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Cutting Edge: Intrathymic Differentiation of Adaptive Foxp3+ Regulatory T Cells upon Peripheral Proinflammatory Immunization

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Thymocytes differentiate into CD4+ Foxp3+ regulatory T cells (T\(_R\)) upon interaction between their TCR and peptide–MHC II complexes locally expressed in the thymus. Conversion of naive CD4+ T cells into T\(_R\) can additionally take place in the periphery under non-inflammatory conditions of Ag encounter. In this study, making use of TCR transgenic models naturally devoid of Foxp3+ cells, we report de novo generation of T\(_R\) upon a single footpad injection of Ag mixed with a classic proinflammatory adjuvant. Abrupt T\(_R\) differentiation upon immunization occurred intrathymically and was essential for robust tolerance induction in a mouse model of spontaneous encephalomyelitis. This phenomenon could be attributed to a specific feature of thymocytes, which, in contrast to mature peripheral CD4+ T cells, were insensitive to the inhibitory effects of IL-6 on the induction of Foxp3 expression. Our findings uncover a pathway for T\(_R\) generation with major implications for immunity and tolerance induction. The Journal of Immunology, 2010, 185: 3829–3833.

Thymocytes expressing TCRs specific for self Ags presented in the thymus die through the process of negative selection (1) or differentiate into Foxp3+ regulatory T cells (T\(_R\)) (2). Autoreactive thymocytes escaping these selection events are exported to peripheral organs and tissues, where T\(_R\) control their activation and pathogenic potential (2). In addition to thymic production of “natural” T\(_R\), peripheral CD4+ T cells can differentiate into “adaptive” Foxp3+ cells in a TGF-β-dependent manner upon Ag encounter in the absence of inflammation in vivo (3, 4). For example, peripheral T\(_R\) conversion has been observed following administration of free Ag by osmotic pumps (5), by the oral route (6), or by specifically targeting the Ag to dendritic cells in the absence of adjuvants (7). Proinflammatory and/or effector Th cell cytokines, such as IL-6, IFN-γ, or IL-4, inhibit peripheral T\(_R\) conversion and promote instead the differentiation into IL-17–, IFN-γ–, or IL-4–producing effector Th cells (8–10).

Negative selection and T\(_R\) differentiation in the thymus are believed to be restricted to T cells interacting with Ags expressed locally, including many tissue-specific proteins ectopically expressed by medullary thymic epithelial cells (11, 12). However, recent studies indicate that abundant blood-borne Ags and peripheral Ag-loaded dendritic cells commonly reach the thymus (13, 14) and participate not only in negative selection but also in differentiation of Foxp3+ cells (15). Yet, it is not known whether intrathymic differentiation of T\(_R\) occurs following peripheral administration of Ag mixed with standard adjuvants and whether this process is affected by ongoing inflammation. Clarifying these issues is essential to determine the full dynamic and outcome of immune responses to self and non-self peripheral Ags.

In this study, using monoclonal TCR transgenic (Tg) mice naturally devoid of Foxp3+ cells, we show that Ags mixed with CFA and administered by a single footpad injection can reach the thymus and locally promote the differentiation of Ag-specific thymocytes into Foxp3+ cells, despite the inflammatory conditions. This event could be attributed to a specific feature of thymocytes, which, in contrast to peripheral mature CD4+ T cells, lack surface IL-6R expression and are thus refractory to the inhibitory effects of IL-6 on the differentiation of Foxp3+ cells. Our findings reveal a previously unrecognized mechanism by which the thymus may establish dominant tolerance to self and non-self Ags during the course of an immune response.

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Abbreviations used in this paper: BM, bone marrow; CD45SP cell, single-positive T cell; EAE, experimental autoimmune encephalomyelitis; LN, lymph node; MBP, monoclonal anti-myelin basic protein; pMBP, MBP peptide; Ptx, pertussis toxin; Tg, transgenic; TgTg, homozygous anti-MBP TCR transgenic mouse; TtR, regulatory T cell; T/R+, monoclonal anti-myelin basic protein TCR-specific transgenic RAG-deficient mouse; TtX, thymectomized; WT, wild-type.

