice at  $p \leq 0.05$ .

### B Foxp3 on gated CD4<sup>+</sup>CD25<sup>+</sup> cells in the LNs



### C Total Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cell number in the LNs



GITR (92%; data not shown) is much greater in euthymic MBP-fed than thymectomized MBP-fed mice (6 and 40%; data not shown). Taken together, these data suggest that MBP-induced CD4<sup>+</sup>CD25<sup>+</sup> T cells resemble natural Treg cells in their expression of cellular markers (intracellular Foxp3 and CTLA4 as well as GITR) and are reduced in the periphery as a result of thymectomy.

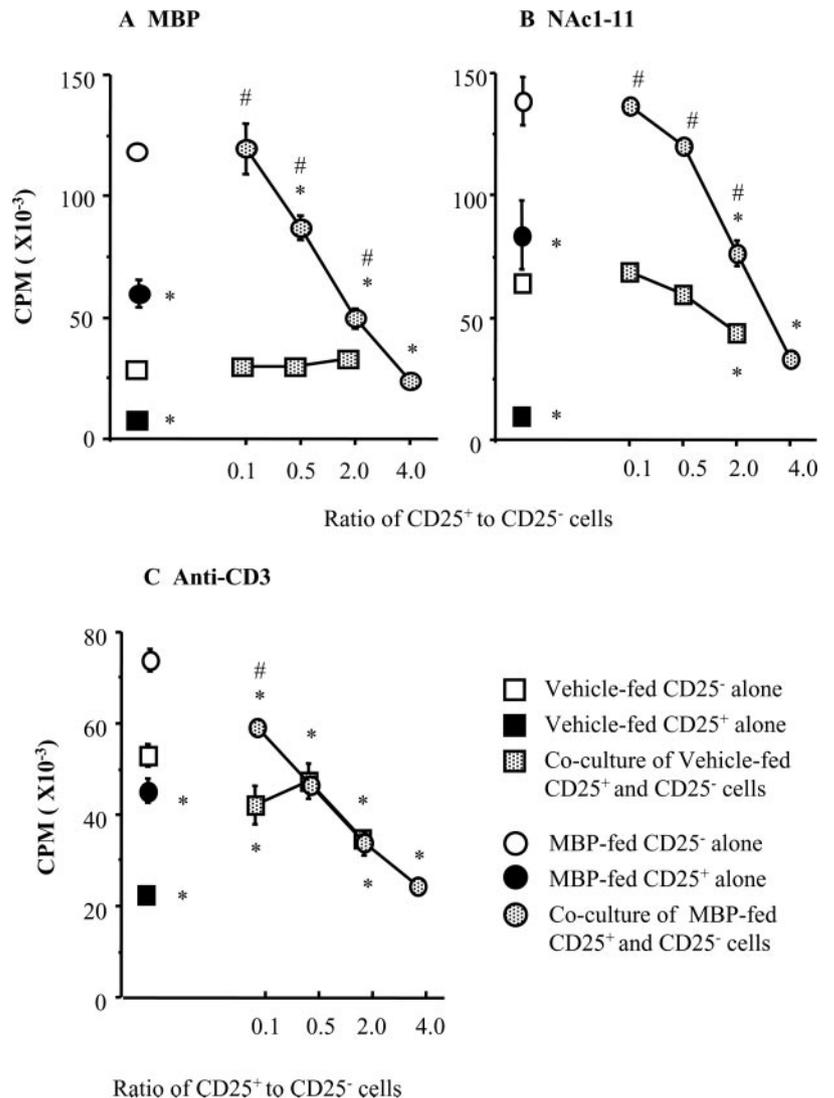
CD4<sup>+</sup>CD25<sup>+</sup> T cells from naive MBP Tg mice are considered Treg cells because adoptive transfer of these CD4<sup>+</sup>CD25<sup>+</sup> cells into Rag<sup>-/-</sup> mice prevent the development of spontaneous EAE (40, 49). To determine whether the CD4<sup>+</sup>CD25<sup>+</sup> T cells exert suppressive activity, their function was assessed in an in vitro proliferation assay. A constant number of responder CD4<sup>+</sup>CD25<sup>-</sup> T cells was cocultured with increasing numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells from MBP-fed or vehicle-fed mice in the presence of specific Ag or anti-CD3. Fig. 4 shows that oral MBP-induced CD4<sup>+</sup>CD25<sup>+</sup> T cells suppress MBP and MBP peptide (NAc1-11)-specific proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells in vitro in a dose-dependent manner (Fig. 4, A and B). In contrast, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from the vehicle-fed group do not markedly suppress MBP-specific or peptide-specific proliferation (Fig. 4, A and B). Similar levels of suppression were observed in anti-CD3-stimulated cultures, likely reflecting the activity of natural Treg cells (Fig. 4C). Because the level of proliferation of either CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> to MBP and NAc1-11 in the MBP-fed group is 2-fold higher than the vehicle-fed group, it is possible that activation is also induced by MBP feeding. Taken

