ANIMAL MODELS

Impaired Expansion of Regulatory T Cells in a Neonatal Thymectomy-Induced Autoimmune Mouse Model

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Neonatal thymectomy in mice is a classic manipulation used to induce the breakdown of immunologic tolerance. Thymectomy in certain strains of mice triggers the onset of organ-specific autoimmune disease. However, the precise mechanism underlying thymectomy-induced organ-specific autoimmune disease remains unclear. Two possible mechanisms have been proposed to explain the association between lymphopenia and autoimmunity in thymectomy mice. One hypothesis is that thymectomy mice cannot sufficiently eliminate autoreactive T cells. A small population of autoreactive T cells is believed to readily interact with self-peptides presented on major histocompatibility complex molecules of professional antigen-presenting cells to become activated and expand. They then migrate to nonlymphoid organs, where autoimmune responses destroy and exert cytotoxic effects on target cells. Thus, the absence of sufficient numbers of Treg cells contributes to the onset of autoimmunity. In support of this hypothesis, CD4+CD25+ Treg cells were observed to differentiate in the thymus and circulate in the periphery after day 3, whereas CD4+CD25+ T cells were produced before day 3 after birth. As a result, fewer Treg cells were present in adult thymectomy mice than in adult nonthymectomy mice. However, recent studies have found that Treg cells were present in the peripheral lymph nodes and spleen before or at day 3 after birth, although the absolute numbers of CD4+CD25+ T cells was lower in thymectomy mice than in nonthymectomy mice. In addition, the immunosuppressive functions of Treg cells from thymectomy mice were maintained. These results support the hypothesis that an insufficient number of Treg cells promotes the onset of autoimmunity. The role of Treg
cells in autoimmunity induced by neonatal thymectomy remains unclear.

Another possibility derives from other recent studies presenting evidence that Treg cells can also be generated outside the thymus. These extrathymically generated Treg cells have been designated induced Treg or peripherally induced Treg (pTreg) cells. pTreg cells are converted from naive CD4⁺ T cells in the presence of transforming growth factor (TGF)-β and interleukin (IL)-2. In vitro, pTreg cells can acquire an immunosuppressive function similar to that of naturally occurring Treg or thymus-derived Treg (tTreg) cells. Several studies have found that the adoptive transfer of pTreg cells generated ex vivo from naive T cells can also prevent the development of autoimmune disease. Thus, if pTreg cells can sufficiently expand in the periphery, they may compensate for effective tTreg cells in neonatal thymectomized mice. The molecular mechanism underlying in vivo expansion of pTreg cells in thymectomy mice is completely obscure.

Sjögren syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltrates and the destruction of salivary and lacrimal glands. We previously established an animal model for SS in NFS/sld mutant mice that were thymectomized at 3 days after birth. These thymectomy NFS/sld mice developed autoimmune lesions in their salivary and lacrimal glands that resembled the lesions of human primary SS. The numbers of peripheral Treg cells in SS patients is significantly lower than that of healthy controls. We previously found that autoreactive CD4⁺ T cells played a pivotal role in the development of autoimmune exocrinopathy in these thymectomy NFS/sld mice. However, whether Treg cells are involved in the molecular pathogenesis of SS remains unknown.

In the present study, we analyzed the effect of neonatal thymectomy on Treg subpopulations relative to numbers and effector functions in the NFS/sld mouse. In addition, the pathogenesis of autoimmunity in this neonatal thymectomy animal model was investigated with respect to expansion and function of Treg cells.

Material and Methods

Ethics

This study was conducted in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government. Our protocols were approved by the Committee on Animal Experiments of Tokushima University.

Mice

Female mice of the NFS/N strain that carried a mutant sld were reared in our specific pathogen-free mouse colony and provided food and water ad libitum. Thymectomy in NFS/sld and C57BL/6 (B6) mice purchased from the Japan SLC Laboratory (Shizuoka, Japan) was performed on day 3 after birth. Neonatal and 8- to 18-week-old mice were used for experiments.

