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

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Running title: Impaired function of T_{reg} cells in a SS model

Abbreviations used in this article: Tx, thymectomy; T_{reg} cell, regulatroty T cell; SS, Sjögren's syndrome; pTreg, peripherally induced T_{reg}; tTreg, thymus-derived T_{reg}

Abstract

Neonatal thymectomy (Tx) 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 Tx 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's syndrome (SS) model, NFS/*sld* mice that underwent neonatal Tx. The ratio of T_{reg} cells to effector memory phenotype T cells in Tx mice was significantly lower than that of non-Tx mice. In addition, *in vitro* induction of peripherally induced T_{reg} cells by transforming growth factor-β (TGF-β) using naïve T cells from SS model mice was severely impaired. The mRNA expression of TGF-β receptor I, II, and Smad3 and -4 in the TGF-β-induced signal transduction pathway of T_{reg} cells in this SS model were lower than those of control mice. In addition, T_{reg} cells in this SS model exhibited an IFN-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.

Introduction

Neonatal thymectomy (Tx) in mice is a classical manipulation used to induce the breakdown of immunological tolerance. Tx in certain strains of mice triggers the onset of organ-specific autoimmune disease.[1-9] However, the precise mechanism underlying Tx-induced organ-specific autoimmune disease remains unclear. Two possible mechanisms have been proposed to explain the association between lymphopenia and autoimmunity in Tx mice. One hypothesis is that Tx 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 (MHC) molecules of professional antigen-presenting cells (APCs) to become activated and expand. They then migrate to non-lymphoid 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 Tx mice than in adult non-Tx mice.[11] However, more recent studies have showed 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 Tx mice than that in non-Tx mice.[12,13] In addition, the immunosuppressive functions of T_{reg} cells from Tx-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} cells in autoimmunity induced by neonatal Tx remains unclear.

Another possibility derives from other recent studies presenting evidence that T_{reg}

cells can also be generated outside of the thymus. These extrathymically generated T_{reg} cells have been designated induced T_{reg} (iT_{reg}) or peripherally induced T_{reg} (pT_{reg}) cells. pT_{reg} cells are converted from naïve $CD4^+$ T cells in the presence of TGF-β and IL-2.[15,16] *In vitro*, pT_{reg} cells can acquire an immunosuppressive function similar to that of naturally occurring T_{reg} (nT_{reg}) or thymus-derived T_{reg} (tT_{reg}) cells.[17,18] Several studies have shown that the adoptive transfer of pT_{reg} cells generated *ex vivo* from naïve 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 Tx mice. The molecular mechanism underlying *in vivo* expansion of pT_{reg} cells in Tx mice is completely obscure.

Sjögren's 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 Tx 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 demonstrated that autoreactive $CD4^+$ T cells played a pivotal role in the development of autoimmune exocrinopathy in these Tx 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 Tx on T_{reg} subpopulations relative to numbers and effector functions in the NFS/*sld* mouse. In addition, the pathogenesis of autoimmunity in this neonatal Tx 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 (Permit Number: toku-09021).

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 from purchased from the Japan SLC Laboratory (Shizuoka, Japan) was performed on day 3 after birth. Neonatal and 8−18-week-old mice were used for experiments.

Histology

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

Confocal microscopic analysis

Frozen sections of the salivary glands from non-Tx and Tx 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 FITC-conjugated antibody against Foxp3. Nuclear DNA was stained with 4′,6-diamino-2-phenylindole dihydrochloride (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.

T_{reg} cell isolation

Single-cell suspensions were prepared from spleen and cervical lymph nodes, 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. Over 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, GITR, 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-CTLA-4, Bcl-xl, Bcl-2 and 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, USA) at a bead to

cell of 1:1 for 20 h. After washing, cells were stained with an anti-CD4 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 cervical lymph nodes were stimulated with 20 ng/ml of phorbol myristate acetate (PMA; Sigma-Aldrich, St. Louis, MO) and 500 ng/ml of ionomycin (Sigma-Aldrich) for 6 h in the presence of brefeldin A (eBioscience) for the last 4 h. Flow cytometric analysis was carried out using a BD FACSCant flow cytometer (BD Biosciences). Data were analyzed using FlowJo FACS Analysis software (Tree Star, Ashland, OR).

