ANIMAL MODELS

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

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Neonatal thymectomy in certain mouse strains is known to induce organ-specific autoimmunity due to impaired functions of T cells, including Foxp3⁺ regulatory T (T_{reg}) cells in the thymus. The precise mechanism underlying the induction of autoimmunity by neonatal thymectomy remains unclear. One possibility is that depletion of T_{reg} cells breaks down peripheral tolerance. We examined the functions of T_{reg} cells by using a murine Sjögren syndrome model of NFS/*sld* mice that underwent neonatal thymectomy. The ratio of T_{reg} cells to effector memory phenotype T cells in thymectomy mice was significantly lower than that of nonthymectomy mice. In addition, *in vitro* induction of peripherally induced T_{reg} cells by transforming growth factor- β (TGF- β) using naive T cells from Sjögren syndrome model mice was severely impaired. The mRNA expression of TGF- β receptor I and II and Smad3 and -4 in the TGF- β -induced signal transduction pathway of T_{reg} cells in this Sjögren syndrome model were lower than those of control mice. In addition, T_{reg} cells in this Sjögren syndrome model exhibited an interferon- γ -producing Th1-like phenotype that resembled effector T cells. In conclusion, these results suggest that abnormal expansion and differentiation of T_{reg} cells and inflammatory cytokines produced by T_{reg} cells contribute to the development of autoimmunity. (*Am J Pathol* 2015, 185: 2886–2897; <http://dx.doi.org/10.1016/j.ajpath.2015.07.007>)

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.^{1–9} 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.¹⁰ Thus, the absence of sufficient numbers of T_{reg} cells contributes to the onset of autoimmunity. In support of this hypothesis, CD4⁺CD25⁺ T_{reg} 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 T_{reg} cells were present in adult thymectomy mice than in adult nonthymectomy mice.¹¹ However, recent studies have found that T_{reg} 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.^{12,13} In addition, the immunosuppressive functions of T_{reg} cells from thymectomy mice were maintained.¹⁴ These results support the hypothesis that an insufficient number of T_{reg} cells promotes the onset of autoimmunity. The role of T_{reg}

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cells in autoimmunity induced by neonatal thymectomy remains unclear.

Another possibility derives from other recent studies presenting evidence that T_{reg} cells can also be generated outside the thymus. These extrathymically generated T_{reg} cells have been designated induced T_{reg} or peripherally induced T_{reg} (pT_{reg}) cells. pT_{reg} cells are converted from naive CD4⁺ T cells in the presence of transforming growth factor (TGF)- β and interleukin (IL)-2.^{15,16} *In vitro*, pT_{reg} cells can acquire an immunosuppressive function similar to that of naturally occurring T_{reg} or thymus-derived T_{reg} (tT_{reg}) cells.^{17,18} Several studies have found that the adoptive transfer of pT_{reg} cells generated *ex vivo* from naive T cells can also prevent the development of autoimmune disease.^{15,19–22} Thus, if pT_{reg} cells can sufficiently expand in the periphery, they may compensate for effective tT_{reg} cells in neonatal thymectomy mice. The molecular mechanism underlying *in vivo* expansion of pT_{reg} 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.²³ We previously established an animal model for SS in NFS/*sld* mutant mice that were thymectomized at 3 days after birth.^{9,24} 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 T_{reg} cells in SS patients is significantly lower than that of healthy controls.²⁵ We previously found that autoreactive CD4⁺ T cells played a pivotal role in the development of autoimmune exocrinopathy in these thymectomy NFS/*sld* mice.²⁶ However, whether T_{reg} cells are involved in the molecular pathogenesis of SS remains unknown.

In the present study, we analyzed the effect of neonatal thymectomy on T_{reg} 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 T_{reg} 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*²⁷ 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 (Biolegend, 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 $\times 400$ magnification. Quick Operation version 3.2 (Carl Zeiss) was used for image acquisition.

T_{reg}-Cell Isolation

Single-cell suspensions were prepared from spleen and cervical lymph nodes (CLNs), after which CD4⁺CD25⁺ T_{reg} 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 T_{reg} 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 Biolegend (San Diego, CA). Anti–cytotoxic T-lymphocyte–associated antigen (CTLA)-4, Bcl-x1, 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-x1 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-x1 or Bcl-2 and

Figure 1 Numbers of T-regulatory cells in the thymus and spleen of neonatal mice at 3 and 7 days after birth. **A** and **B**: CD4/CD8 and CD4⁺Foxp3⁺ cell populations in the thymus (**A**) and spleen (**B**) from nonthymectomy (non-Tx) NFS/*sld* mice at 3 and 7 days after birth was assessed by flow cytometry. **C**: Numbers of CD4⁺ T cells and Foxp3⁺CD4⁺ T cells in the spleen of non-Tx mice were determined. Results are expressed as means $\pm$ SD (**C**). $n = 5$ mice per condition (**C**).