together, the MBP-induced/activated CD4<sup>+</sup>CD25<sup>+</sup> population contains some Treg cells, which express markers of Treg cells (intracellular Foxp3 and CTLA4 as well as GITR), and suppress Ag-specific proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells in vitro.

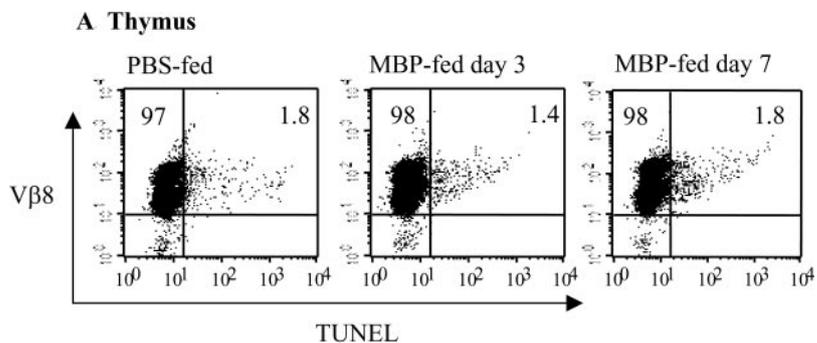
*The thymus plays a role in deletion of autoreactive T cells in the periphery following orally administered MBP*

We observed previously that the oral administration of Ag induces T cell activation with early TCR down-regulation in the spleen and pLNs that precedes deletion (32). In the thymus, we found that expression of the Tg TCR is decreased 3 and 5 days after MBP feeding (data not shown) accompanied by a significant decrease in the total thymocyte number on day 3 after feeding relative to thymocyte numbers in unfed mice (data not shown). The recovery of the TCR on day 7 in the thymus most likely represents the emergence of new lymphocyte precursors (data not shown). Assessment of apoptosis in the thymus 3 days after MBP feeding showed no significant increase in Vβ8<sup>+</sup>TUNEL<sup>+</sup> cells (Fig. 5A). Taken together, our data suggest that the thymus does not serve as a site for clonal deletion following orally administered Ag. It is possible that lymphocyte precursors in the thymus may traffic to the periphery where they could be deleted by activation-induced cell death.

We also examined whether there was evidence in support of the hypothesis that the thymus may play a role in deletion of T cells specific for mucosally administered Ag in the periphery. We found

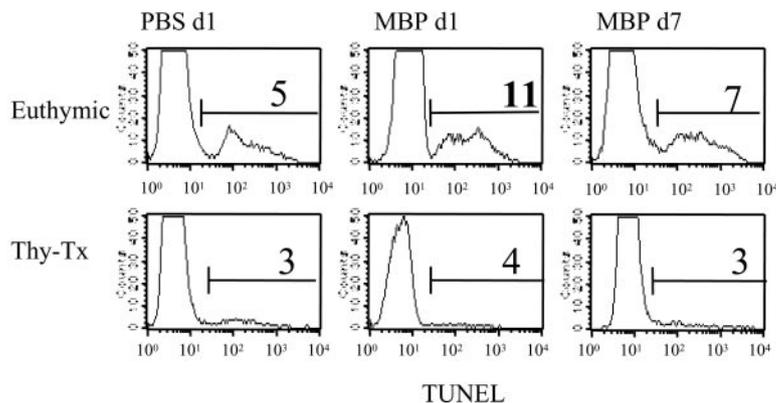


**FIGURE 4.** CD4<sup>+</sup>CD25<sup>+</sup> cells from MBP-fed mice suppress autoantigen-specific proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells. CD4<sup>+</sup>CD25<sup>-</sup> cells ( $2.5 \times 10^4$ ) purified from euthymic MBP-fed or control mice were cultured with APCs ( $7.5 \times 10^4$ ) and MBP (A), NAc1-11 (B), or anti-CD3 (C) in the presence or absence of varying numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells, which were purified from the same group of mice for 72 h. \*, Different between CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup>; #, different between MBP-fed and vehicle-fed mice at  $p < 0.05$ – $0.001$  by ANOVA.