Suppression assay

Cell proliferation was determined using 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular probes, Eugene, OR, USA). Magnetically isolated $CD4^+CD25^-$ conventional T cells (T_{conv} cells) were labeled with CFSE according to the manufacturer's instructions. CFSE-stained $CD4^+CD25^-$ T_{conv} cells (2.5×10^4/well) were incubated with purified T_{reg} cells (2.5×10^4/well) for 3 days in the presence of Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) at bead to 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-stained and magnetically isolated T_{reg} cells (5×10^4 cells/well) were cultured with Dynabeads Mouse T-Activator CD3/CD28 at a bead to 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/well) were incubated for 7 days with Dynabeads Mouse T-Activator CD3/CD28 at a bead to T_{reg} cell ratio of 2:1 and 2000 U/ml of IL-2.[28]

Induction of T_{reg} cells

$CD44^{low}CD62L^+$ naïve $CD4^+$ T cells were purified from spleens using EasySep purification kits (Stemcell Technologies). Purified naïve $CD4^+$ T cells (5 × 10^4 cells/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 naïve $CD4^+$ cells from spleen in non-Tx and Tx NFS/*sld* mice were expanded for 3 weeks as described in T_{reg} Expansion assay. 1× 10^6 T_{reg} cells/mouse were transferred intravenously into Tx 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 histological examination. Formalin-fixed tissue sections were subjected to hematoxylin-eosin (HE) staining, and three pathologists independently evaluated the histology without being informed of the condition of each mouse. The number of infiltrating lymphocytes in the salivary gland specimens was counted per 0.25 mm^2.

Quantitative RT-PCR analysis

Total RNA was isolated from splenic naïve $CD4^+$ 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′-AACTGAAACACCGTGGGAAC-3′ and reverse, 5′-TGGGAAGCTTTCAGTTGACC-3′; TGF-βRII: forward, 5′-CCCAGTCTGGAAATGAAAGC-3′ and reverse, 5′-ACTTTTGTCGTGGGTTCTGG-3′; TGF-βRIII: forward, 5′-TCAGATTTGTGCCTGTCTCG-3′ and reverse, 5′-CTGGGTGTTCTGCATTTGTG-3′; Smad2: forward, 5′-TCCCATTCCTGTTCTGGTCC-3′ and reverse, 5′-ACTGCCCACACAAACCTTTC-3′; Smad3: forward, 5′-TGATGGCTTCTGTTCTGGTG-3′ and reverse, 5′-GGCACTTTCCTTTTGGACTG-3′; Smad4: forward, 5′-ATGGCTATGTGGATCCTTCG-3′ and reverse, 5′-TCAATCGCTTCTGTCCTGTG-3′; β-actin: forward, 5′-GTGGGCCGCTCTAGGCACCA-3′ and reverse, 5′-CGGTTGGCCTTAGGGTTCAGGGGG-3′. Relative mRNA expression of each transcript was normalized against β-actin mRNA.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of IFN-γ in supernatants was measured by ELISA. 96-well flat-bottomed plates were pre-coated 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 (OPD; 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 ± standard deviation (SD). Comparisons of the results between two groups were made by unpaired, two-tailed Student's *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 Tx. There were no differences in the proportions of $Foxp3^+$ T_{reg} cells in the thymus between 3 and 7 day after birth (Figure 1A). The proportions of $Foxp3^+$ T_{reg} cells in the spleen of both 3-day- and 7-day-old mice were approximately 12−13% (Figure 1B), although the numbers of $Foxp3^+$ T_{reg} cells in the spleen of 7-day-old mice were dramatically 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.

Number of Peripheral T_{reg} Cell in the Tx SS Model

Next, we investigated T_{reg} cells in Tx 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 Tx mice was higher than that in non-Tx control mice (Figure 2A), the actual number of T_{reg} cells in both the spleen and cervical lymph nodes (CLNs) of Tx mice was significantly lower than that in non-Tx mice (Figure 2C). In addition, the number of $CD4^+$ T cells in Tx mice was significantly lower than that in non-Tx mice (Figure 2C).

The proportion of $CD44^{high}CD62L^-$ effector memory phenotype $CD4^+$ T cells of the spleen and CLNs was dramatically higher in Tx mice than in non-Tx mice (Figure 2D). In addition, the ratio of T_{reg} cells to effector memory T cells in the spleen and CLNs of Tx mice was significantly lower than that of non-Tx mice (Figure 2E).

To assess whether T_{reg} cells had migrated into the salivary glands as a target organ in Tx mice, $Foxp3^+T_{reg}$ cells were examined using a confocal microscope. $Foxp3^+CD4^+T_{reg}$ cells had infiltrated into the salivary glands of Tx 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 Tx mice.