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 (2.5×10^4 per well) were incubated with purified T_{reg} cells (2.5×10^4 per well) for 3 days in the presence of Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) at bead/T_{reg} cell ratios of 0.05, 0.1, and 0.25.

T_{reg} Expansion Assay

T_{reg} cell expansion was assessed by CFSE dilution and cell counts. For the proliferation assay, CFSE-labeled and magnetically isolated T_{reg} cells (5×10^4 cells per well) were cultured with Dynabeads Mouse T-Activator CD3/CD28 at a bead/T_{reg} cell ratio of 0.25:1 and 100 U/mL of IL-2 (eBioscience) for 3 days. For cell counts, isolated T_{reg} cells (1×10^5 cells per well) were incubated for 7 days with Dynabeads Mouse T-Activator CD3/CD28 at a bead/T_{reg} cell ratio of 2:1 and 2000 U/mL of IL-2.²⁸

Induction of T_{reg} Cells

CD44^{low}CD62L⁺ naive CD4⁺ T cells were purified from spleens using EasySep purification kits (Stemcell Technologies). Purified naive CD4⁺ T cells (5×10^4 cells

per well) were cultured with Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) at a bead/T_{reg} 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 T_{reg} 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 T_{reg} expansion assay. A total of 1×10^6 T_{reg} 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- β RI), forward, 5'-AACTGAAACACCGTGGGAAC-3' and reverse, 5'-TGGAAGCTTTTCAGTTGACC-3'; TGF- β RII, forward, 5'-CCCAGTCTGGAAATGAAAGC-3' and reverse, 5'-ACTTTTGTCTGGGTTCTGG-3'; TGF- β RIII, forward, 5'-TCAGATTTGTGCCTGTCTCG-3' and reverse,

Figure 2 Numbers of T regulatory (T_{reg}) cells in peripheral lymphoid tissues. **A** and **B**: Frequency of Foxp3⁺ cells gated on CD4⁺ cells in spleen and cervical lymph nodes (CLNs) of nonthymectomy (non-Tx) and Tx mice at 10 weeks of age was determined by flow cytometry. **C**: Numbers of CD4⁺ and Foxp3⁺CD4⁺ T cells in the spleen and CLNs of non-Tx and Tx mice were determined by flow cytometry. **D**: Effector memory phenotype of CD4⁺ T cells in spleen and CLNs was analyzed by flow cytometry. **E**: Ratio of T_{reg} to effector to memory phenotype T cells. **F**: Histologic analysis of salivary glands (SGs) from Tx mice (hematoxylin and eosin staining; left). CD4⁺ T cells (red), Foxp3⁺ cells (green), and cell nuclei (blue) in SGs were observed under a confocal microscope (right). Data are expressed as means ± SD. *n* = five mice per each group (**A–F**). Results are presented as the means ± SD of five mice per group (**A–D**). **P* < 0.05, ***P* < 0.01 versus non-Tx mice. Scale bar = 100 μm (**F**).

5'-CTGGGTGTTCTGCATTTGTG-3'; Smad2, forward, 5'-TCCCATTCTGTTCTGGTCC-3' and reverse, 5'-ACTGCCACACAAACCTTTC-3'; Smad3, forward, 5'-TGATGCTTCTGTTCTGGTG-3' and reverse, 5'-GGCACTTTCCTTTGGACTG-3'; Smad4, forward, 5'-ATGGCTATGTGATCCTTCG-3' and reverse, 5'-TCAATCGCTTCTGTCCTGTG-3'; β-actin, forward, 5'-GTGGGCCGCTCTAGGCACCA-3' and reverse, 5'-CGGTTGGCCTTAGGGTTCAGGGG-3'. Relative mRNA expression of each transcript was normalized against β-actin mRNA.