Histologic Analysis

Salivary glands were removed from thymectomy and non-thymectomy mice, fixed with 10% phosphate-buffered formaldehyde (pH 7.2), and prepared for histologic examination. Sections were stained with hematoxylin and eosin.

Confocal Microscopic Analysis

Frozen sections of the salivary glands from nonthymectomy and thymectomy mice were fixed with cold acetone, blocked with the M.O.M. blocking reagent (Vector Laboratories, Inc., Burlingame, CA), and then stained with a biotinylated antibody against CD4 (Biologend, San Diego, CA) and Alexa Fluor 568—conjugated streptavidin (Invitrogen, Carlsbad, CA) as a secondary antibody. After permeabilization with 0.2% Triton, sections were stained with a fluorescein isothiocyanate—conjugated antibody against Foxp3. Nuclear DNA was stained with DAPI (Invitrogen). Sections were observed with a laser scanning confocal microscope (Carl Zeiss, Jena, Germany) at ×400 magnification. Quick Operation version 3.2 (Carl Zeiss) was used for image acquisition.

Treg⁻Cell Isolation

Single-cell suspensions were prepared from spleen and cervical lymph nodes (CLNs), after which CD4⁺CD25⁻ Treg cells were isolated using the EasySep purification kit (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. More than 90% of the isolated Treg cells expressed intracellular Foxp3.

Flow Cytometric Analysis

The following monoclonal antibodies were purchased from eBioscience (San Diego, CA): anti-mouse CD4, CD8, glucocorticoid-induced tumor necrosis factor receptor, CD25, Foxp3, and IL-17. Anti-Helios, IL-2, IL-4, IL-10, CD44, CD62L, and CD304 were purchased from Biologend (San Diego, CA). Anti—cytotoxic T-lymphocyte—associated antigen (CTLA)-4, Bcl-xl, Bcl-2, and interferon (IFN)-γ were purchased from BD Biosciences (San Jose, CA). Intracellular Foxp3 expression was analyzed using an intracellular Foxp3 detection kit (eBioscience), according to the manufacturer’s instructions. For Bcl-xl and Bcl-2 staining, CD4⁺ T cells purified from spleen were stimulated with Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies, Carlsbad, CA) at a bead to cell of 1:1 for 20 hours. After washing, cells were stained with an anti-CD4 monoclonal antibody (mAb), fixed in fixation/permeabilization solution, permeabilized in permeabilization buffer, and stained with anti-Bcl-xl or Bcl-2 and
Foxp3 mAbs. For intracellular cytokine staining, lymphocytes from CLNs were stimulated with 20 ng/mL of phorbol myristate acetate (Sigma-Aldrich, St. Louis, MO) and 500 ng/mL of ionomycin (Sigma-Aldrich) for 6 hours in the presence of brefeldin A (eBioscience) for the last 4 hours. Flow cytometric analysis was performed using a BD FACSCant flow cytometer (BD Biosciences). Data were analyzed using FlowJo FACS Analysis software version 7.6.3 (Tree Star, Ashland, OR).

Suppression Assay

Cell proliferation was determined using 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). Magnetically isolated CD4⁺CD25⁻ conventional T cells were labeled with CFSE according to the manufacturer’s instructions. CFSE-labeled CD4⁺CD25⁻ conventional T cells (2.5 × 10⁴ per well) were incubated with purified Treg cells (2.5 × 10⁴ per well) for 3 days in the presence of Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) at bead/Treg cell ratios of 0.05, 0.1, and 0.25.