Suppressor Function of T_{reg} Cells in Tx Mice

Next, the regulatory function of T_{reg} cells in Tx mice was investigated. To assess the phenotypes of T_{reg} cells, we examined the expression of hallmark proteins in T_{reg} cells, including cytotoxic T-lymphocyte-associated antigen (CTLA)-4 and glucocorticoid-induced tumor necrosis factor-receptor (GITR). The CTLA-4 expression levels on $Foxp3^+$ $CD4^+$ T_{reg} cells from the spleen and CLNs of Tx mice were similar to those of cells derived from non-Tx mice (Figure 3A). In addition, there was no difference in GITR expression on T_{reg} cells from the spleen and CLNs from Tx and non-Tx 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 non-Tx mice were labeled with CFSE and cultured for 72 h together with unlabeled $CD4^+CD25^+$ T_{reg} cells in the presence of anti-CD3/CD28 mAbs. T_{reg} cells from both non-Tx and Tx mice suppressed the proliferation of effector T cells (Figure 3B). In addition, there was no difference in T_{reg} cell suppression activity between cells derived from Tx and non-Tx mice when using $CD25^-CD4^+$ T cells from Tx mice as effector cells (Supplementary Figure 1). Furthermore, there was no significant difference in the suppression function of T_{reg} cells from non-Tx and Tx mice by *in vitro* suppression assay with different ratios of effector and T_{reg} cells (Supplementary Figure 2). These results suggest that T_{reg} cells from Tx mice have the same *in vitro* immunosuppressive ability as those from non-Tx mice.

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

T_{reg} Cell Expansion in Tx Mice

To investigate the peripheral expansion of T_{reg} cells in Tx 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 Tx mice was significantly lower than that of T_{reg} cells from non-Tx mice (Figure 4A). After 7 days of T_{reg} cell culture, the total number of T_{reg} cells from Tx mice was markedly lower than that from non-Tx 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 Tx and non-Tx mice (Figure 4C).

We next examined the expression of Bcl-xl and Bcl-2, anti-apoptotic 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 Tx or non-Tx mice (Figure 4D, E). These results suggest that the impaired expansion of T_{reg} cells in Tx mice may involve a pathway independent of survival signaling by the Bcl-2 family.

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

While tT_{reg} cells are derived from thymic differentiation, it is now apparent that conventional peripheral naïve $CD4^{+}$ 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 Tx 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 Tx mice were significantly lower than those of non-Tx mice

(Figure 5A). In contrast, the proportion of tT_{reg} cells in the spleen and CLNs of Tx mice was considerably higher than that of non-Tx mice (Figure 5B). The ratio of pT_{reg} cell to tT_{reg} cells in the spleen and CLNs of Tx mice was significantly lower than that of non-Tx 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 Tx 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 Tx mice than that in non-Tx mice (Figure 5C). These findings suggest that the differentiation of pT_{reg} cells is impaired in Tx mice.

In Vitro Induction of pT_{reg} Cells

Although a reduction in tT_{reg} cell numbers was expected in Tx 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 $CD44^{low}CD62L^{+}$ naïve $CD4^{+}$ T cells from the spleen of non-Tx and Tx 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 naïve $CD4^{+}$ T cells to $Helios^{-}Foxp3^{+}$ pT_{reg} cells from Tx mice was significantly suppressed compared with that of naïve $CD4^{+}$ T cells from non-Tx mice (Figure 6A, B).

Whether expansion of pT_{reg} cells in Tx-treated C57BL/6 (B6) mice is impaired was unclear. Purified naive T cells of spleen from non-Tx and Tx 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 Tx B6 mice were also significantly suppressed compared with that of naïve $CD4^{+}$ T cells from non-Tx B6 mice (Supplemental Figure 3). The result suggests that Tx induces impaired differentiation and expansion of pT_{reg}

cells in any mouse strain.

TGF-β Receptor and Smad Expression on Naïve T Cells from Tx Mice

Because TGF-β signaling is important for the generation of pT_{reg} cells,[15] we examined the mRNA expression of TGF-βRI, TGF-βRII, and TGF-βRIII in $CD4^+CD25^-$ T cells. TGF-βRI and TGF-βRII mRNA expression levels in these cells from Tx mice were significantly lower than those in cells from non-Tx mice (Figure 7). In addition, the mRNA expression of Smad3 and -4, key molecules for TGF-β receptor signaling during pT_{reg} expansion,[33-35] in cells from Tx mice were also significantly lower in cells from Tx mice than in those from non-Tx 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 Tx mice was impaired in addition to the decreased generation of tT_{reg} cells in the thymus.

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

T_{reg} cells employ 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 pro-inflammatory 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 Tx mice, we examined intracellular cytokine production in purified $CD4^+$ cells after stimulation with PMA/ionomycin.

$Foxp3^-CD4^+$ effector cells from Tx 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 Tx mice (Figure 8A). However, T_{reg} cells, including tT_{reg} and pT_{reg} cells, from Tx 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 IL10 production between cells from non-Tx and Tx mice (Figure 8B). IFN-γ production by T_{reg} cell subsets was analyzed using flow cytometry, indicating that IFN-γ production by $CD62L^{-}IFN\text{-}\gamma^{+}$ cells in both pT_{reg} and tT_{reg} cells stimulated with PMA/ionomycin was significantly higher in those from Tx mice than from non-Tx mice (Figure 8C, D).

