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Thymic Regulation of Autoimmune Disease by Accelerated Differentiation of Foxp3+ Regulatory T Cells through IL-7 Signaling Pathway

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The exact role of adult thymus in autoimmune disease state is poorly understood. We show here that thymus regulated experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, as evidenced by loss of spontaneous recovery in thymectomized EAE mice. There was progressive enrichment for CD4 single-positive Foxp3+ regulatory T cells in thymocytes during the course of EAE and they suppressed the disease when adoptively transferred. Thymus was shown to undergo an active process characterized by accelerated differentiation and proliferation of regulatory T (Treg) cells through a mechanism involving increased expression of IL-7 in stromal cells and dynamic expression of IL-7 receptor in thymic Treg cells. This process preceded the development of Foxp3+ regulatory T cells in thymus and peripheral immune system in mounting as well as regulating autoimmune responses. The study reveals a novel role of thymus in self-regulation of autoimmune condition. The Journal of Immunology, 2009, 183: 6135–6144.

As a central lymphoid organ, thymus is known for its role in T cell development among other newly discovered functions (1–4). The central functions of thymus are critical to immune tolerance under normal or physiologic conditions through mechanism such as clonal deletion or clonal anergy of self-reactive T cells (5–8). More recent studies indicated that thymus-derived CD4+CD25+Foxp3+ regulatory T (Treg) cells play an important role in peripheral tolerance of autoreactive T cells (9–14). Multiple signals, such as TCR and costimulatory molecules CD28, are required for the thymic development of Treg cells (15–18). Common γ-chain (γc) cytokines, IL-2, and, to a lesser extent, IL-7 and IL-15 are required for thymic Treg cell development. Gene knockout of γc in mice leads to complete absence of Foxp3+ Treg cells in thymus and peripheral immune system (19–25). In this process, transcription factor STAT5 is critically involved in the activation of γc cytokine receptors and is shown to bind to Foxp3 promoter and the Foxp3-CNS2 element (26, 27). STAT5 conditional knockout in double-positive thymocytes results in a remarkable reduction in CD4 single-positive (SP) Foxp3+ thymocytes (26, 28).

However, it remains elusive as to whether and how thymus plays a role in an autoimmune disease state with respect to thymic differentiation and output of Treg cells. There are some preliminary indications suggesting that thymectomy in adult patients with myasthenia gravis is associated with increased risks for the development of systemic autoimmune conditions, supporting the potential role of thymus in immune regulation (29). In addition to its suggested role in spontaneous remission in rat experimental autoimmune encephalomyelitis (EAE) (30), thymus is potentially responsible for deficit in the function of the peripheral Treg pool in patients with multiple sclerosis (31). Thus, it is of importance to elucidate the exact regulatory role and underlying mechanism of thymus in autoimmune disease state in the context of interplay between thymus and the peripheral immune system in mounting as well as regulating autoimmune responses.

This study was undertaken to investigate the potential regulatory role of thymus in autoimmune disease, using EAE as an animal model for MS. The study was prompted by our initial observation that EAE worsened as disease severity was sustained in thymectomized mice. We hypothesized that autoimmune attack triggered widespread and profound effects that potentially involved the thymus, beyond the peripheral immune system, to regulate autoimmune response. In this study, we systemically analyzed the regulatory component of thymus with respect to differentiation and output of CD4SP Foxp3+ Treg cells during the course of EAE and its impact in the numeric and functional outcome of the peripheral Treg pool in the disease. The emphasis was given to addressing the thymic mechanism whereby cytokine milieu characteristic of EAE induced specific changes in differentiation and output of Treg cells. The study provides new evidence supporting the critical role of thymus in the regulation of autoimmune T cell responses and clinical course of autoimmune conditions.