Treg Expansion Assay

Treg cell expansion was assessed by CFSE dilution and cell counts. For the proliferation assay, CFSE-labeled and magnetically isolated Treg cells (5 × 10⁴ cells per well) were cultured with Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) at a bead/Treg cell ratio of 0.25:1 and 100 U/mL of IL-2 (eBioscience) for 3 days. For cell counts, isolated Treg cells (5 × 10⁵ cells per well) were incubated for 7 days with Dynabeads Mouse T-Activator CD3/CD28 at a bead/Treg cell ratio of 2:1 and 2000 U/mL of IL-2.²⁸

Induction of Treg Cells

CD44⁻CD62L⁺ naive CD4⁺ T cells were purified from spleens using EasySep purification kits (Stemcell Technologies). Purified naive CD4⁺ T cells (5 × 10⁴ cells per well) were cultured with Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) at a bead/Treg cell ratio of 0.25:1 and 30 U/mL of IL-2 (eBioscience) in the presence of 1, 5, or 10 ng/mL of human TGF-β (PeproTech Inc., Rocky Hill, NJ).

Adoptive Transfer of Treg Cells into SS Model

Isolated naive CD4⁺ cells from spleen in nonthymectomy and thymectomy NFS/sld mice were expanded for 3 weeks as described in the Treg expansion assay. A total of 1 × 10⁶ Treg cells per mouse were transferred intravenously into thymectomy NFS/sld mice at 10 weeks of age. At 8 weeks after the transfer, the salivary gland tissues were removed, fixed with 10% phosphate-buffered formaldehyde (pH 7.2), and prepared for histologic examination. Formalin-fixed tissue sections were subjected to hematoxylin and eosin staining, and three pathologists independently evaluated the histologic findings without being informed of the condition of each thymectomy mouse. The number of infiltrating lymphocytes in the salivary gland specimens was counted per 0.25 mm².

Quantitative RT-PCR Analysis

Total RNA was isolated from splenic naive CD4⁺ T cells using RNeasy Plus Micro kits (Qiagen, Hilden, Germany). Total RNA was reverse transcribed using a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). This cDNA was used as the PCR template. Transcript levels were determined using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara). The primer sequences used were as follows: TGF-β receptor I (TGF-βRI), forward, 5'-AAGCAACCGTGGAGAAC-3' and reverse, 5'-GGGAGGCAGTTGTGACC-3'; TGF-βRII, forward, 5'-AAGCAACCGTGGAGAAC-3' and reverse, 5'-GGGAGGCAGTTGTGACC-3'; TGF-βIII, forward, 5'-ACTTTTGTCTGTGATTGTTCTGG-3' and reverse, 5'-TCAGATTGCGCTCTGCTCG-3' and reverse,
Enzyme-Linked Immunosorbent Assay

The concentration of IFN-γ in supernatants was measured by enzyme-linked immunosorbent assay. Then 96-well flat-bottomed plates were precoated with capture antibodies, and diluted samples or standard recombinant cytokines were added to each well. After the plates were washed, biotinylated antibodies were added, and the wells were incubated with horseradish peroxidase—labeled streptavidin. A solution of 3,3′,5′-tetramethylbenzidine was added to each well as the substrate. The optimal density at 490 nm was measured using a microplate reader (model 680; Bio-Rad, Richmond, CA).

Statistical Analysis

Results are presented as the means ± SD. Comparisons of the results between the two groups were made by the unpaired, two-tailed t-test. *P < 0.05 was considered significant.

Results

T<sub>reg</sub> Cells in the Thymus and Spleen of Neonatal Mice at 3 and 7 Days after Birth

We first analyzed Foxp3<sup>+</sup> T<sub>reg</sub> cells in the thymus and spleen of neonatal NFS/sld mice without thymectomy. No differences were found in the proportions of Foxp3<sup>+</sup> T<sub>reg</sub> cells in the thymus between 3 and 7 days after birth (Figure 1A). The proportions of Foxp3<sup>+</sup> T<sub>reg</sub> cells in the spleen of both 3-day-old and 7-day-old mice were approximately 12% to 13%
Peripheral T<sub>reg</sub> Cell in the Thymectomy SS Model