To measure IFN-γ secretion from expanded T_{reg} cells of Tx mice, purified T_{reg} cells were stimulated with anti-CD3/28 mAbs in the presence of IL-2 for 10 days. The supernatants from 0−3 and 7−10 days were analyzed by ELISA. The IFN-γ concentration in the supernatant from T_{reg} cells of Tx mice was significantly higher than that of non-Tx mice (Figure 8E). These results suggest that T_{reg} cells in Tx 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 Tx mice to determine the relationship between T_{reg} cells and autoimmunity in these mice. Our results showed that a reduction in T_{reg} cell numbers in the periphery of Tx 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 Tx mice was insufficient to

compensate for the shortage in T_{reg} cell numbers.

We showed that induction of T_{reg} cells from peripheral naive $CD4^+$ T cells was suppressed in Tx 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, SLE, type 1 diabetes, gastritis, colitis, and MS.[15,19-22,45] The results of the present study demonstrate that a reduced ratio of T_{reg} cells to effector T cells may influence the onset or development of autoimmunity in Tx mice and suggest that neonatal thymectomy interferes with the differentiation and expansion of pT_{reg} cells from naïve 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 Tx 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 naïve T cells.[15] The binding of TGF-β to TGF-βR 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-βR 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-βRII, TGF-βRIII, Smad2, and Smad3 in naïve 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-βR and Smad are expressed in naïve T cells in this model.

We also found that T_{reg} cells from Tx mice had different cytokine production profiles compared with those from control mice. The cells from Tx mice preferentially produced a pro-inflammatory cytokine, IFN-γ, similar to activated conventional $CD4^+$ T cells. Recent studies have suggested that T_{reg} cells are capable of secreting pro-inflammatory 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 demonstrated 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 Tx T_{reg} cells and transfer of non-Tx T_{reg} cells even though there was no difference in *in vitro* suppression between non-Tx and Tx T_{reg} cells. Thus, it is possible that T_{reg} cells in Tx mice exhibit an effector cell-like phenotype that does not fully promote autoimmunity in addition to the reduced T_{reg} cell numbers observed in Tx 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 potently contributes 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 Tx.[9,25] We investigated the pathogenesis of 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 relationship between T_{reg} cells and organ-specific autoimmunity.

In conclusion, T_{reg} cells in Tx mice were insufficient for regulating effector T cells and protecting autoimmunity with respect to cell numbers and function. The results of this study will be useful for understanding the pathogenesis of Tx-induced autoimmune diseases and the development of new autoimmunity therapies with T_{reg} cells.

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Figure legends

Figure 1 Numbers of T_{reg} cells in the thymus and spleen of neonatal mice at 3 and 7 days after birth. **A, B**: CD4/CD8 and $CD4^+Foxp3^+$ cell populations in the thymus (**A**), and spleen (**B**) from 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 presented as the mean ± SD for 5 mice per condition.

Figure 2 Numbers of T_{reg} cells in peripheral lymphoid tissues. **A**: Frequency of $Foxp3^+$ cells gated on $CD4^+$ cells in spleen and cervical lymph nodes (CLNs) of non-Tx and Tx mice at 10 weeks of age was determined by flow cytometry. Results are representative of 5 mice per each group. Results are presented as the mean ± SD of 5 mice per group. *p <0.05 vs. non-Tx mice. **B**: Numbers of $CD4^+$ and $Foxp3^+CD4^+$ T cells in the spleen and CLNs of non-Tx and Tx mice were determined by flow cytometry. Results are presented as the mean ± SD of 5 mice per group. *p <0.05, **p <0.01 vs. non-Tx mice. **C**: Effector memory phenotype of $CD4^+$ T cells in spleen and CLNs was analyzed by flow cytometry. Results are representative of 5 mice. Ratio of effector/memory to naïve T cells is presented as the mean ± SD of 5 mice per group. *p <0.05, **p <0.01 vs. non-Tx mice. **D**: Ratio of T_{reg} to effector/memory T cells is presented as the means ± SD of 5 mice per group. *p <0.05 vs. non-Tx mice. **E**: Histological analysis of salivary glands (SGs) from Tx mice (H&E staining; left). Scale bar: 100μm. $CD4^+$ T cells (red), $Foxp3^+$ cells (green), and cell nuclei (blue) in SGs were observed under a confocal microscope (right). Results are representative of 5 mice. Scale bar: 10 μm.

Figure 3 Surface phenotypes and immunosuppressive functions of T_{reg} cells. **A**: CTLA-4 and GITR expressions on $CD4^+Foxp3^+$ cells in the spleen and CLNs of non-Tx and Tx mice at 10−12 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. Representative results of 3 independent experiments are shown. **B**: CFSE-labeled $CD4^+CD25^-$ T cells (2.5×10^4 cells/well) from Tx mice were cultured with unlabeled T_{reg} cells (2.5×10^4/well) from non-Tx and Tx mice along with anti-CD3/28 mAb-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. Representative results of 3 independent experiments are shown. **C**: T_{reg} cells (1×10^6 cells) expanded from naïve $CD4^+$ T cells of spleen in non-Tx and Tx mice were transferred intravenously into Tx mice. 8 weeks later salivary gland tissues of the recipient mice were histopathologically analyzed. Photos are representative of 5 mice per group. Scale bar: 100 μm. **D**: The number of infiltrating lymphocytes in 0.25 mm^2 of salivary gland tissue was counted, and results are presented as the mean ± SD of 5 mice per group. *p <0.05, ***p <0.005 vs. Tx mice.