Materials and Methods

EAE and thymectomy

C57BL/6 (B6) and EGFP transgenic mice (C57BL/6 background) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. EAE was induced by myelin oligodendrocyte glycoprotein (MOG)35-55 immunization using a standard protocol (32). Clinical signs of
EAE were assessed daily using a scoring system described previously (32). For adoptive transfer, 2 × 10^7 thymocytes and 2 × 10^7 CD4SP thymocytes or their CD25^+ subset (CD4SP CD25^+ thymocytes), which were obtained from EAE mice on day 24 postimmunization (p.i.), were injected i.v. into thymectomized mice on day 7 p.i., respectively. PBS injection was used as a control. For the cytokine blocking assay, EAE mice were i.p. injected with 200 µg of polyclonal IL-7 Ab (R&D Systems) every 48 h for four doses, starting from day 14 to day 21 p.i. For thymectomy, upper median sternotomy was performed on anesthetized mice (6 wk of age), and both lobes of the thymus were removed. The thoracic cage was sutured closed and skin was secured. Control mice underwent the same procedure with thymus left intact. Mice were allowed to recover for 7 days before experiments. Mice were kept in a conventional, pathogen-free facility at the Institute of Health Sciences. All animal procedures were approved by the Institutional Review Board of the Institute of Health Sciences.

**Histology**

Tissues were removed from EAE mice on days 16, 24, and 31 and immediately fixed in 4% paraformaldehyde. Paraffin-embedded 5- to 10-µm spinal cord sections were stained with Luxol fast blue and H&E.

**Flow cytometry**

For the surface marker staining, cells were incubated with fluorochrome-conjugated Abs to the indicated markers (eBioscience) at the recommended dilution or isotype control Abs for 30 min on ice. For intracellular staining of Foxp3 and Ki67, cells were fixed and permeabilized with fixation/permeabilization buffer (eBioscience) for 1 h and then stained with anti-Foxp3 mAb (eBioscience) at 4°C and anti-Ki67 mAb (BD Biosciences) at room temperature in permeabilization buffer (eBioscience) for 30 min. For pSTAT5 staining, cells were fixed with 2% paraformaldehyde for 10 min at 37°C and permeabilized with 90% methanol for 30 min on ice and then stained with pSTAT5 (BD Biosciences) and CD25 (eBioscience) mAbs. For the cell apoptosis assay, cells were stained using the annexin V/PI apoptosis detection kit (BD Biosciences). Stained cells were analyzed by a FACSArria instrument (BD Biosciences).

**Cell purification**

Thymic stromal cells were isolated as described previously (33). In brief, thymic fragments from B6 mice were subjected to a series of digestions in 0.125% (v/v) collagenase/disappease (Roche) and 0.1% (w/v) DNase I (Sigma-Aldrich) in RPMI 1640 medium at 37°C. Cell suspension was incubated with anti-CD90.2 microbeads (Miltenyi Biotec) to remove T cells. The purity of the CD90.2^− cell fraction was always >95% with exclusion of dead cells using propidium iodide. Infiltrating mononuclear cells from spinal cord were purified using a Percoll gradient (70%/37%) (PerkinElmer) (32). For the suppression assay or TCR excision circle (TREC) analysis, CD4^+CD25^− T cells or CD4^+CD25^+ T cells were purified using the CD4^+CD25^+ regulatory T cell isolation kit (Miltenyi Biotech). For thymocyte subsets, thymic single-cell suspension was first depleted of CD8^+ T cells using the mouse CD8 selection kit (StemCell Technologies) and then MACS-sorted using CD4 microbeads (Miltenyi Biotec) or FACS-sorted using PE-anti-CD4 and allophycocyanin-anti-CD25 by MoFlo XDP cell sorter (Dako). The purity of the CD4^+CD25^− or CD4^+CD25^+ T cell fractions was always >95%. The purified CD4SP CD25^+ or CD4^+CD25^+ Treg cells were >90% Foxp3^+.

**Proliferation and suppression assays**

For the suppression assay, freshly isolated splenic CD4^+CD25^− T cells from sham surgery mice were used as responder (4 × 10^4 per well) with 2 µg/ml Con A (Sigma-Aldrich) and 8 × 10^4 irradiated (1500 rad) syngeneic splenic APCs from sham surgery mice (as responder or presence of CD4^+CD25^+ T cells or CD4SP CD25^+ thymocytes at a density of 4 × 10^5/well). During the last 16 h, cells were pulsed with 1 µCi of [3H]thymidine (PerkinElmer) before harvest. [3H]thymidine incorporation was determined using a beta counter (PerkinElmer).