Next, we investigated T<sub>reg</sub> cells in thymectomy NFS/sld mice (Tx mice) used as an SS model at 10 weeks of age. Although the proportion of Foxp3<sup>+</sup> T<sub>reg</sub> cells among CD4<sup>+</sup> T cells in thymectomy mice was higher than that in nonthymectomy mice (Figure 2A and B), the actual number of T<sub>reg</sub> cells in both the spleen and CLNs of thymectomy mice was significantly lower than that in nonthymectomy mice (Figure 2C). In addition, the number of CD4<sup>+</sup> T cells in thymectomy mice was significantly lower than that in non thymectomy mice (Figure 2C).

The proportion of CD4<sup>+</sup>CD62L<sup>+</sup> effector memory phenotype CD4<sup>+</sup> T cells of the spleen and CLNs was notably higher in thymectomy mice than in nonthymectomy mice (Figure 2D). In addition, the ratio of T<sub>reg</sub> cells to effector memory T cells in the spleen and CLNs of thymectomy mice was significantly lower than that of non thymectomy mice (Figure 2E).

To assess whether T<sub>reg</sub> cells had migrated into the salivary glands as a target organ in thymectomy mice, Foxp3<sup>+</sup> T<sub>reg</sub> cells were examined using a confocal microscope. Foxp3<sup>+</sup>CD4<sup>+</sup> T<sub>reg</sub> cells had infiltrated into the salivary glands of thymectomy mice (Figure 2F). These results indicate that the number of T<sub>reg</sub> cells may be insufficient to suppress effector T cells for the induction of autoimmune lesions in target organs of thymectomy mice.

Suppressor Function of T<sub>reg</sub> Cells in Thymectomy Mice

Next, the regulatory function of T<sub>reg</sub> cells in thymectomy mice was investigated. To assess the phenotypes of T<sub>reg</sub> cells, we examined the expression of hallmark proteins in T<sub>reg</sub> cells, including CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor. The CTLA-4 expression levels on Foxp3<sup>+</sup> CD4<sup>+</sup> T<sub>reg</sub> cells from the spleen and CLNs of thymectomy mice were similar to those of cells derived from nonthymectomy mice (Figure 3A). In addition, there was no difference in glucocorticoid-induced tumor necrosis factor receptor expression on T<sub>reg</sub> cells from the spleen and CLNs from thymectomy and nonthymectomy mice (Figure 3A).

Next, we used an in vitro suppression assay to evaluate the function of T<sub>reg</sub> cells. CD4<sup>+</sup>CD25<sup>-</sup> effector T cells from non-Tx (non-Tx) and Tx mice were transferred intravenously into Tx mice. Eight weeks later salivary gland tissues of the recipient mice were histopathologically analyzed. The number of infiltrating lymphocytes in 0.25 mm<sup>2</sup> of salivary gland tissue was counted. Data are expressed as means ± SD (D). n = 3 independent experiments (A and B) and 5 mice (C and D). *P < 0.05, **P < 0.005 versus Tx mice. Scale bar = 100 μm (C).
nonthymectomy mice were labeled with CFSE and cultured for 72 hours together with unlabeled CD4^+ CD25^+ Treg cells in the presence of anti-CD3/CD28 mAbs. Treg cells from both non-thymectomy and thymectomy mice suppressed the proliferation of effector T cells (Figure 3B). In addition, no difference was found in Treg cell suppression activity between cells derived from thymectomy and nonthymectomy mice when using CD25^−/CD4^+ T cells from thymectomy mice as effector cells (Supplemental Figure S1). Furthermore, no significant difference was found in the suppression function of Treg cells from nonthymectomy and thymectomy mice by in vitro suppression assay with different ratios of effector and Treg cells (Supplemental Figure S2). These results suggest that Treg cells from thymectomy mice have the same in vitro immunosuppressive ability as those from nonthymectomy mice.