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 *o*-phenylenediamine (Sigma-Aldrich) 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_{reg} Cells in the Thymus and Spleen of Neonatal Mice at 3 and 7 Days after Birth

We first analyzed Foxp3-expressing T_{reg} cells in the thymus and spleen of neonatal NFS/*sld* mice without thymectomy. No differences were found in the proportions of Foxp3⁺ T_{reg} cells in the thymus between 3 and 7 days after birth (Figure 1A). The proportions of Foxp3⁺ T_{reg} cells in the spleen of both 3-day-old and 7-day-old mice were approximately 12% to 13%

(Figure 1B), although the numbers of Foxp3⁺ T_{reg} cells in the spleen of 7-day-old mice were notably increased, along with an increase in CD4⁺ T cells (Figure 1C). These results suggest that the expansion of peripheral T_{reg} cells during the neonatal period may be involved in the maintenance of the peripheral tolerance by T_{reg} cells.

Peripheral T_{reg} Cell in the Thymectomy SS Model

Next, we investigated T_{reg} cells in thymectomy NFS/*sld* mice (Tx mice) used as an SS model at 10 weeks of age. Although the proportion of Foxp3⁺ T_{reg} cells among CD4⁺

T cells in thymectomy mice was higher than that in non-thymectomy control mice (Figure 2, A and B), the actual number of T_{reg} cells in both the spleen and CLNs of thymectomy mice was significantly lower than that in non-thymectomy mice (Figure 2C). In addition, the number of CD4⁺ T cells in thymectomy mice was significantly lower than that in non-thymectomy mice (Figure 2C).

The proportion of CD44^{high}CD62L⁺ effector memory phenotype CD4⁺ 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_{reg} 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_{reg} cells had migrated into the salivary glands as a target organ in thymectomy mice, Foxp3⁺T_{reg} cells were examined using a confocal microscope. Foxp3⁺CD4⁺ T_{reg} cells had infiltrated into the salivary glands of thymectomy mice (Figure 2F). These results indicate that the number of T_{reg} 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_{reg} Cells in Thymectomy Mice

Next, the regulatory function of T_{reg} cells in thymectomy mice was investigated. To assess the phenotypes of T_{reg} cells, we examined the expression of hallmark proteins in T_{reg} cells, including CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor. The CTLA-4 expression levels on Foxp3⁺ CD4⁺ T_{reg} 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_{reg} 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_{reg} cells. CD4⁺ CD25[−] effector T cells from

Figure 3 Surface phenotypes and immunosuppressive functions of T-regulatory (T_{reg}) cells. **A:** Cytotoxic T-lymphocyte-associated antigen (CTLA)-4 and glucocorticoid-induced tumor necrosis factor receptor (GITR) expressions on CD4⁺Foxp3⁺ cells in the spleen and cervical lymph nodes (CLNs) of non-thymectomy (non-Tx) and Tx mice at 10 to 12 weeks of age were determined by flow cytometry. Solid lines indicate CD4⁺Foxp3⁺ cells from Tx mice, and dotted lines indicate these cells from non-Tx mice. The isotype control is shaded on each graph. **B:** 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled CD4⁺CD25[−] T cells (2.5×10^4 cells per well) from Tx mice were cultured with unlabeled T_{reg} cells (2.5×10^4 per well) from non-Tx and Tx mice along with anti-CD3/28 monoclonal antibody-coated beads at bead to cell ratios of 0.05:1, 0.1:1, and 0.25:1. After culture for 3 days, CD4⁺CD25[−] T-cell division was determined by CFSE dilution. **C:** T_{reg} cells (1×10^6 cells) expanded from naive CD4⁺ T cells of spleen in non-Tx and Tx mice were transferred intravenously into Tx mice. Eight weeks later salivary gland tissues of the recipient mice were histopathologically analyzed. **D:** The number of infiltrating lymphocytes in 0.25 mm² of salivary gland tissue was counted. Data are expressed as means $\pm$ 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 μ m (**C**).

Figure 4 T-regulatory (T_{reg}) cell expansion in thymectomy (Tx) mice. **A:** 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE)—labeled purified T_{reg} cells (5×10^4 cells per well) from non-Tx and Tx mice were cultured with anti-CD3/28 monoclonal antibody (mAb)—coated beads at a bead/cell ratio of 0.25:1 and 100 U/mL of interleukin (IL)-2. After culture for 3 days, T_{reg} cell division was determined by CFSE dilution. **B:** T_{reg} cells (1×10^5 cells per well) from the spleen of non-Tx and Tx mice were stimulated with anti-CD3/28 mAb-coated beads at a 2:1 bead/cell ratio and 2000 U/mL of IL-2 for 7 days. Open circles indicate CD4⁺Foxp3⁺ cells from non-Tx mice, and closed circles indicate those from Tx mice. **C:** CD25 expression on CD4⁺Foxp3⁺ cells from the spleen and cervical lymph nodes (CLNs) of non-Tx (dotted lines) and Tx (solid lines) mice was determined by flow cytometry. **D** and **E:** CD4⁺ T cells (2×10^5 cells per well) from the spleen of non-Tx and Tx mice were cultured with anti-CD3/28 mAb-coated beads at a 1:1 bead/cell ratio. After culture for 20 hours, Bcl-xl and Bcl-2 expression in Foxp3⁺CD4⁺ and Foxp3⁺CD4⁺ cells was determined by flow cytometry. Results are expressed as means $\pm$ SD of three mice per group (**A** and **B**) and five mice per group (**C**). $n = 3$ independent experiments (**B**, **D**, and **E**). * $P < 0.05$, ** $P < 0.01$ versus non-Tx mice.