**Cytokine stimulation assay**

CD-SP CD25^+ or CD4SP CD25^+ thymocytes (1 × 10^5/well) were stimulated with mlmL-7 (5 ng/ml; R&D Systems) for 72 h to determine cell proliferation or for 1 h to detect phospho-STAT5 (pSTAT5) levels by FACS staining as described above. For the STAT5 inhibitor assay, CD4SP CD25^+ and CD4SP CD25^+ thymocytes from EAE mice on day 24 p.i. were incubated with or without 100 µM STAT5 inhibitor (Calbiochem) in the presence of 5 ng/ml IL-7 for 72 h to determine cell proliferation. CD4SP thymocytes from naïve mice were stimulated with rmTNFα, IL-6, IL-12, IL-1β, or IFN-γ at 25 ng/ml (R&D Systems) for 24 h to analyze CD127 expression or for 48 h to determine Foxp3 expression by flow cytometry.

**BrdU incorporation**

Mice were injected i.p. with 200 µl of BrdU (3 mg/ml; Sigma-Aldrich) every 12 h for 4 days. Thymocytes were surface stained with anti-CD4, anti-CD8, and then fixed. Cells were incubated in DNsase solution (300 µg/ml) for 1 h at 37°C and stained with FITC-anti-BrdU (BD Biosciences) and allophycocyanin-anti-Foxp3 (eBioscience) for 20 min at room temperature before FACS analysis.

**Thymic reentry assay**

CD4^+CD25^− and CD4^+CD25^+ T cells were purified from spleenocytes of EAE EGFP transgenic mice on day 16 p.i. The resulting cells (2 × 10^6 each population) were mixed and transferred into EAE wild-type (WT) mice on day 16 p.i. After 4 days, thymocytes, spleenocytes, and CNS-infiltrating cells from recipients were analyzed for Foxp3 expression by FACS.

**Quantitative real-time RT-PCR**

Total RNA was isolated from cell pellets using a RNeasy Mini Kit (Qiagen) and first-strand cDNA was synthesized using a Sensiscript RT Kit (Qiagen) according to the manufacturer’s instructions. mRNA expression was determined by real-time PCR using SYBR Green Master mix under standard thermocycler conditions (Applied Biosystems). Data were collected and quantitatively analyzed on an ABI Prism 7900 sequence detection system (Applied Biosystems). Sequences of PCR primer pairs were: B-actin (internal control), forward 5′-TGTCACCTTCCAGCA GAGT-3′ and reverse 5′-AGCTCAGTAACAGTCCGCCCTA-3′; IFN-γ, forward 5′-TACAGCAGTGCCCTGAC-3′ and reverse 5′-TGTCCTCC GCTGTAGACTCTG-3′; IL2, forward 5′-GGCTGTACCATCTGTA G-3′ and reverse 5′-TTCCTGTGATTTCGCAAATTC-3′; IL15, forward 5′-CATCCTACCTGTCATCTTGTT-3′ and reverse 5′- CATCTATCCAGTGGCCTCTGTT-3′.

**TREC assay**

TREC assay was performed as described (34). Cell lysates were prepared by incubation in the proteinase K solution at 55°C for 2 h, followed by inactivation at 95°C for 15 min. Lysate from 20,000 cells was subjected to real-time quantitative PCR. Sequences of PCR primer pairs were: mRoRec, 5′-GGGCGACAGACGCTGTG-3′; ϕIA, 5′-GGAGGTTTTTGTAAAG GTGCTCA-3′; ϕIB, forward 5′-TACAGAYGACCTGCAC-3′ and reverse 5′-CAGCAGTGGCAACAGAAGCG-3′; ϕID, forward 5′-GACCTACCATCTGTA G-3′ and reverse 5′-TTCCTGTGATTTCGCAAATTC-3′; ϕID, forward 5′-CATCCTACCTGTCATCTTGTT-3′ and reverse 5′- CATCTATCCAGTGGCCTCTGTT-3′.

**Statistics**

One-way ANOVA, where applicable, was performed to determine whether a value of p < 0.05 was considered statistically significant.