**FIGURE 5.** MBP feeding increases apoptosis in the periphery of euthymic but not thymectomized mice. On the indicated day after MBP feeding, Tg cells ( $CD4^+V\beta8^+$ ) in the thymuses (A) and the pLNs (B) from euthymic and thymectomized TCR Tg mice were analyzed by flow cytometry.  $CD4^+V\beta8^+$  thymocytes and pLN lymphocytes were also analyzed for apoptosis using TUNEL staining. Two to five mice per group were analyzed at each time point.

### B pLNs: TUNEL on $CD4^+V\beta8^+$ cells



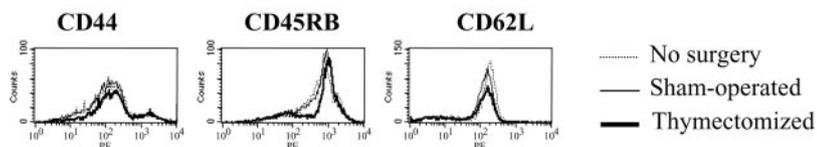
decreased  $CD4^+V\beta8^+$  T cells in the lymph node of both euthymic and thymectomized Tg mice 1 day after feeding (Ref. 32; data not shown). Assessment of apoptosis in the lymph node showed an increase in apoptotic  $CD4^+V\beta8^+$  cells on day 1 in euthymic but not thymectomized MBP-fed mice (Fig. 5B). A moderately increased level of apoptosis was also observed 7 days after MBP feeding. The data suggest that the thymus plays a role in induction of apoptosis of oral Ag-activated autoreactive T cells ( $CD4^+V\beta8^+$ ) in the periphery following MBP feeding.

### Thymectomy does not result in homeostatic proliferation and the generation of memory cells

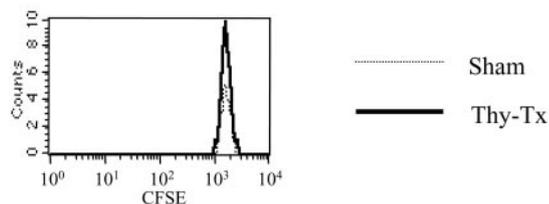
We (32) and others (45–48) have reported that thymectomy results in lymphopenia. This lymphopenic state may lead T cells to undergo homeostatic proliferation with the resultant generation of

memory cells, which are difficult to tolerate (45–48). To test for homeostatic proliferation, we purified MBP-specific TCR Tg  $CD4^+$  T cells from thymectomized or sham-operated mice; the cells were labeled with CFSE and transferred i.v. into syngeneic B10.PL recipients. We observed no homeostatic proliferation of the CFSE-labeled donor cells from either thymectomized or sham-operated donors 8 days after transfer to recipients (Fig. 6B). In addition, we observed very similar levels of expression of CD44, CD45RB, and CD62L ( $CD44^{low}CD45RB^{high}CD62L^{high}$ ) on  $CD4^+$  T cells (donor cells) in both thymectomy and sham-operated groups before oral Ag administration (Fig. 6A). Taken together, thymectomy does not appear to result in homeostatic proliferation or the generation of memory cells in this model, although thymectomy does result in lymphopenia, particularly in  $CD4^+V\beta8.2^+$  T cells (Ref. 32; data not shown).

### A Donor cells



### B Recipient



**FIGURE 6.** Thymectomy did not increase memory T cells ( $CD44^{high}CD35RB^{low}CD62L^{low}$ ) in the donor nor did it induce homeostatic proliferation in the recipient mice. Two weeks after surgery, CD44, CD45RB, and CD62L expression on  $CD4^+$  T cells in the pLNs of donor were analyzed by flow cytometry (A). Eight days after transfer, CFSE expression on  $CD4^+CFSE^+$  donor cells were analyzed by flow cytometry (B); three to four mice per group.