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Materials and Methods

Mice

C57BL/10.PL, C57BL/10.PL-TgI-L, C57BL/10.PL RAG1−/−-MBP-TCR Tg (T/R−), C57BL/6, and Foxp3GFP reporter knock-in mice were bred at the Instituto Gulbenkian de Ciência Animal Facility. Foxp3null, IL-6−/−, IL-6+/-, and wild-type (WT) homozygote anti-MBP TCR Tg C57BL/10.PL mice were bred at the Skirball Institute Central Animal Facility, New York University Medical Center, New York, NY. Mouse experimental protocols were approved by the Institutional Ethical Committee and the Portuguese Veterinary General Division. Foxp3GFP knock-in mice (16) were kindly provided by A. Rudensky (University of Washington, Seattle, WA).

Immunizations, experimental autoimmune encephalomyelitis scoring, and chimera generation

For immunizations, mice received 100 μl (50 μl in each footpad) of peptide or protein emulsified in CFA (ready-made CFA; Difco/BD Biosciences, San Jose, CA). When indicated, pertussis toxin (PTX; List Biological Laboratories, Campbell, CA) was administrated i.v. in two doses of 200 ng at a 1-d interval. Experimental autoimmune encephalomyelitis (EAE) was monitored as previously described (17). For adoptive transfer, purified cells suspended in 100 μl PBS were injected into the retro-orbital plexus. For mixed bone marrow (BM) chimeras, recipient mice were lethally irradiated (900 rads) and constituted the following day with T cell-depleted BM cells.

Cell purification and analysis

Cell suspensions from spleen, blood, thymus, or lymph node (LN; popliteal, inguinal, axillary, mesenteric, and brachial) were incubated with a saturating amount of Fc-block (anti-CD16/CD32) before staining. Nuclear Foxp3 was detected according to the manufacturer’s (eBioscience, San Diego, CA) instructions. For intracellular cytokine staining, cells were stimulated for 4 h with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (500 ng/ml; Calbiochem, San Diego, CA). Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 2 h of stimulation. Data were acquired on a FACS-Calibur or Aria (BD Biosciences) and analyzed inside a lymphocyte gate with CellQuest (BD Biosciences) and Flowjo (Tree Star, Ashland, OR) software. Cell purification was performed using Aria or MoFlo high-speed cell sorters (DakoCytomation, Carpinteria, CA).

Cell culture

For Foxp3 induction assays, cells were plated at 2.5 × 10^4 cells/well in flat-bottom 96-well plates with 3 μg/ml plate-bound anti-CD3 mAb, 1 μg/ml soluble anti-CD28 mAb (eBioscience), ∼10 U/ml IL-2 (X63-IL-2 supernatant), 0.2 ng/ml TGF-β1, and 20 ng/ml IL-6 (R&D Systems, Minneapolis, MN). Cultures were set in triplicates in a final volume of 200 μl for 72 h. For Stat3 phosphorylation assays, cells were stimulated for 15 min with 100 ng/ml IL-6 and stained with anti- phospho Stat3-AlexaFluor 647 Ab (4/1-P-Stat3; BD Biosciences), according to the manufacturer’s instructions.

Statistical analysis

Statistical significance was determined using the two-tailed Student t test and the log rank test. A p value <0.05 was considered significant (p < 0.05; **p < 0.01; ***p < 0.001).

Results and Discussion

Immunization promotes tolerance induction through differentiation of Treg

Anti-myelin basic protein (MBP) TCR-specific Tg RAG-deficient mice (T/R−) or homozygotes for the Tg-TCR (TgTg) spontaneously develop severe progressive EAE by 2 mo of age and succumb in their third month of life (17). As these mice are naturally devoid of Foxp3+ cells, they offer an ideal system to test protocols that may induce the de novo generation of Treg. Administration of the nominal Ag together with IFA has been shown to prevent EAE occurrence in T/R− mice, presumably through the induction of T cell anergy (18). We first tested whether immunization protocols known to induce various degrees of inflammation would differently affect disease onset and progression in T/R− mice. A single footpad injection of 100 μg of the agonist N-terminal Ac1-17 MBP peptide (pMBP) mixed with IFA or CFA protected T/R− for at least 3 mo, whereas administration of CFA alone did not alter the course of the disease (Fig. 1A). Protection from EAE was associated with the emergence of Foxp3+ cells, readily detectable in PBLs as early as 6 d postinjection and undetectable in control animals (Fig. 1B, 1C). Foxp3+ cells reached ∼14% of CD4+ T cells by 6 wk postimmunization and remained at ≥4% for an additional 6–7 wk. Full EAE protection lasted 3 mo, after which immunized mice developed a chronic and mild disease that coincided with a significant decline in peripheral Foxp3+ cell frequency. Renewed injection of CFA-pMBP 3 mo after the first immunization prolonged protection to more than 7 mo of age. As CFA is a more potent adjuvant than IFA, it was selected to further analyze the function and origin of Foxp3+ cells generated upon proinflammatory immunization.