Figure 4 T_{reg} cell expansion in Tx mice. **A**: CFSE-labeled purified T_{reg} cells (5×10^4 cells/well) from non-Tx and Tx mice were cultured with anti-CD3/28 mAbs-coated beads at a bead: to cell ratio of 0.25:1 and 100 U/ml of IL-2. After culture for 3 days, T_{reg} cell division was determined by CFSE dilution. Results are presented as the mean ± SD of 3 mice per group. *p <0.05 vs. non-Tx mice. **B**: T_{reg} cells (1×10^5 cells/well) from the spleen of non-Tx and Tx mice were stimulated with anti-CD3/28 mAbs-coated beads at a 2:1 bead to 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. Results are presented as the mean ± SD of 3 independent experiments. **p <0.01 vs. non-Tx mice. **C**: CD25 expression on $CD4^+Foxp3^+$ cells from the spleen and CLNs of non-Tx (dotted lines) and Tx (solid lines) mice was determined by flow cytometry. Results are representative of 5 mice per group. **D, E**: $CD4^+$ T cells (2×10^5 cells/well) from the spleen of non-Tx and Tx mice were cultured with anti-CD3/28 mAbs-coated beads at a 1:1 bead to cell ratio. After culture for 20 h, Bcl-xl and Bcl-2 expression in $Foxp3^-CD4^+$ and $Foxp3^+CD4^+$ cells was determined by flow cytometry. Results are representative of 3 independent experiments.

Figure 5 pT_{reg} cells in Tx mice. **A**: Numbers of $Foxp3^+CD4^+$ cells, $Foxp3^+Helios^+CD4^+$ cells, and $Foxp3^+Helios^-CD4^+$ cells in the spleen and CLNs of non-Tx and Tx mice at 10 weeks of age were determined by flow cytometry. Results are presented as the mean ± SD of 3 mice per group. *p <0.05 vs. non-Tx mice. **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. Profiles are representative of 5 mice per group. Ratio of $Helios^-Foxp3^+$ to $Helios^+Foxp3^+$ cells is presented as the mean ± SD of 3 mice per group. *p <0.05 vs. non-Tx mice. **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. Profiles are representative of 3 mice per group. Ratio of $CD304^{-/low}Foxp3^+$ to $CD304^{high}Foxp3^+$ cells is presented as the mean ± SD of 3 mice per group. *p <0.05 vs. non-Tx mice.

Figure 6 *In vitro* induction of pT_{reg} cells. **A**: Freshly isolated naïve T cells (5×10^4 cells/well) from the spleen of non-Tx and Tx mice were stimulated with anti-CD3/28

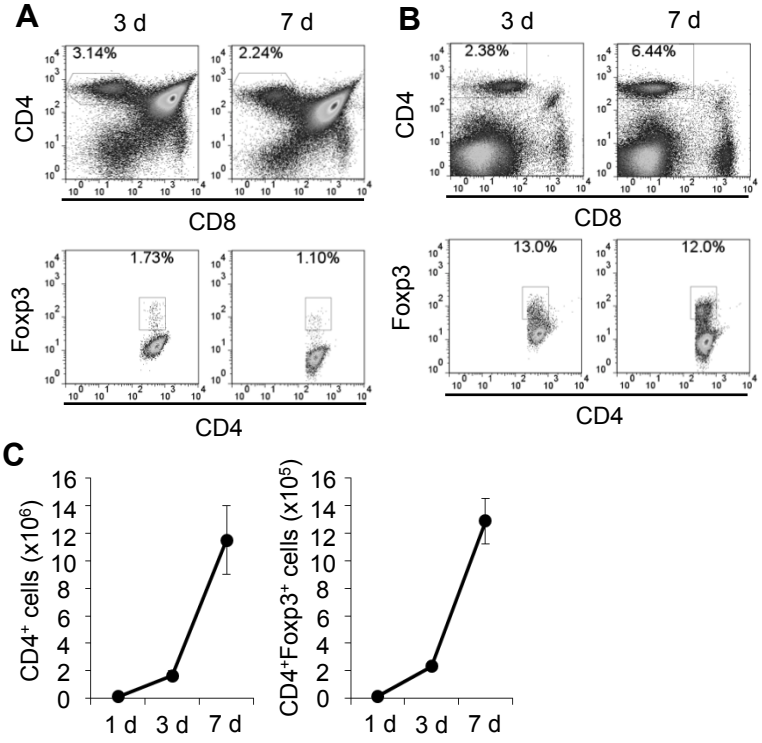
mAbs-coated bead to cell ratio of 0.25:1 and 30 U/ml of IL-2 in the presence of 1, 5, or 10 ng/ml of TGF-β. After culture for 4 days, intracellular Foxp3 and Helios expression were determined by flow cytometry. Representative results of 3 independent experiments are shown. **B**: Proportions of $Helios^{-}Foxp3^{+}$ cells in non-Tx and Tx mice. Results are presented as the mean ± SD of triplicates from 3 independent experiments. *p <0.05, **p <0.01 vs. non-Tx mice.