To determine the in vivo suppression activity of Treg cells in thymectomy mice, adoptive transfer of Treg cells from nonthymectomy and thymectomy mice was performed using thymectomy mice as recipients, and the presence of autoimmune salivary gland lesions was evaluated. The number of lymphocytes infiltrating into the target tissue in thymectomy mice was significantly reduced by the transfer of Treg cells from nonthymectomy mice while the inflammatory lesion was not completely protected (Figure 3, C and D). Although the number of infiltrating lymphocytes in thymectomy Treg cell–transferred mice was significantly lower than that of nontransferred thymectomy mice, the number of infiltrating lymphocytes in the thymectomy Treg cell–transferred mice was significantly higher than that of nonthymectomy Treg cell–transferred mice (Figure 3, C and D). This result suggests that the suppression function of Treg cells of thymectomy mice may be not sufficient for controlling autoimmunity in vivo.

**Treg-Cell Expansion in Thymectomy Mice**

To investigate the peripheral expansion of Treg cells in thymectomy mice, CFSE-labeled Treg cells were stimulated with anti-CD3/28 mAbs in the presence of IL-2 for 3 days. Interestingly, the proliferation of Treg cells from thymectomy mice was significantly lower than that of Treg cells from nonthymectomy mice (Figure 4A). After 7 days of Treg cell culture, the total number of Treg cells from thymectomy mice was markedly lower than that from nonthymectomy mice.
Because the IL-2–IL-2R signaling pathway plays a pivotal role in the generation, homeostasis, and expansion of T<sub>reg</sub> cells, we examined IL-2R receptor (CD25 protein) expression on the surface of T<sub>reg</sub> cells. Surface CD25 expression on T<sub>reg</sub> cells did not differ between cells from thymectomy and nonthymectomy mice (Figure 4C).

We next examined the expression of Bcl-xL and Bcl-2, antiapoptotic members of the Bcl-2 family. Stimulation with anti-CD3/28 mAbs up-regulated Bcl-xL and Bcl-2 protein levels in Foxp3<sup>+</sup>CD4<sup>+</sup> T cells, although there was no increase in Bcl-xL and Bcl-2 expression in T<sub>reg</sub> cells of either thymectomy or nonthymectomy mice (Figure 4D and E). These results suggest that the impaired expansion of T<sub>reg</sub> cells in thymectomy mice may involve a pathway independent of survival signaling by the Bcl-2 family.

### pT<sub>reg</sub>-Cell Expansion in Thymectomy NFS/sld Mice

Although tT<sub>reg</sub> cells are derived from thymic differentiation, it is now apparent that conventional peripheral naive CD4<sup>+</sup> T cells can be converted to Foxp3<sup>+</sup> T<sub>reg</sub> cells, called pT<sub>reg</sub> cells. The transcription factor Helios is one of the markers that can be used to discriminate between tT<sub>reg</sub> cells and pT<sub>reg</sub> cells. Thus, we analyzed these T<sub>reg</sub> cell subsets in thymectomy mice using Foxp3 and Helios expression. The numbers of both Helios<sup>+</sup> Foxp3<sup>+</sup> tT<sub>reg</sub> cells and Helios<sup>−</sup> Foxp3<sup>+</sup> pT<sub>reg</sub> cells in the spleen and CLNs of thymectomy mice were significantly lower than those of thymectomy mice (Figure 5B). Because the IL-2–IL-2R signaling pathway plays a pivotal role in the generation, homeostasis, and expansion of T<sub>reg</sub> cells, we examined IL-2R receptor (CD25 protein) expression on the surface of T<sub>reg</sub> cells. Surface CD25 expression on T<sub>reg</sub> cells did not differ between cells from thymectomy and nonthymectomy mice (Figure 4C).