**Results**

Role of thymic Treg cells in spontaneous EAE recovery

The functional role of thymus in the course of EAE was first examined in thymectomized mice compared with sham surgery controls. As shown in Fig. 1A, control mice developed a typical course of EAE characterized by a disease onset at day 7 p.i., rapidly reaching the peak of severity on day 16 and followed by a characteristic recovery phase (day 16 to day 24). In contrast, the spontaneous recovery phase was not evident when EAE mice had been thymectomized. Consistent with the observed clinical score was severe inflammation and demyelination in affected spinal cord in thymectomized EAE mice compared with that of sham surgery controls.
EAE mice (Fig. 1B). At the level of thymus, total thymocytes isolated from EAE mice at recovery (day 24) were found to exert significant regulatory properties when adoptively transferred to thymectomized EAE mice on day 7 p.i. (Fig. 1C). The resulting CD4SP T cell subsets were purified from thymus of EAE mice on day 24 and were found to exhibit similar regulatory properties as those of total thymocytes (Fig. 1D). The observed regulatory effect was attributable to CD4SP CD25− T cells, as the effect was abolished when CD4SP CD25− T cells were depleted from the CD4SP T cell population (Fig. 1D).

Role of thymic Treg cells in spontaneous recovery of EAE

At the level of the peripheral immune system, in sham surgery controls, the percentage of splenic Treg cells was not significantly altered before day 16 and progressively increased from day 16 through day 24, at which time period the level of CD4+ CD25− Foxp3+ Treg cells failed to rise in thymectomized EAE mice (Fig. 2A). In parallel, there was significantly impaired regulatory function of purified splenic T cells from thymectomized EAE mice (Fig. 2B). It was evident that the impaired function was directly attributable to decreased levels of newly emigrant Treg cells as determined by reduced frequency of TRECs in purified splenic CD4+ CD25− Treg cells from thymectomized EAE mice during the recovery phase as compared with those from control mice (Fig. 2C).

We further compared the thymic output of T cells between Treg and effector T (Teff) cell populations by quantitatively measuring the TREC number in purified CD4+ CD25− and CD4+ CD25+ T cells. As shown in Fig. 2D, there was a progressive increase in absolute TREC number in both Treg cell and Teff cell subsets in control EAE mice between day 16 and day 24 as opposed to thymectomized EAE mice. We compared the rate of thymic output between Treg and Teff cells by measuring the ratio of the difference in TREC number during EAE recovery over that at baseline in the respective cell populations. Thymic output of Treg cells significantly exceeded that of Teff cells by nearly 5-fold (10.8 vs 2.3, Table I). Additionally, the proliferation rate of Treg cells was significantly higher than that of Teff cells in control EAE mice (day16 to day 24) as measured by Ki67 staining (Fig. 2E). The analysis of both TREC number and the proliferation rate seemingly correlated with the increased percentage of Treg cells but not Teff cells in CD4+ splenic T cells as described above. Consistently, there were reduced levels and impaired regulatory function

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**FIGURE 1.** Role of thymic Treg cells in spontaneous recovery of EAE. A. Sham surgery and thymectomized mice were immunized with MOG35-55. B. Spinal cord sections from sham surgery and thymectomized EAE mice on days 16, 24, and 31 p.i. were stained for degree of demyelination by Luxol fast blue and for inflammation by H&E. Original magnification, ×100. C. Total thymocytes purified from EAE mice on day 24 p.i. were adoptively transferred to thymectomized EAE mice (2 × 10⁶ cells/mouse) on day 7 p.i. D. Two million CD4SP thymocytes or their CD25− subpopulation was purified from EAE mice on day 24 p.i. and transferred to thymectomized mice as in C. Thymectomized mice given PBS were included as a reference. Data are mean clinical scores ± SEM from three separate experiments with similar results in A, C, and D, or are shown as representative images of four experiments in B. *, p < 0.05 and **, p < 0.01, t test.
of CD4<sup>+</sup>CD25<sup>+</sup> T cells derived from spinal cord tissue of thymectomized EAE mice as compared with those of control EAE mice (Fig. 3). Thus, these data indicate that thymic regulatory T cells play an important role in replenishing the peripheral Treg pool, which has direct functional consequences in the clinical course of EAE.