Foxp3+ cells emerging upon CFA-pMBP immunization were phenotypically and functionally bona fide Treg. About 60% of them expressed CD25, and most CD25+ cells were Foxp3+, GITR+, and CD103+. They efficiently suppressed the proliferation of conventional CD4+ CD25− cells in vitro and displayed regulatory activity in vivo, as shown by adoptive transfer experiments (Supplemental Fig. 1). Finally, immunization did not prevent or delay severe EAE in TgTg bearing a Foxp3 null mutation (Foxp3scurfy) (Fig. 1A), formally demonstrating that dominant tolerance through de novo induction of Foxp3+ Treg, and not T cell anergy (18), is the mechanism of disease prevention following immunization of T/R− mice.

![FIGURE 1.](http://www.jimmunol.org/)

CFA-peptide immunization promotes tolerance mediated by de novo generated Foxp3+ Treg. A. For each protocol, treatment of mice was initiated at 1 mo of age and mice were scored for EAE at least twice per week, until 3 mo of age. Data represent pooled results of two to eight independent experiments. B and C, T/R− mice 1 mo old were immunized with CFA-PBS, CFA-pMBP (B) or with IFA-pMBP (C). Percentage of Foxp3+ cells in a CD4+ cell gate in PBLs (mean ± SD; n = 3 per group; ○, ‡) and cumulative EAE incidence (●, ○) along time. D, TgTg IL-6+/− or IL-6−/− mice 1 mo-old were immunized with CFA-pMBP into the footpad and their draining LNs analyzed 4 or 14 d later. Representative histogram for Foxp3 and percentage of Foxp3+ cells measured in a CD4+ cell gate. n = 2–6 in each group. Data are representative of two independent experiments.
Concomitant differentiation of effect Th cells and Tr upon proinflammatory immunization

The above observations needed to be reconciled with the notion that CFA is an adjuvant used to induce EAE in WT mice. Our immunization regimen promoted T cell expansion, activation, and differentiation into IFN-γ- and IL-17-producing cells (Supplemental Fig. 2), all readily detectable by day 4 postinjection and concomitant with Foxp3+ cell emergence. Protocols for efficient induction of EAE in WT animals commonly rely on the co-administration of PTX and the immunogen. Consistently, administration of PTX abrogated the protective effect of CFA-pMBP (Fig. 1A), amplified cellular expansion, increased the number of IFN-γ-producing cells, and reduced both Foxp3+ cell frequency and number (Supplemental Fig. 2). We conclude that whereas CFA-pMBP promotes Th1, Th17, and Tr differentiation, PTX inhibits the induction, migration, and/or expansion of Foxp3+ cells, a role reminiscent of its effect on Tr survival and function (19).

As expected, CFA administration also provoked rapid and vigorous production of innate cytokines, including IL-6 (not shown). Intriguingly, this cytokine has been shown to play a key role in preventing peripheral Tr conversion, notably upon CFA administration (8). Consistent with this notion, immunized IL-6−/− TgTg mice displayed a 3- to 5-fold increased frequency of peripheral Foxp3+ cells when compared with control IL-6+/− TgTg mice similarly treated (Fig. 1D). As unimmunized IL-6−/− and IL-6+/− TgTg mice were devoid of Foxp3+ cells, we conclude, as previously shown (8), that inflammation driven by IL-6 interfered with Tr conversion. Collectively, our results suggest that a subset of T cells in IL-6–competent mice is insensitive to the inhibitory effect of IL-6 and consequently can convert to Tr despite ongoing inflammation.