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**Figure 5** Peripherally induced T-regulatory (pT<sub>reg</sub>) cells in thymectomy (Tx) mice. A: Numbers of Foxp3<sup>+</sup>CD4<sup>+</sup> cells, Foxp3<sup>+</sup>Helios<sup>−</sup>CD4<sup>+</sup> cells, and Foxp3<sup>+</sup>Helios<sup>−</sup>CD4<sup>+</sup> cells in the spleen and cervical lymph nodes (CLNs) of non-Tx and Tx mice at 10 weeks of age were determined by flow cytometry. B: Foxp3 and Helios expression by CD4<sup>+</sup> T cells in the spleen and CLNs of non-Tx and Tx mice were determined by flow cytometry. C: Foxp3 and CD304 expression by CD4<sup>+</sup> T cells in the spleen and CLNs of non-Tx and Tx mice were determined by flow cytometry. Results are expressed as means ± SD (A and C), n = 3 mice per group (A–C); n = 5 mice (B) and 3 mice (C) per group for profiles. *P < 0.05 versus non-Tx mice. tT<sub>reg</sub>, thymus-derived T regulatory.
nonthymectomy mice (Figure 5A). In contrast, the proportion of tTreg cells in the spleen and CLNs of thymectomy mice was considerably higher than that of nonthymectomy mice (Figure 5B). The ratio of pTreg cell to tTreg cells in the spleen and CLNs of thymectomy mice was significantly lower than that of nonthymectomy mice (Figure 5B). Another marker, CD304 (neuropilin-1), expressed in the Treg cell subset,32 was used to distinguish pTreg and tTreg cells in thymectomy mice. The ratio of CD304\textsuperscript{low}/Foxp3\textsuperscript{+} pTreg cells to CD304\textsuperscript{high}/Foxp3\textsuperscript{+} tTreg cells of both spleen and CLNs was significantly lower in thymectomy mice than that in non-thymectomy mice (Figure 5C). These findings suggest that the differentiation of pTreg cells is impaired in thymectomy mice.

In Vitro Induction of pTreg Cells

Although a reduction in tTreg cell counts was expected in thymectomy mice, it was unclear why the expansion or maintenance of pTreg cells was impaired. To elucidate the mechanism underlying pTreg cell maintenance, purified CD44\textsuperscript{low}/CD62L\textsuperscript{+} naive CD4\textsuperscript{+} T cells from the spleen of nonthymectomy and thymectomy mice were cultured with anti-CD3/CD28 mAbs in the presence of IL-2 and TGF-\beta to differentiate and expand them into pTreg cells. The differentiation of naive CD4\textsuperscript{+} T cells to Helios\textsuperscript{+}/Foxp3\textsuperscript{+} pTreg cells from thymectomy mice was significantly suppressed compared with that of naive CD4\textsuperscript{+} T cells from non-thymectomy mice (Figure 6, A and B).

Whether expansion of pTreg cells in thymectomy-treated C57BL/6 (B6) mice was impaired was unclear. Purified naive T cells of spleen from nonthymectomy and thymectomy B6 mice were cultured with anti-CD3/CD28 mAbs in the presence of IL-2 and TGF-\beta for 7 days. Helios\textsuperscript{+}/Foxp3\textsuperscript{+} pTreg cells from thymectomy B6 mice were also significantly suppressed compared with that of naive CD4\textsuperscript{+} T cells from nonthymectomy B6 mice (Supplemental Figure S3). The result suggests that thymectomy induces impaired differentiation and expansion of pTreg cells in any mouse strain.

TGF-\betaR and Smad Expression on Naive T Cells from Thymectomy Mice

Because TGF-\beta signaling is important for the generation of pTreg cells,15 we examined the mRNA expression of TGF-\betaRI, TGF-\betaRII, and TGF-\betaRIII in CD4\textsuperscript{+}/CD25\textsuperscript{-} T cells. TGF-\betaRI and TGF-\betaRII mRNA expression levels in these...
cells from thymectomy mice were significantly lower than those in cells from nonthymectomy mice (Figure 7). In addition, the mRNA expression of Smad3 and -4, key molecules for TGF-βR signaling during pTreg expansion,33-35 in cells from thymectomy mice was also significantly lower in cells from thymectomy mice than in those from nonthymectomy mice (Figure 7). These results suggest that the differentiation and expansion of pTreg cells via the TGF-β/Smad pathway in the periphery of thymectomy mice was impaired in addition to the decreased generation of tTreg cells in the thymus.