Active enrichment and output of thymic Treg cells in EAE recovery

To understand the role of thymic regulatory mechanism in EAE, we characterized specific changes in a CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell population in relationship to the disease course. Flow cytometric analysis of thymic T cells isolated at peak (day 16) of EAE and recovery (day 24), as compared with those at baseline (day 0), showed that the percentage of Foxp3<sup>+</sup> T cells in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells increased progressively from 2.9 ± 0.2% at baseline to 8.3 ± 0.7% at peak and, more profoundly, to 15.5 ± 1.1% at EAE recovery (Fig. 4A). Consistent with the finding described above was the increased absolute number of thymic CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in EAE recovery (2.6 ± 0.5 × 10<sup>5</sup> vs 4.0 ± 0.4 × 10<sup>5</sup>, p < 0.05). Taken together, the data indicate rapid enrichment of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in total thymocytes during the recovery phase.
Furthermore, as illustrated in Fig. 4B, thymic CD4SP Foxp3+ T cells during EAE recovery underwent a characteristic phenotypic shift from Qa2lowCD24high seen at baseline to predominantly Qa2highCD24low, a combined phenotype consistent with thymocyte maturation and thymic output (35, 36). Importantly, these thymic CD4SP CD25+ T cells isolated at EAE recovery exhibited marked regulatory function comparable to that of matched splenic CD4+CD25+ T cells or baseline thymic CD4SP CD25+ T cells (Fig. 4C). These findings confirm that thymus underwent an active process leading to maturation and enrichment of functional CD4SP Foxp3+ T cells during the recovery phase of EAE.

Cellular mechanism underlying active enrichment of thymic Treg cells in EAE

We further investigated whether enrichment of thymic Treg cells during EAE recovery was due to enhanced differentiation and proliferation or some other mechanisms, such as thymic reentry of peripheral CD4+Foxp3+ T cells or reduced apoptosis relative to CD4SP Foxp3+ T cells. As shown in Fig. 5A, there was markedly elevated proliferation of CD4SP Foxp3+ thymocytes in EAE recovery, as evidenced by increased levels of BrdU incorporation in CD4SP Foxp3+ thymocytes in EAE mice compared with those in nonimmunized controls (18.2 ± 1.6% vs 8.5 ± 1.0%, p < 0.01). In contrast, the proliferation rate did not differ significantly in CD4SP Foxp3+ T cells between the two groups (32.3 ± 0.1% vs 32.1 ± 2.5%). Moreover, there were no significant differences in the levels of apoptotic cells (percentage annexin V+ cells) among the CD4SP CD25+ and CD4SP CD25+ populations in the same timeframe (Fig. 5B), discounting differential apoptosis as an explanation for the enrichment of thymic Treg cells. To rule out the possibility of thymic reentry, we transferred equal numbers of CD4−CD25− and CD4+CD25+ splenocytes from EAE mice of the EGFP transgenic background into WT EAE recipients. Subsequent flow cytometric analysis confirmed that although EGFP+Foxp3+ and EGFP−Foxp3+ T cells could be detected in the spleen and spinal cord of recipient mice, EGFP+Foxp3+ T cells were not found in the thymus, excluding thymic reentry of peripheral Treg cells as a possibility (Fig. 5C). Collectively, the data are indicative of increased differentiation or proliferation of thymic CD4SP Foxp3+ T cells during EAE recovery.

Table I. Thymic output of Treg and Teff cells

<table>
<thead>
<tr>
<th></th>
<th>Baseline (day 0 vs day 8)</th>
<th>EAE Recovery (day 16 vs day 24)</th>
<th>Rate of ΔTREC (baseline vs recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treg cells</td>
<td>3.37 ± 0.29</td>
<td>0.87 ± 0.06</td>
<td>10.8</td>
</tr>
<tr>
<td>(TREC × 10⁴)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTREC × 10⁴</td>
<td>0.99</td>
<td>0.97</td>
<td>2.3</td>
</tr>
<tr>
<td>Treg cells</td>
<td>47.4 ± 6.7</td>
<td>14.1 ± 2.1</td>
<td>2.3</td>
</tr>
<tr>
<td>(TREC × 10⁴)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTREC × 10⁴</td>
<td>3.5 ± 1.9</td>
<td>32.2 ± 4.9</td>
<td>18.1</td>
</tr>
</tbody>
</table>

*Treg cells (Treg) and CD4+CD25+ T cells (Teff) were obtained from euthymic naive mice (day 0) and thymectomized naive mice at day 8 after surgery (baseline) or EAE mice on days 16 and 24 p.i. (EAE recovery) and subjected to TREC analysis. The thymic output was calculated as the ΔTREC number.