## Discussion

Our strategy uses adult-thymectomized mice to define the role of the thymus in oral tolerance induction at the cellular level. The thymus plays an essential role in the induction of oral tolerance because no protection from EAE and no reduction in DTH were observed in adult-thymectomized mice fed MBP or OVA, respectively (Fig. 1). Our studies outlined here allow an evaluation of this important organ in the development of regulatory cells, clonal deletion of autoreactive T cells, and the generation of memory T cells as participants in the mucosal tolerance pathway in EAE. Recently, in experimental autoimmune uveitis, Avichezer et al. (50) reported an enhanced response to the self-Ag, interphotoreceptor retinoid-binding protein (IRBP), in mice with an IRBP-deficient thymus. In that study, mice deprived of thymus-dependent CD25<sup>+</sup> regulatory cells by thymectomy and treatment with anti-CD25-neutralizing Ab developed more aggressive experimental autoimmune uveitis. The authors interpreted their results as the thymus was responsible for culling of IRBP-reactive precursor-effector cells, as well as the generation of CD25<sup>+</sup> Treg cells specific to IRBP (50).

Recently, Bluestone and Abbas (51) proposed a classification scheme for Treg cells in which two subsets were differentiated based on their development, specificity, mechanism of action and dependence on TCR, and costimulatory signaling (51). Natural Treg cells that develop in the thymus are CD28-CD80/86 dependent, IL-2 dependent, express CD25, are specific for self-Ags, are contact dependent, and cytokine independent. Induced Treg cells that develop in the periphery are CD28-CD80/86 independent, IL-2 dependent, vary in their expression of CD25, are specific for tissue or foreign Ags, and are cytokine dependent (51). While a large proportion of regulatory cell populations can be placed in one subset or the other, there are exceptions to these classifications.

Based on our observations, the abrogation of oral tolerance in adult-thymectomized mice (Fig. 1) is correlated with a significant increase in Th1 response in the periphery relative to euthymic MBP-fed mice (Fig. 2). The increase in Th1 reactivity most likely explains the increase in EAE clinical score observed in thymectomized vehicle-fed mice relative to euthymic vehicle-fed mice (Fig. 1, A and B). Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells significantly increased in the periphery of euthymic MBP-fed mice (Fig. 3C) compared with thymectomized MBP-fed mice. We propose that "natural" Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells may exist at similar levels in euthymic and adult-thymectomized vehicle-fed mice. However, thymus-derived Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells induced by oral Ag are only present in euthymic MBP-fed mice. The thymus-derived populations may exert suppressive function because the levels of proliferative response to MBP peptide in euthymic MBP-fed mice are significantly lower than thymectomized MBP-fed mice (data not shown). The ability of the CD4<sup>+</sup>CD25<sup>+</sup> T cells to suppress the proliferation of the CD4<sup>+</sup>CD25<sup>-</sup> population in an Ag-specific and dose-dependent manner (Fig. 4) confirms their identity as T regulatory cells. These results support the observations by Hori et al. (49) that specificity to MBP is required for effector functions but is not sufficient for thymic selection/commitment of "natural" CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in preventing spontaneous EAE.

Furtado et al. (52) reported that spontaneous EAE in MBP TCR Tg (Rag<sup>-/-</sup>) mice could be prevented by administration of purified CD4<sup>+</sup>CD8<sup>-</sup> thymocytes from wild-type syngeneic mice. Our results showed that returning thymocytes to a thymectomized mouse restores the capacity for oral tolerance, and reconstitution of thymocytes cannot restore tolerance and protection without oral Ag (Table I). These observations suggest that thymocytes are required for reconstitution of tolerance as well as oral tolerance in Th1-mediated EAE. On the other hand, Mucida et al. (53) reported that

oral tolerance could be effectively induced in the absence of thymus-derived natural Treg cells in a mouse model of Th2-mediated hyper-IgE and asthma. It has been noted recently that human thymus-derived Treg cells exert a different suppressive activity on Th1 and Th2 effectors (54). It is reasonable that Th2 cells are less susceptible than Th1 cells to the suppressive activity of thymus-derived Treg cells (54). Taken together, the thymus is required for natural Treg cells, as well as oral Ag-induced/activated Treg cells in oral tolerance in Th1-mediated disease.