Peripheral immunization induces intrathymic differentiation of Ag-specific Tr

We next examined whether Foxp3+ cell differentiation in immunized Tr mice occurred in the thymus. Remarkably, kinetic analysis in Tr mice immunized with a single footpad injection of CFA-pMBP revealed that Foxp3+ cells represented ~12% of CD4+ single-positive T (CD4SP) cells in the thymus by day 3 postimmunization (Fig. 2A–C). Noteworthy, Foxp3+ cells were not detectable in LNs before day 4, excluding the possibility that thymic Foxp3+ cells were circulating peripherally differentiated Tr (Fig. 2A). Despite an increasing frequency of thymic Tr during 7 d postimmunization, the total number of Foxp3+ thymocytes was highest at day 3 and gradually decreased following the reduction in total CD4SP. In contrast, peripheral Foxp3+ cell frequency increased at least until day 14 postimmunization (Fig. 2B, 2C). Intrathymic differentiation of Tr following footpad immunization was Ag specific and not a singularity of anti-MBP Tg mice, or of small synthetic self-peptides, as similar results were obtained upon CFA-peptide or CFA-protein, but not CFA-PBS, administration to anti-OVA DO11.10 Rag1−/− TCR-Tg mice (Supplemental Figs. 3, 4). Together, these results demonstrate that not only blood-borne (15) but also peripheral Ags administered s.c. in a stable water-in-oil emulsion can enter the thymus and be presented locally to promote both deletion and Tr differentiation.

**FIGURE 2.** Peripheral proinflammatory immunization leads to abrupt Ag-specific Tr differentiation in the thymus. A–C, Tr mice 1 mo old were immunized with CFA-pMBP into the footpad and thymus, and LNs were analyzed at different time points. A, Representative dot plot for Foxp3 versus CD4+ analysis at different time points.

A: Percentage of CD4+ cells in a Foxp3+ gate from PBLs over time; Student t test. **p < 0.01, E and F; One group of 1-mo-old euthymic Tr mice was left untreated (V), n = 12, although both thymus (A; n = 7) and sham-PTX (B;k n = 5) were immunized with CFA-pMBP 3 d post surgery. E, Percentage of Foxp3+ cells in a CD4+ cell gate from PBLs over time; Student t test. F, Percentage of survival over time (p < 0.01, untreated versus TXT CFA-pMBP; p < 0.01, TXT CFA-pMBP versus sham CFA-pMBP; log rank test). **p < 0.01; ***p < 0.001.
We next ascertained that Ag-specific T<sub>R</sub> differentiate in the thymus following immunization of mice bearing a polyclonal repertoire of lymphocytes. BM from T<sup>R</sup> (Thy1.2<sup>+</sup>) and WT (Thy1.1<sup>+</sup>) mice were co-injected into lethally irradiated WT (Thy1.1<sup>+</sup>) animals. These mixed BM chimeras were immunized with CFA-pMBP at 2 mo postreconstitution when they contained 5–10% of Tg CD4<sup>SP</sup> cells. Analysis of their thymic 4 d postimmunization revealed both negative selection and T<sub>R</sub> differentiation of Ag-specific CD4<sup>SP</sup> thymocytes, as indicated by a 100-fold reduction in number and the emergence of ∼7% Foxp3<sup>+</sup> cells among Thy1.2<sup>+</sup> cells. Importantly, among the WT polyclonal Thy1.1<sup>+</sup> cells, neither thymocyte number nor Foxp3<sup>+</sup> cell frequency was affected by immunization (Fig. 2D). These results demonstrate that intrathymic T<sub>R</sub> differentiation upon peripheral proinflammatory immunization is restricted to Ag-specific T cells and can occur in the context of a normal polyclonal repertoire of lymphocytes.

To directly evaluate the contribution of the thymus to tolerance induction and to the accumulation of peripheral T<sub>R</sub> upon immunization, healthy 4-wk-old T<sup>R</sup> mice were thymectomized (TXT) and immunized with CFA-pMBP 3 d later. Strikingly, Foxp3<sup>+</sup> cells were undetectable in peripheral blood of TXT mice for the first 2 wk postimmunization. In addition, T<sub>R</sub> frequency was significantly lower in TXT than in euthymic mice at any time point of the 10-wk-long kinetic (Fig. 2E). These results are in agreement with our kinetic analysis above, indicating that early and efficient differentiation of T<sub>R</sub> is restricted to the thymus. Nevertheless, immunization delayed disease progression in TXT mice, possibly as a result of peripheral generation of functional Foxp3<sup>+</sup> cells. However, although all TXT T<sup>R</sup> mice succumbed to EAE by 30 wk after immunization, at this same time, euthymic animals were alive and only 40% of them showed signs of mild disease (score ≤2) (Fig. 2F). Together, our data demonstrate that the thymus, most likely owing to intrathymic T<sub>R</sub> differentiation, is essential for induction of potent and long-lasting tolerance.