*The rate of ΔTREC is expressed as the increased thymic output during EAE recovery over that of baseline.
Role of IL-7 in the enrichment of thymic Treg cells in EAE

We hypothesized that the cytokine milieu characteristically associated with EAE was attributable to accelerated proliferation and differentiation of thymic Treg cells. As IL-2, IL-7, and IL-15 are the key players in thymic Treg cell differentiation and development (19–25), we first examined their potential involvement in this process by measuring the expression levels in thymocytes or thymic stromal cells derived from EAE mice. Only the level of II7 was significantly elevated as detected in thymic stromal cells isolated from EAE mice at peak of EAE and recovery compared with that seen in baseline control mice (Fig. 6A). The functional role of IL-7 in sustaining in vivo proliferation of CD4SP Foxp3+ and CD4SP Foxp3− thymic T cells was demonstrated in BrdU incorporation assay in EAE mice treated with IL-7 blocking Ab. As shown in Fig. 6B, the percentage of BrdU+ T cells dropped in the CD4SP Foxp3+ T cell population from 15% to 9%, and it did so at a similar rate in CD4SP Foxp3− cells from 30% to 18% during the recovery phase. Furthermore, there were significantly decreased TREC frequency and total TREC number in purified splenic Treg cells from EAE mice treated with IL-7 Ab as compared with those in control mice (Fig. 6C), which was not accounted for by their proliferation rate (percentage Ki67+, 24.2 ± 0.8% vs 24.3 ± 0.6%, p > 0.05). Consistently, the regulatory function of Treg cells was found to be impaired in Ab-treated EAE mice (Fig. 6D). The findings collectively suggest that the increased thymic proliferation of IL-7 in the course of EAE is responsible for the enrichment and output of thymic Treg cells and consequently the functional outcome of the peripheral Treg pool.

Preferential response of CD4SP Foxp3+ thymocytes in relationship to differential IL-7R expression

We further addressed whether the preferential responses of thymic CD4SP Foxp3+ cells to IL-7 over CD4SP Foxp3− T cells were associated with the dynamic expression of IL-7 receptor, whose expression was influenced by proinflammatory cytokine milieu characteristic of EAE. Toward this end, IL-7 receptor α-chain (CD127) expression was analyzed in thymic CD4SP Foxp3+ and CD4SP Foxp3− cell populations during the course of EAE. As illustrated in Fig. 7A, in CD4SP Foxp3+ cells, CD127 expression was low at baseline and markedly increased at EAE peak and recovery (12% at baseline vs 52% and 64%, respectively), while it did not change significantly in CD4SP Foxp3− T cells. Purified CD4SP CD25+ and CD4SP CD25− T cells were stimulated by exogenous IL-7 at a predetermined concentration. Using STAT5 phosphorylation as readout for IL-7 signaling, we found that thymic Treg cells isolated from EAE recovery mice were significantly more responsive to IL-7 than those derived at baseline (Fig. 7B). Moreover, the IL-7-dependent proliferation of CD4SP CD25+ T cells was significantly increased at EAE recovery compared with that at baseline or of CD4SP CD25− T cells at recovery and could be inhibited by treatment with a STAT5 inhibitor (Fig. 7, C and D). We further examined the possibility that the preferential expression of CD127 in thymic CD4SP Foxp3+ T cells in the course of EAE was associated with proinflammatory cytokines induced in EAE. Among the proinflammatory cytokines characteristic of EAE, only TNF-α significantly enhanced CD127 expression in CD4SP Foxp3+ and CD4SP Foxp3− T cells (Fig. 8A). Consistently, TNF-α, together with IL-7, markedly promoted the percentage and proliferation of CD4SP Foxp3+ T cells as seen in Fig. 8, B and C. Thus, the results indicate that preferential proliferation/expansion and enrichment of thymic Treg cells in EAE is associated with dynamic expression of IL-7 and IL-7 receptor in susceptible CD4SP Foxp3+ T cells.