Since we observed a decrease in Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells in the thymus 1 day after MBP feeding (Fig. 3A), the possibility exists that the Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> thymocytes may traffic into the periphery or be deleted 1 day after MBP feeding. The increase in Foxp3<sup>+</sup> lymphocyte numbers on day 7 in the thymus most likely represents new, natural/induced Treg cells. Alternatively, another type of thymus-derived Treg cell may be involved in the induction of oral tolerance. Two major populations of  $\alpha\beta$  TCR Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> phenotype; and NK1.1, CD4<sup>-</sup> or <sup>+</sup>, CD8<sup>-</sup> phenotype) have been implicated in the induction of acquired thymic tolerance (7, 55, 56). Both are important sources of neonatal/natural "suppressor" cells and contribute to the prevention of organ-specific autoimmune disorders (57). Because we did not observe the presence of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells in the thymus after MBP feeding, it is possible that the NK1.1 regulatory population is responsible for the suppressive influence of the thymus in oral tolerance. We are currently investigating this possibility. Alternatively, it is possible that the thymus-derived Treg cells may not express Foxp3<sup>+</sup> but rather carry negative regulatory signaling molecules, including CTLA4 (data not shown), programmed death-1, or B and T lymphocyte attenuation. We observed that MBP-induced CD4<sup>+</sup>CD25<sup>+</sup> T cells significantly suppress the proliferative response of CD4<sup>+</sup>CD25<sup>-</sup> cells to MBP and MBP peptide NAc1-11 in vitro (Fig. 4). Our observations suggest that oral Ag-induced Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> Treg cells are at least partly of recent thymic origin.

Our data presented here shows that the thymus does not appear to be a site for clonal deletion of autoreactive T cells induced by the oral administration of autoantigen. The decreased expression of the Tg TCR in the thymus and lymph node was accompanied by an overall decrease in thymocyte number (data not shown). However, TUNEL analysis conducted either before or at the time of decreased numbers of thymocytes shows no differences in TUNEL staining between MBP-fed and vehicle-fed mice (Fig. 5A). However, the increased apoptotic autoreactive (CD4<sup>+</sup>V $\beta$ 8<sup>+</sup>) T cells in the periphery were related to the presence of the thymus and exposure to orally administered Ag (Fig. 5B). It is suggested that the thymus plays a role in induction of apoptosis of oral Ag-activated autoreactive T cells in the lymph node following MBP feeding. We reasoned that the fluctuation in cell numbers observed in the lymph node and thymus (data not shown) may be due to apoptosis in the lymph node and the export of cells out of the thymus in the latter instance.

We also investigated the notion that the thymus serves as a distribution point for naive T cells, and in its absence, T cell numbers are maintained through homeostatic proliferation. This results in the generation of memory T cells, which are difficult to tolerize (45-48). Tough et al. (58) reported that recent thymic emigrants to the lymph node and spleen in mice expressed a naive phenotype, i.e., CD45RB<sup>high</sup>CD44<sup>low</sup>CD62L<sup>high</sup> that was absent in thymectomized mice as demonstrated using BrdU labeling. In contrast, cells in the lymph node and spleen of thymectomized mice expressed a memory (CD45RB<sup>low</sup>CD44<sup>high</sup>CD62L<sup>low</sup>) phenotype. Rosa et al. (59) also reported that the thymus is important to the survival of the naive cell pool because such cells disappear as measured by

phenotype and function when the thymus is removed. Our previous studies, as well as the data presented here, show that thymectomy results in lymphopenia. However, we observed no evidence for homeostatic proliferation or for the generation of memory cells in thymectomized mice (Fig. 6).

In summary, the present study provides direct evidence that the thymus plays an essential role in the induction of oral tolerance using two different models. We show that the thymus is an important site for the development of regulatory cells following oral administration of Ag. Its role as a site for clonal deletion of autoreactive T cells or as a source for naive T cell distribution is less significant in oral tolerance. Understanding the roles of the thymus in the induction of tolerance pathways in EAE may provide new insights into immunotherapy for the treatment of MS.

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## Disclosures

The authors have no financial conflict of interest.

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