Figure 7 mRNA expression of TGF-βR and Smad in naïve $CD4^{+}$ T cells from Tx mice. Naïve $CD4^{+}$ T cells from the spleen of non-Tx and Tx mice at 10−12 weeks of age were used to determine TGF-βRI, II, III, Smad2, Smad3, and Smad4 mRNA expression by quantitative RT-PCR. Target mRNA expression is relative to β-actin ($\times 10^{-3}$). Results are presented as the mean ± SD of 4 mice per group. *p <0.05, **p <0.01 vs. non-Tx mice.

Figure 8 Cytokine production by T_{reg} cells from Tx mice. **A, B**: $Foxp3^{-}CD4^{+}$ (**A**) and $Foxp3^{+}CD4^{+}$ (**B**) cells among $CD4^{+}$ T cells from the CLNs of non-Tx and Tx mice at 10 weeks of age were stimulated with 20 ng/ml of PMA and 500 ng/ml of ionomycin. After culture for 6 h, IFN-γ-, IL-4-, IL-2-, IL-17-, and IL-10-producing cells were detected by intracellular staining. **C, 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. Results are representative of 2 independent experiments. Results are presented as the mean ± SD of 4 mice per group. **p <0.01 vs. non-Tx mice. **E**: Purified T_{reg} cells (1×10^{5} cells/well) from the spleen of non-Tx and Tx mice were stimulated with anti-CD3/28 mAbs-coated beads at a 2:1 bead to cell ratio and 2000 U/ml of IL-2 for 10 days. IFN-γ concentrations of the

culture supernatants at 0−3 and 7−10 days were analyzed by ELISA. Results are presented as the mean ± SD of 3 mice per group. **p <0.01 vs. non-Tx mice.

Figure 1


A
3 d
7 d
3.14%
2.24%
CD4
CD8
1.73%
1.10%
Foxp3
CD4
B
3 d
7 d
2.38%
6.44%
CD4
CD8
13.0%
12.0%
Foxp3
CD4
C
CD4+ cells (x10^6)
16
14
12
10
8
6
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2
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1 d
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CD4+Foxp3+ cells (x10^5)
16
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1 d
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7 d