nonthymectomy mice were labeled with CFSE and cultured for 72 hours together with unlabeled CD4⁺CD25⁺ T_{reg} cells in the presence of anti-CD3/CD28 mAbs. T_{reg} cells from both nonthymectomy and thymectomy mice suppressed the proliferation of effector T cells (Figure 3B). In addition, no difference was found in T_{reg} 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 T_{reg} cells from nonthymectomy and thymectomy mice by *in vitro* suppression assay with different ratios of effector and T_{reg} cells (Supplemental Figure S2). These results suggest that T_{reg} cells from thymectomy mice have the same *in vitro* immunosuppressive ability as those from nonthymectomy mice.

To determine the *in vivo* suppression activity of T_{reg} cells in thymectomy mice, adoptive transfer of T_{reg} 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 T_{reg} 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 T_{reg} cell—transferred mice was significantly lower than that of nontransferred thymectomy mice, the number of infiltrating lymphocytes in the thymectomy T_{reg} cell—transferred mice was significantly higher than that of nonthymectomy T_{reg} cell—transferred mice (Figure 3, C and D). This result suggests that the suppression function of T_{reg} cells of thymectomy mice may be not sufficient for controlling autoimmunity *in vivo*.

T_{reg}-Cell Expansion in Thymectomy Mice

To investigate the peripheral expansion of T_{reg} cells in thymectomy mice, CFSE-labeled T_{reg} cells were stimulated with anti-CD3/28 mAbs in the presence of IL-2 for 3 days. Interestingly, the proliferation of T_{reg} cells from thymectomy mice was significantly lower than that of T_{reg} cells from nonthymectomy mice (Figure 4A). After 7 days of T_{reg} cell culture, the total number of T_{reg} cells from thymectomy mice was markedly lower than that from nonthymectomy

Figure 5 Peripherally induced T-regulatory (pT_{reg}) cells in thymectomy (Tx) mice. **A:** Numbers of Foxp3⁺CD4⁺ cells, Foxp3⁺Helios⁺CD4⁺ cells, and Foxp3⁺Helios⁻CD4⁺ 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⁺ 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⁺ 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_{reg}, thymus-derived T regulatory.

mice (Figure 4B). Because the IL-2–IL-2R signaling pathway plays a pivotal role in the generation, homeostasis, and expansion of T_{reg} cells,^{29,30} we examined IL-2R receptor (CD25 protein) expression on the surface of T_{reg} cells. Surface CD25 expression on T_{reg} 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⁺CD4⁺ T cells, although there was no increase in Bcl-xl and Bcl-2 expression in T_{reg} cells of either thymectomy or nonthymectomy mice (Figure 4, D and E). These results suggest that the impaired expansion of T_{reg} cells in thymectomy mice

may involve a pathway independent of survival signaling by the Bcl-2 family.

pT_{reg}-Cell Expansion in Thymectomy NFS/*sld* Mice

Although tT_{reg} cells are derived from thymic differentiation, it is now apparent that conventional peripheral naive CD4⁺ T cells can be converted to Foxp3⁺ T_{reg} cells, called pT_{reg} cells.¹⁷ The transcription factor Helios is one of the markers that can be used to discriminate between tT_{reg} cells and pT_{reg} cells.³¹ Thus, we analyzed these T_{reg} cell subsets in thymectomy mice using Foxp3 and Helios expression.