Treg Cells from Thymectomy Mice Produce IFN-γ

Treg cells use several mechanisms, including cell-to-cell contact, for achieving immune suppression.36,37 In contrast, the anti-inflammatory cytokine IL-10 is required for in vivo suppression.38-40 In addition, several studies have indicated that Treg cells produce proinflammatory effector cytokines, such as IFN-γ and IL-17, under certain specific conditions and in some autoimmune diseases.41-44 To explore the cytokine production profile of the peripheral total CD4⁺ T cells, including Treg cells and effector T cells in thymectomy mice, we examined intracellular cytokine production in purified CD4⁺ cells after stimulation with phorbol myristate acetate/ionomycin.

Foxp3⁺ CD4⁺ effector cells from thymectomy mice produced elevated levels of IFN-γ and IL-4 (Figure 8A). There was no evidence for increased expression of IL-2, IL-17, or IL-10 by these cells from thymectomy mice (Figure 8A). However, Treg cells, including tTreg and pTreg cells, from thymectomy mice also produced large amounts of IFN-γ and IL-4, resembling the profile of effector cells (Figure 8B). There were no differences in IL-2, IL-17, or IL-10 production between cells from nonthymectomy and thymectomy mice (Figure 8B). IFN-γ production by Treg cell subsets was analyzed using flow cytometry, indicating that IFN-γ production by CD62L⁻ IFN-γ⁺ cells in both pTreg and tTreg cells stimulated with phorbol myristate acetate/ionomycin was significantly higher in those from thymectomy mice than in those from nonthymectomy mice (Figure 8).
thymectomy mice than from nontronymocyte mice (Figure 8, C and D).

To measure IFN-γ secretion from expanded Treg cells of thymectomy mice, purified Treg cells were stimulated with anti-CD3/28 mAbs in the presence of IL-2 for 10 days. The supernatants from 0 to 3 and 7 to 10 days were analyzed by enzyme-linked immunosorbent assay. The IFN-γ concentration in the supernatant from Treg cells of thymectomy mice was significantly higher than that of nontronymocyte mice (Figure 8E). These results suggest that Treg cells in thymectomy mice may have an effector-like phenotype that is associated with the development of autoimmunity.

Discussion

Peripheral T-cell expansion, including that by Treg cells, is vigorously promoted during the neonatal period. In this study, we focused on the dynamics and functions of Treg cells derived from neonatal thymectomy mice to determine the association between Treg cells and autoimmunity in these mice. Our results indicate that a reduction in Treg cell counts in the periphery of thymectomy mice influenced the onset of autoimmune disease in our model, which was in accordance with the results of previous reports of other thymectomized models.1–8 Thus, the expansion ability of Treg cells in thymectomy mice was insufficient to compensate for the shortage in Treg cell counts.

We found that induction of Treg cells from peripheral naive CD4+ T cells was suppressed in thymectomy mice. Although the in vivo function of pTreg cells remains uncertain, several studies have reported that pTreg cells suppress inflammation in autoimmune and/or inflammatory diseases, such as asthma, systemic lupus erythematosus, type 1 diabetes, gastritis, colitis, and multiple sclerosis.15,19–22,45 The results of the present study indicate that a reduced ratio of Treg cells to effector T cells may influence the onset or development of autoimmunity in thymectomy mice and suggest that neonatal thymectomy interferes with the differentiation and expansion of pTreg cells from naive T cells. Several markers of Treg cell subsets have been reported, including Helios and CD304.31,32,46 In the present study, a reduction of the ratio of pTreg cells to tTreg cells using Helios and CD304 was observed in thymectomy mice. Helios is known to be one of the ikaros family of transcription factors important for the differentiation of tTreg cells.31 CD304 is also a surface marker of tTreg cells.32