Figure 2

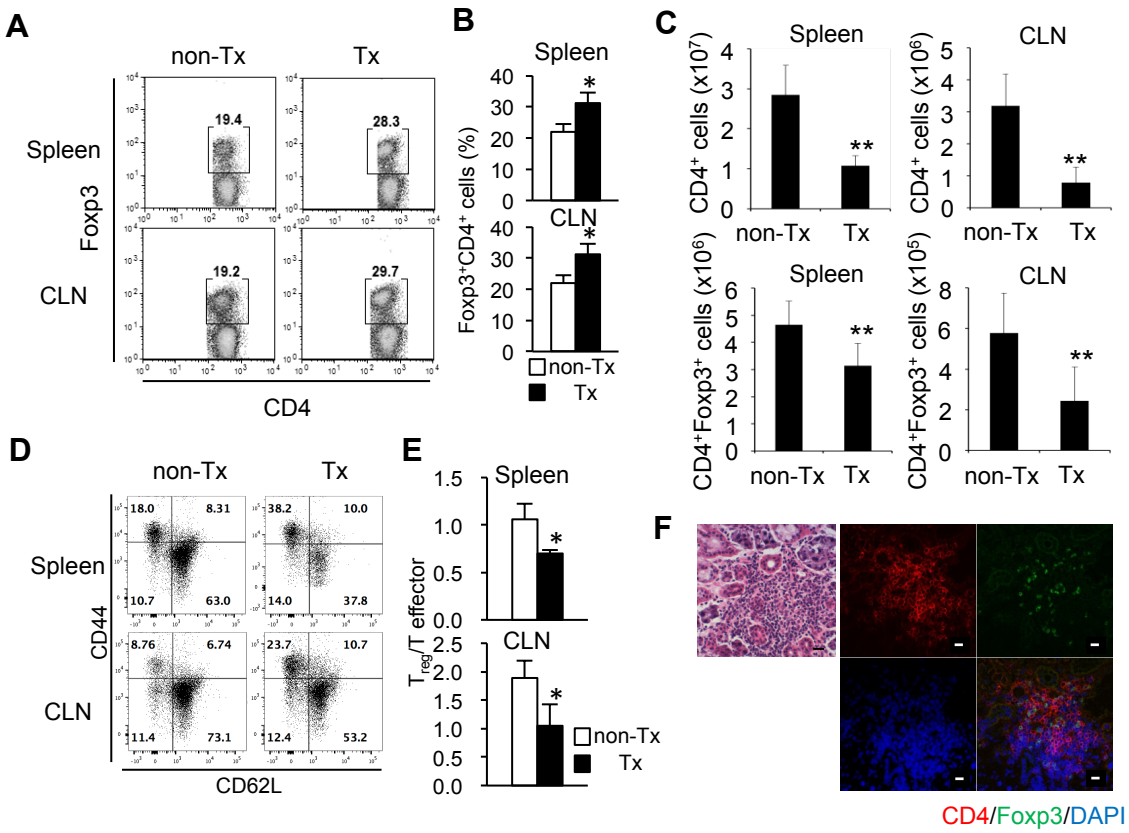

A
non-Tx
Tx
Spleen
CLN
Foxp3
CD4
19.4
28.3
19.2
29.7
B
Spleen
CLN
Foxp3+CD4+ cells (%)
non-Tx
Tx
C
Spleen
CLN
CD4+ cells (x10^7)
CD4+ cells (x10^6)
CD4+Foxp3+ cells (x10^6)
CD4+Foxp3+ cells (x10^5)
non-Tx
Tx
D
non-Tx
Tx
Spleen
CLN
CD44
CD62L
18.0
8.31
10.7
63.0
38.2
10.0
14.0
37.8
8.76
6.74
11.4
73.1
23.7
10.7
12.4
53.2
E
Spleen
CLN
Treg/T effector
non-Tx
Tx
F
CD4/Foxp3/DAPI