The numbers of both Helios⁺ Foxp3⁺ tT_{reg} cells and Helios⁻ Foxp3⁺ pT_{reg} cells in the spleen and CLNs of thymectomy mice were significantly lower than those of

Figure 6 *In vitro* induction of peripherally induced T-regulatory (pT_{reg}) cells. **A:** Freshly isolated naive T cells (5×10^4 cells per well) from the spleen of nonthymectomy (non-Tx) and Tx mice were stimulated with anti-CD3/28 monoclonal antibody-coated bead/cell ratio of 0.25:1 and 30 U/mL of interleukin (IL)-2 in the presence of 1, 5, or 10 ng/mL of transforming growth factor (TGF)- β . After culture for 4 days, intracellular Foxp3 and Helios expression was determined by flow cytometry. **B:** Proportions of Helios⁺Foxp3⁺ cells in non-Tx and Tx mice. Results are expressed as means $\pm$ SD. $n = 3$ independent experiments (**A** and **B**). * $P < 0.05$, ** $P < 0.01$ versus non-Tx mice.

nonthymectomy mice (Figure 5A). In contrast, the proportion of tT_{reg} cells in the spleen and CLNs of thymectomy mice was considerably higher than that of nonthymectomy mice (Figure 5B). The ratio of pT_{reg} cell to tT_{reg} 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 T_{reg} cell subset,³² was used to distinguish pT_{reg} and tT_{reg} cells in thymectomy mice. The ratio of CD304^{low} Foxp3⁺ pT_{reg} cells to CD304^{high} Foxp3⁺ tT_{reg} 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 pT_{reg} cells is impaired in thymectomy mice.

In Vitro Induction of pT_{reg} Cells

Although a reduction in tT_{reg} cell counts was expected in thymectomy mice, it was unclear why the expansion or maintenance of pT_{reg} cells was impaired. To elucidate the mechanism underlying pT_{reg} cell maintenance, purified CD4^{low}CD62L⁺ naive CD4⁺ 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- β to differentiate and expand them into pT_{reg} cells. The differentiation of naive CD4⁺ T cells to Helios⁺Foxp3⁺ pT_{reg} cells from thymectomy mice was significantly suppressed compared with that of naive CD4⁺ T cells from nonthymectomy mice (Figure 6, A and B).

Whether expansion of pT_{reg} cells in thymectomy-treated C57BL/6 (B6) mice is 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- β for 7 days. Helios⁺Foxp3⁺ pT_{reg} cells from thymectomy B6 mice were also significantly suppressed compared with that of naive CD4⁺ T cells from

nonthymectomy B6 mice (Supplemental Figure S3). The result suggests that thymectomy induces impaired differentiation and expansion of pT_{reg} cells in any mouse strain.

TGF- β R and Smad Expression on Naive T Cells from Thymectomy Mice

Because TGF- β signaling is important for the generation of pT_{reg} cells,¹⁵ we examined the mRNA expression of TGF- β R1, TGF- β R2, and TGF- β R3 in CD4⁺CD25⁺ T cells. TGF- β R1 and TGF- β R2 mRNA expression levels in these

Figure 7 mRNA expression of transforming growth factor (TGF)- β receptors (TGF- β R) and Smads in naive CD4⁺ T cells from Tx mice. Naive CD4⁺ T cells from the spleen of non-Tx (white bars) and Tx (black bars) mice at 10 to 12 weeks of age were used to determine TGF- β R1, -R2, -R3, Smad2, Smad3, and Smad4 mRNA expression by quantitative RT-PCR. Target mRNA expression is relative to β -actin ($\times 10^{-3}$). Results are expressed as means $\pm$ SD. $n = 4$ mice per group. * $P < 0.05$, ** $P < 0.01$ versus non-Tx mice.

Figure 8 Cytokine production by T-regulatory (T_{reg}) cells from thymectomy (Tx) mice. **A** and **B**: Foxp3⁻CD4⁺ (**A**) and Foxp3⁺CD4⁺ (**B**) cells among CD4⁺ T cells from the cervical lymph nodes (CLNs) of non-Tx and Tx mice at 10 weeks of age were stimulated with 20 ng/mL of phorbol myristate acetate and 500 ng/mL of ionomycin. After culture for 6 hours, interferon (IFN)-γ, interleukin (IL)-4, IL-2, IL-17, and IL-10-producing cells were detected by intracellular staining. **C** and **D**: Intracellular IFN-γ production and CD62L expression of T_{reg} (Foxp3⁺CD4⁺), pT_{reg} (Foxp3⁺Helios⁻CD4⁺), and tT_{reg} (Foxp3⁺Helios⁺CD4⁺) cells from non-Tx and Tx mice were analyzed by flow cytometry. **E**: Purified T_{reg} cells (1 × 10⁵ cells per well) from the spleen of non-Tx and Tx mice were stimulated with anti-CD3/28 monoclonal antibody-coated beads at a 2:1 bead/cell ratio and 2000 U/mL of IL-2 for 10 days. IFN-γ concentrations of the culture supernatants at 0 to 3 and 7 to 10 days were analyzed by enzyme-linked immunosorbent assay. Results are expressed as means ± SD (**A–E**). *n* = 4 mice per group (**A–E**); *n* = 3 mice per group (**E**) from two independent experiment (**A–E**). ***P* < 0.01 versus non-Tx mice. pT_{reg}, peripherally induced T regulatory; tT_{reg}, thymus-derived T regulatory.