**Immature CD4<sup>SP</sup> thymocytes are refractory to IL-6–mediated inhibition of Foxp3 induction**

Taken together, the above results indicated that intrathymic, in contrast to peripheral, T<sub>R</sub> differentiation is insensitive to the inhibitory effects of inflammation. We next tested whether these differences could be attributed to intrinsic features of developing T cells by performing in vitro T<sub>R</sub> differentiation assays. As expected, addition of IL-6 to T<sup>R</sup> peripheral CD4<sup>+</sup> T cells inhibited by ∼70% the generation of Foxp3<sup>+</sup> cells induced by TGF-β (Fig. 3A). However, this inhibition was significantly lower (∼40%) for CD4<sup>SP</sup> thymocytes. Similar results were obtained when testing WT CD4<sup>+</sup> CD8<sup>+</sup> Foxp3<sup>+</sup> cells isolated from Foxp3<sup>+</sup> knock-in mice (Fig. 3B, 3C). Strikingly, purified HSA<sup>high</sup> Foxp3<sup>+</sup> CD4<sup>SP</sup> thymocytes, purged of recirculating and more mature cells (see Ref. 16), were totally refractory to the inhibitory effect of IL-6. The sensitivity of each cell subset to IL-6 directly correlated with the level of both surface IL-6Rα expression (Fig. 3D) and proximal IL-6R signaling, determined by intracellular phosphorylated Stat3 detection upon IL-6 exposure (Fig. 3E). Together, these results indicate that newly formed T cells are insensitive to IL-6–mediated inhibition of Foxp3 induction owing to their very limited responsiveness to this cytokine. These findings are in agreement with the notion that maturation stage is one key factor controlling the predisposition of T cells toward T<sub>R</sub> differentiation (20) and provide a molecular basis for our observation that de novo generation of T<sub>R</sub> takes place in the thymus rather than in the periphery upon proinflammatory immunization.

In conclusion, we show that a single footpad injection of Ags mixed with a highly inflammatory adjuvant promotes the de novo generation of protective Ag-specific T<sub>R</sub>. This unexpected finding is explained by the demonstration that vigorous T<sub>R</sub> differentiation occurred intrathymically early following immunization. The latter phenomenon occurred despite the elevated levels of inflammatory mediators in circulation, notably IL-6, most likely owing to the specific resistance of newly formed T cells to the inhibitory effects of IL-6 on T<sub>R</sub> differentiation.

An essential role of the thymus in ensuring induced peripheral tolerance has been occasionally reported, and mostly attributed to a property of recent thymic emigrants (e.g., see Ref. 21). In view of our findings, it is tempting to speculate that immature T cells exported to the periphery may be the preferential precursors of peripherally differentiated T<sub>R</sub>. A recent study reported increased T<sub>R</sub> frequency in the thymus of
non-Tg mice upon induction of EAE by immunization, a phenomenon associated with elevated intrathymic IL-7 expression (22). These thymic TR allowed disease remission, suggesting that they were immunogen specific. Together, this work and ours indicate that intrathymic TR differentiation upon peripheral immunization is a robust event, because of both microenvironment modifications and, as we demonstrate in this study, intrinsic properties of thymocytes.

Tolerance induction to peripheral Ags that gain access to the thymus may be essential not only to purge the repertoire of self-reactive T cells specific to Ags that are not expressed by thymic APCs (13) but also to broaden the repertoire of Foxp3+ TR, assuring robust dominant tolerance to peripheral tissues. In addition, our evidence that intrathymic differentiation of TR can take place under strong and systemic inflammatory conditions uncover a potential mechanism for the emergence of pathogen-specific TR during infections, provided pathogen-derived Ags gain access to the thymus. Noteworthy is that microbe-specific TR have been shown to be essential for protection against a secondary challenge to the microbe (23). Thus, specific TR differentiation upon Ag access to the thymus may play an essential role not only in the establishment of self-tolerance but also in immunity to reinfection. Finally, our observations have major implications for the design of vaccines for preventing or treating infectious diseases, cancer, or autoimmunity.

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Disclosures
The authors have no financial conflicts of interest.