TGF-β plays a key role in the generation and expansion of Treg cells. TGF-β signaling is required for the development of tTreg cells in the thymus, possibly by promoting their survival.15 In addition, TGF-β promotes the differentiation and expansion of pTreg cells by promoting Foxp3 expression in naive T cells.15 The binding of TGF-β to TGF-βR activates the structurally similar transcription factors Smad2 and Smad3 in target cells.27 Activated Smad2 or Smad3 heterodimerizes with Smad4 followed by translocation into the nucleus to regulate target gene expression.48 However, there are also Smad-independent pathways that mediate TGF-βR signaling.48 TGF-β induces the rapid activation of TGF-βR-activated kinase 1, Ras-Erk, and PI3K-Akt pathways.48 In the present study, mRNA expression of TGF-βRII, TGF-βRIII, Smad2, and Smad3 in naive T cells was reduced after neonatal thymectomy, suggesting that there may be many mechanisms for maintaining the expression of key genes for T-cell survival and function in the neonatal thymus. The present study suggests an important possibility that effector T cells may be resistant to the Treg cell-mediated suppression, which is consistent with the finding that lower levels of TGF-βR and Smad are expressed in naive T cells in this model.

We also found that Treg cells from thymectomy mice had different cytokine production profiles compared with those from control mice. The cells from thymectomy mice preferentially produced a proinflammatory cytokine, IFN-γ, similar to activated conventional CD4+ T cells. Recent studies have suggested that Treg cells are capable of secreting proinflammatory cytokines for maintaining Foxp3 expression.41,44,49 In contrast, an IFN-γ–induced molecule, interferon regulatory factor 1, reportedly bound to the Foxp3 promoter region and inhibited Foxp3 expression.52 Furthermore, recent reports have found that Foxp3 expression in Treg cells is unstable and that Treg cells not expressing Foxp3 can be converted to effector cells when they encounter an inflammatory milieu.53–56 In the present study, the number of infiltrating lymphocytes differed between transfer of thymectomy Treg cells and transfer of nontronymocyte Treg cells even though there was no difference in in vitro suppression between nontronymocyte and thymectomy Treg cells. Thus, it is possible that Treg cells in thymectomy mice exhibit an effector cell–like phenotype that does not fully promote autoimmunity in addition to the reduced Treg cell counts observed in mice.

Neonatal immune responses are considered to be immunologically immature.37 In addition, the neonatal T-cell repertoire is more pathogenic than that of adults.56 Thus, T-cell differentiation and development in the neonatal thymus potently contribute to maintaining immune tolerance in the periphery. Neonatal thymectomy is one of the traditional manipulations used to induce organ-specific autoimmune diseases in various organs, including stomach, testis, prostate, ovary, thyroid gland, salivary gland, and lacrimal gland.1–9 We established a murine model for SS using the NFS/sld mouse strain that underwent neonatal thymectomy.9,25 We investigated the pathogenesis of existing and new therapeutic strategies for SS using this model.58–60 Impaired Treg cells are known to contribute to the onset or development of SS.61 However, whether the expansion or function of pTreg cells influences the pathogenesis of SS remains unclear. In the present study, we focused on the unique phenotype and function of Treg cells in this SS model to elucidate the association between Treg cells and organ-specific autoimmunity.
In conclusion, $T_{reg}$ cells in thymectomy mice were insufficient for regulating effector T cells and protecting autoimmunity with respect to cell counts and function. The results of this study will be useful for understanding the pathogenesis of thymectomy-induced autoimmune diseases and the development of new autoimmunity therapies with $T_{reg}$ cells.

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Supplemental Data

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References

transcription factor family member. differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol 2010, 184:3433−3441


41. Bertiou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, Hafler DA: IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood 2009, 113:4240−4249