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 pT_{reg} 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 pT_{reg} cells via the TGF-β/Smad pathway in the periphery of thymectomy mice was impaired in addition to the decreased generation of tT_{reg} cells in the thymus.

T_{reg} Cells from Thymectomy Mice Produce IFN-γ

T_{reg} 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 T_{reg} 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 T_{reg} 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, T_{reg} cells, including tT_{reg} and pT_{reg} 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 T_{reg} cell subsets was analyzed using flow cytometry, indicating that IFN-γ production by CD62L⁺IFN-γ⁺ cells in both pT_{reg} and tT_{reg} cells stimulated with phorbol myristate acetate/ionomycin was significantly higher in those from

thymectomy mice than from nonthymectomy mice (Figure 8, C and D).

To measure IFN- γ secretion from expanded T_{reg} cells of thymectomy mice, purified T_{reg} 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 T_{reg} cells of thymectomy mice was significantly higher than that of nonthymectomy mice (Figure 8E). These results suggest that T_{reg} 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 T_{reg} cells, is vigorously promoted during the neonatal period. In this study, we focused on the dynamics and functions of T_{reg} cells derived from neonatal thymectomy mice to determine the association between T_{reg} cells and autoimmunity in these mice. Our results indicate that a reduction in T_{reg} 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.^{1–9} Thus, the expansion ability of T_{reg} cells in thymectomy mice was insufficient to compensate for the shortage in T_{reg} cell counts.

We found that induction of T_{reg} cells from peripheral naive CD4⁺ T cells was suppressed in thymectomy mice. Although the *in vivo* function of pT_{reg} cells remains uncertain, several studies have reported that pT_{reg} 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 T_{reg} 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 pT_{reg} cells from naive T cells. Several markers of T_{reg} cell subsets have been reported, including Helios and CD304.^{31,32,46} In the present study, a reduction of the ratio of pT_{reg} cells to tT_{reg} 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 tT_{reg} cells.³¹ CD304 is also a surface marker of tT_{reg} cells.³²

TGF- β plays a key role in the generation and expansion of T_{reg} cells. TGF- β signaling is required for the development of tT_{reg} cells in the thymus, possibly by promoting their survival.¹⁵ In addition, TGF- β promotes the differentiation and expansion of pT_{reg} cells by promoting Foxp3 expression in naive T cells.¹⁵ The binding of TGF- β to TGF- β R activates the structurally similar transcription factors Smad2 and Smad3 in target cells.⁴⁷ 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- β R signaling.⁴⁸

TGF- β induces the rapid activation of TGF- β -activated kinase 1, Ras-Erk, and PI3K-Akt pathways.⁴⁸ In the present study, mRNA expression of TGF- β RII, TGF- β RIII, 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 T_{reg} cell-mediated suppression, which is consistent with the finding that lower levels of TGF- β R and Smad are expressed in naive T cells in this model.

We also found that T_{reg} 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 T_{reg} cells are capable of secreting proinflammatory cytokines for maintaining Foxp3 expression.^{41,44,49–51} 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 T_{reg} cells is unstable and that T_{reg} 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 T_{reg} cells and transfer of nonthymectomy T_{reg} cells even though there was no difference in *in vitro* suppression between nonthymectomy and thymectomy T_{reg} cells. Thus, it is possible that T_{reg} cells in thymectomy mice exhibit an effector cell-like phenotype that does not fully promote autoimmunity in addition to the reduced T_{reg} cell counts observed in mice.

Neonatal immune responses are considered to be immunologically immature.⁵⁷ 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 potentially 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 T_{reg} cells are known to contribute to the onset or development of SS.⁶¹ However, whether the expansion or function of pT_{reg} cells influences the pathogenesis of SS remains unclear. In the present study, we focused on the unique phenotype and function of T_{reg} cells in this SS model to elucidate the association between T_{reg} 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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