Discussion

Thymus has been considered traditionally as the central immune organ/site for T cell development and maturation (37, 38). The role
of adult thymus in autoimmune disease process is not well understood. In this study, we provide compelling new evidence indicating that thymus is actively involved in regulating autoimmune responses and markedly alters the clinical course of EAE. Importantly, thymus appears to affect the clinical outcome of EAE after the disease is established, as evidenced by the loss of spontaneous recovery in thymectomized EAE mice and by marked regulatory properties of thymocytes isolated from EAE mice at a time window preceding EAE recovery. The observed thymic changes are specifically induced by the autoantigen. This is supported by the observations that the same adjuvant or pertussis toxin treatment alone does not have similar effects and

![FIGURE 6. Role of IL-7 in the enrichment of thymic Treg cells.](image)

A. IL7 and IL15 mRNA abundance were assessed by real-time PCR analysis of sorted Thy1.2+ thymic stromal cells. IL2 mRNA level was analyzed by real-time PCR in total thymocytes. B. Flow cytometry of thymocytes from nonimmunized and EAE mice treated with IL-7 Ab or PBS (starting from day 14 to day 21 p.i.). Nonimmunized mice were injected with BrdU i.p. for 4 days, and EAE mice were treated with BrdU i.p for 4 days (starting from day 17 to day 21 p.i.; Recovery). Data represent BrdU+ cells among CD4SP Foxp3+ or CD4SP Foxp3 population. C. TREC frequency (left) and TREC number (right) of sorted CD4+CD25+ splenocytes from PBS and IL-7 Ab-treated mice as in B were measured by real-time PCR. D. CD4+CD25+CD25- regulatory T cells from indicated PBS and IL-7 Ab-treated mice as suppressor were cocultured with Con A and APC. Data are presented as the mean cpm ± SEM of triplicate cultures. *, p < 0.05 and **, p < 0.01, t test. Data are representative of three separate experiments with at least three mice per group.

![FIGURE 7. Preferential responses of CD4SP Foxp3+ thymocytes in relation to IL-7R expression.](image)

A. Flow cytometry of the expression of CD127 by CD4SP Foxp3+ and CD4SP Foxp3- thymocytes. The shaded area and solid line represent isotype control and CD127, respectively. B. Purified cells were assessed for phospho-STAT5 stimulated with IL-7. The shaded area and solid line represent unstimulated and stimulated cells, respectively. C. Proliferation of CD4SP CD25+ (CD25+) and CD4SP CD25- (CD25-) from EAE mice on days 0 and 24 p.i. were assessed by [3H]thymidine incorporation stimulated with medium and IL-7 for 72 h. Data represent the mean cpm ± s.e.m. of triplicates. D. Proliferation of indicated cells from EAE mice on day 24 p.i. incubated with or without STAT5 inhibitor in the presence of IL-7 for 72 h. Data represent the mean cpm ± SEM of triplicates. *, p < 0.05 and **, p < 0.01, t test. Data are representative of three separate experiments with at least three mice per group.
that the thymic changes are not caused by stress-related events (data not shown). Our analysis reveals that there is progressive expansion of the thymic CD4SP Foxp3\(^+\) Treg cell pool, resulting from markedly accelerated differentiation and proliferation of thymic CD4SP Foxp3\(^+\) T cells over the CD4SP Foxp3\(^-\) T cell population. Alternative explanations for the enrichment of thymic Treg cells in relationship to EAE, such as reentry of peripheral Treg cells or reduced apoptosis of thymic Treg cells, were ruled out in parallel experiments.

There is evidence that peripheral Treg cells are functionally impaired in autoimmune disease state, such as MS (39–42) and rheumatoid arthritis (43, 44), potentially through decreased thymic output of Treg cells (31). Here we provide supporting evidence that rapid replenishment by newly emigrated Treg cells of thymic origin into preexisting peripheral Treg cell pool in an autoimmune state is critical to self-regulation of the disease process and thus its clinical outcome. In the absence of new Treg cell emigrants, that is, in thymectomized mice, recovery from EAE is severely impaired, suggesting that the preexisting peripheral Treg pool alone is not sufficient for complete regulation of pathogenic T cells. It is conceivable that maintenance of peripheral Treg cells is largely dependent on IL-2 (25, 45), while the thymic development and output of Treg cells is regulated by IL-7. As described herein, IL-7 expression in thymic stromal cells is not up-regulated until peak of EAE, which coincides with the increased TREC levels in peripheral Treg cells. The data further suggest that the process of differentiation and maturation of thymic Treg cells in EAE is driven by IL-7/IL-7 receptor signaling to directly affect the peripheral Treg pool and the clinical outcome in EAE.