References
Legends to supplemental data

Supplemental Figure 1. Foxp3+ cells emerging upon CFA-pMBP immunization resemble *bona fide* Treg. Upper panel: 1-month-old T/R- mice were immunized with CFA-pMBP. LN cells were analyzed 14 days after immunization. Representative dot-plot for Foxp3 versus CD25 within CD4+ cells and histograms for GITR and CD103 within CD4+ CD25+ cells (solid line) or CD4+ CD25- cells (dotted line). Middle panel: CD4+ CD25+ cells from CFA-pMBP-treated T/R- mice 14 days post-immunization (open squares) or from naïve WT mice (filled circles) were plated at different ratios with CD4+ CD25- cells from WT mice at 2.5x10^4 cell/well in U-bottom 96-well plates for 72 h together with 10^5 irradiated (30 Gy) splenocytes as APC and 1 µg/ml anti-CD3 mAb (clone 145.2C11; home-made). Cultures were set in triplicates in a final volume of 200 µl. Proliferation was monitored by addition of [3H] thymidine for the last 6 h of culture. Lower panels: RAG1−/− mice received 2.5x10^5 CD4+ cells isolated from either 1-month-old T/R- mice (naive, n=7), or from CFA-pMBP-immunized T/R- mice 60 days after immunization (CFA-pMBP, n=3) or 2.5x10^5 CD4+ cells of each population (Co-T, n=5). EAE score of each mouse 40 days after transfer (p<0.05: naïve versus CFA-pMBP, and p<0.01: naïve versus Co-T; Pearson’s Chi-square exact test). Right, Percentage of survival over time (p<0.05: naïve versus CFA-pMBP, and p<0.01: naïve versus Co-T; logrank test).

Supplemental Figure 2. Simultaneous differentiation of T_R and effector T helper cells upon proinflammatory immunization. 1-month-old T/R- mice were immunized with CFA-pMBP (white bars) or CFA-pMBP and PTX (black bars, PTX on day 0 and 2) and their draining LN analyzed at different time points after immunization. A, Representative dot-plot for CD25 versus Foxp3 and IL-17 versus IFN-γ within CD4+
T cells. 

**B.** Number of total CD4+ T cells and percentage and number of Foxp3+ cells, IFN-γ- and IL-17-producing cells measured in a CD4+ gate. The dotted line corresponds to untreated mice. Data are representative of three independent experiments with n=3 per group.

**Supplemental Figure 3.** No noticeable difference between untreated and CFA-PBS-injected mice. 1-month-old T/R− mice were either untreated or immunized with CFA-PBS or CFA-pMBP into the footpad and thymus and LN were analyzed 4 days later. 

A. Representative dot-plots for CD4 versus CD8 inside a live lymphocyte gate, for CD4 versus Foxp3 inside a CD4+ CD8− gate from the thymus or from draining LN and for IL-17 versus IFN-γ from draining LN. 

**B.** Number of total thymocytes, Foxp3+ thymocytes or Foxp3+ LN cells.

**Supplemental Figure 4.** Intrathymic Foxp3+ cell differentiation in DO11.10 RAG1−/− mice upon CFA-OVA peptide or whole OVA protein immunization. 6-week-old DO11.10 RAG1−/− were either untreated or injected with CFA mixed with PBS or 100 μg of OVA peptide or 10 mg of OVA protein into the footpad and analyzed 4 days later. Percentage (upper panel) and number (lower panel) of Foxp3+ cells among CD4+ CD8− thymocytes or LN cells, n=2-6 in each group.
Supplemental Figure 1

- Graph showing the relationship between CD25 and GITR.
- Bar graph illustrating the percentage of survival over weeks after transfer.
- Line graph depicting EAE scores over weeks.
Supplemental Figure 3

A

Thymus

CD4

Untreated  CFA-PBS  CFA-pMBP

CD8

CD4

CD8

LN

Foxp3

IFN-γ

IL-17

B

Number of Thymocytes (x10⁶)

Number of Foxp3⁺ thymocytes (x10⁵)

Number of Foxp3⁺ LN cells (x10⁵)

Untreated  CFA  PBS  CFA  pMBP

Untreated  CFA  PBS  CFA  pMBP

Untreated  CFA  PBS  CFA  pMBP
Supplemental Figure 4

The figure shows the percentage of Foxp3+ cells in LN and Thymus after treatment with different substances:

- **Untreated**
- **CFA-PBS**
- **CFA-OVA peptide**
- **CFA-OVA protein**

The x-axis represents the number of Foxp3+ cells (×10^5), and the y-axis represents the percentage of Foxp3+ cells. The data is indicated by bars and error bars for each condition.