The thymic process discussed above appears selective for CD4SP Foxp3\(^+\) Treg cells over CD4SP Foxp3\(^-\) T cells. The observed thymic changes are characteristic of Treg cells and are not seen in Teff cells in both thymic and peripheral compartments during the course of EAE. This is further indicated by the overall regulatory function of the thymus in EAE through rapid enrichment of CD4SP Foxp3\(^+\) cells while the thymic CD4SP Foxp3\(^-\) population neither regulates nor increases encephalitogenicity. An important aspect of this study is to elucidate the underlying mechanism for the selectivity. We demonstrate that the selective differentiation and expansion of CD4SP Foxp3\(^+\) over CD4SP Foxp3\(^-\) T cells in the same thymic environment is attributable, in part, to the level and dynamic expression of IL-7.

![Figure 8](https://www.jimmunol.org/)

**Figure 8.** Role of TNF-\(\alpha\) in up-regulated expression of CD127 in CD4SP thymocytes. A. Flow cytometry of the expression of CD127 by CD4SP Foxp3\(^+\) and CD4SP Foxp3\(^-\) thymocytes from naive mice stimulated with TNF-\(\alpha\), IL-6, IFN-\(\gamma\), IL-1\(\beta\), and IL-12 for 24 h. The shaded area and solid line represent isotype control and CD127, respectively. B, CD4SP thymocytes purified from naive mice were cultured in the presence of TNF-\(\alpha\) or IL-7 for 48 h. Data represent expression of Foxp3 in these cells. C, Flow cytometry of Ki67 expression by CD4SP Foxp3\(^+\) and CD4SP Foxp3\(^-\) thymocytes from naive mice stimulated with TNF-\(\alpha\), IL-7, or IL-7 and TNF-\(\alpha\) for 24 h. Data are representative of three separate experiments with at least three mice per group.
receptor in CD4SP Foxp3\(^+\) cells, thus the favored response of CD4SP Foxp3\(^+\) cells to IL-7 as compared with those of CD4SP Foxp3\(^-\) cells during the course of EAE. This is of particular interest, as IL-7 receptor is minimally expressed in CD4SP Foxp3\(^-\) cells under normal physiologic conditions. This is consistent with the lack of IL-7-dependent response in Treg cells in the absence of EAE. Strikingly, the response of CD4SP Foxp3\(^+\) cells to IL-7 for differentiation and expansion is gained through rapid up-regulation of IL-7 receptor in the disease state. There is evidence described herein that this process is mediated through an IL-7-dependent JAK/STAT-5 pathway. The findings collectively support the conclusion that the selectivity for thymic Treg cell differentiation and expansion in EAE is associated with a dynamic process in both the production of IL-7 and the expression of IL-7 receptor in CD4SP Foxp3\(^+\) cells in thymus.

One of the key questions addressed herein is how the dynamic expression of IL-7 receptor in EAE is triggered. Many proinflammatory cytokines produced systemically as a result of EAE may be responsible for triggering this process. We demonstrate that among the key inflammatory cytokines characteristic of EAE examined, TNF-\(\alpha\) is found to induce up-regulation of IL-7 receptor expression in thymic Treg cells and leads to subsequent Treg cell proliferation and expansion. Thus, thymus receives the inflammatory signal in autoimmune disease state through key inflammatory cytokines, such as TNF-\(\alpha\), to rapidly expand the thymic Treg cell pool through increased IL-7 receptor expression and IL-7-dependent Treg cell response. Consequently, CD4SP Foxp3\(^-\) T cells undergo accelerated differentiation and output as part of the attempt of the immune systems to self-regulate autoimmune responses and disease activity, which may partially explain why, in most cases, EAE is a self-limiting condition. Taken together, the study provides new evidence supporting the role of thymus in self-regulation of autoimmune conditions, which may have important implications in the understanding of its functional role in other immune-related pathologies.

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Disclosures
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References