Figure 3

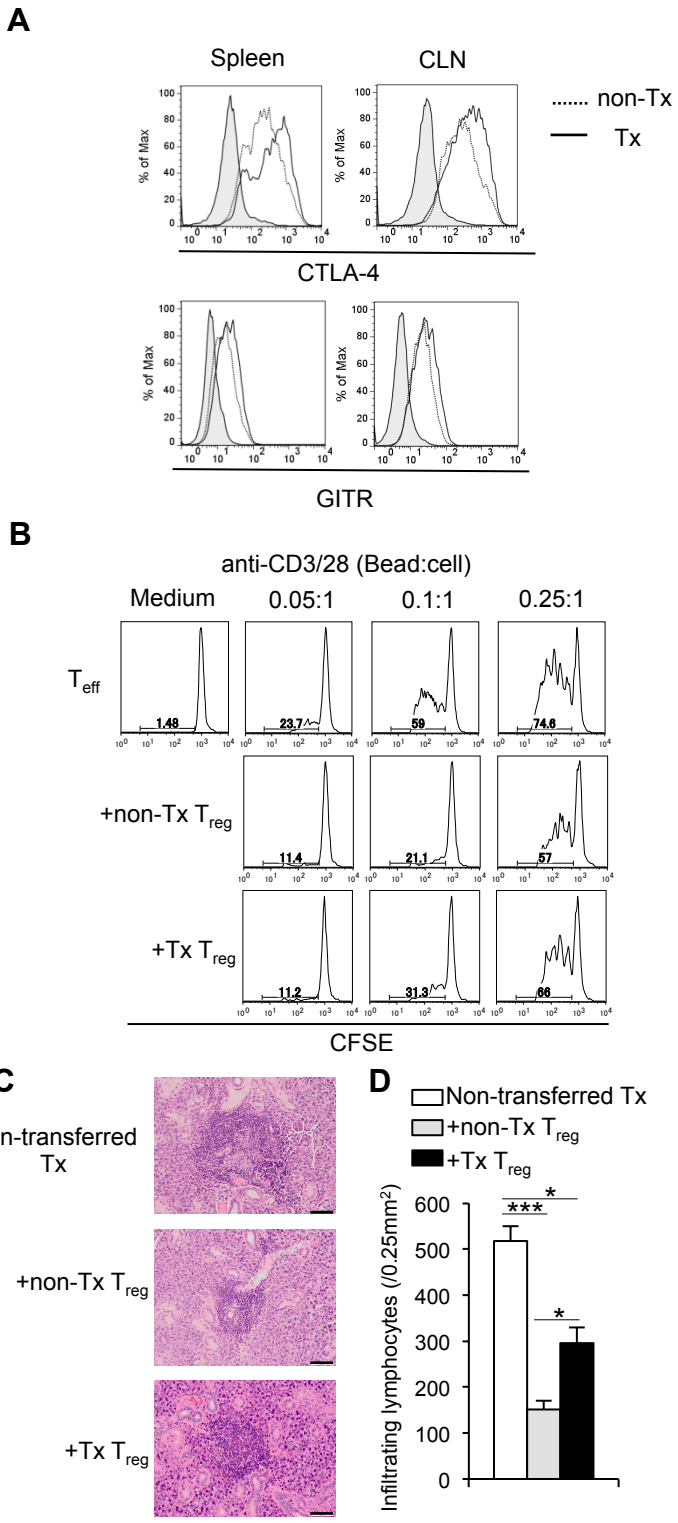

A
Spleen
CLN
..... non-Tx
Tx
% of Max
CTLA-4
GITR
B
anti-CD3/28 (Bead:cell)
Medium
0.05:1
0.1:1
0.25:1
T_eff
+non-Tx T_reg
+Tx T_reg
1.48
23.7
59
74.6
11.4
21.1
57
11.2
31.3
66
CFSE
C
Non-transferred Tx
+non-Tx T_reg
+Tx T_reg
D
Non-transferred Tx
+non-Tx T_reg
+Tx T_reg
Infiltrating lymphocytes (/0.25mm²)
600
500
400
300
200
100
0
***
*
*

Figure 4

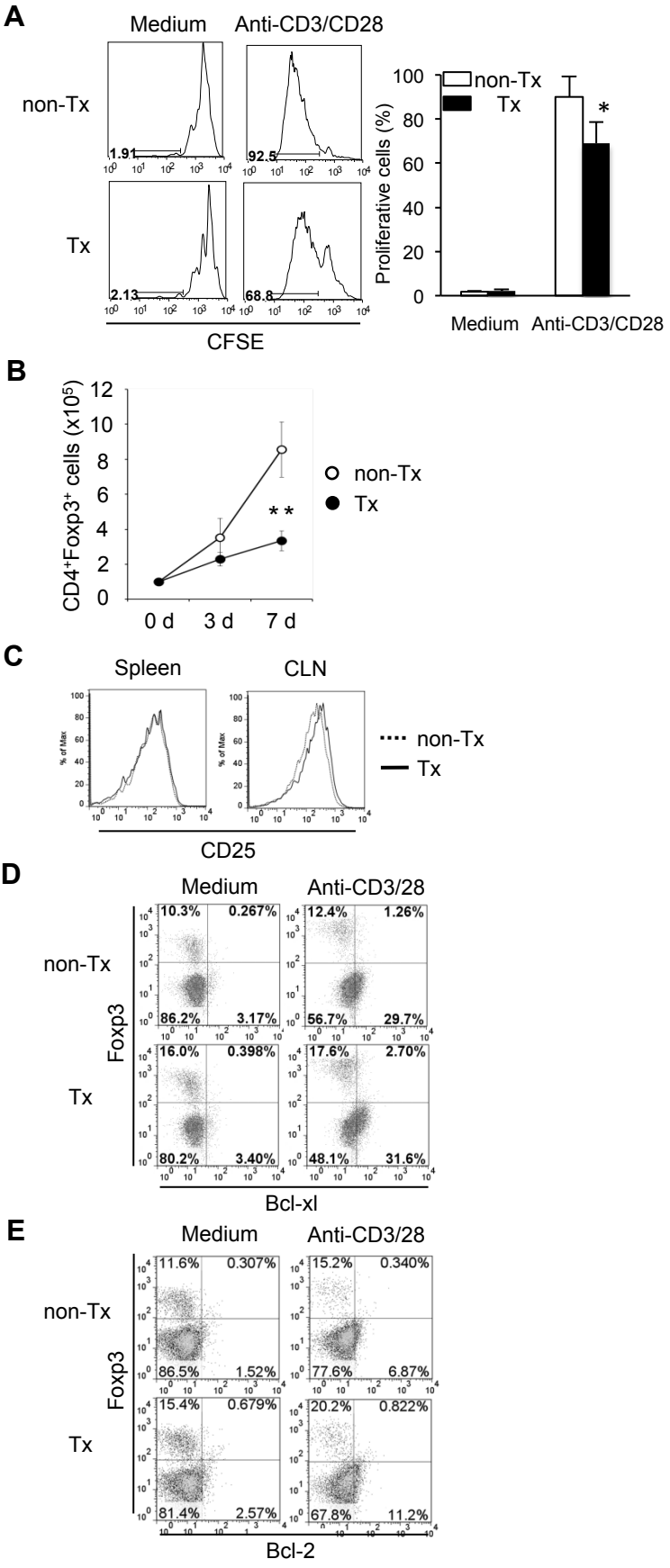

A
Medium
Anti-CD3/CD28
non-Tx
Tx
1.91
92.5
2.13
68.8
CFSE
Proliferative cells (%)
non-Tx
Tx
*
Medium
Anti-CD3/CD28
B
CD4+Foxp3+ cells (x10^5)
non-Tx
Tx
**
0 d
3 d
7 d
C
Spleen
CLN
% of Max
non-Tx
Tx
CD25
D
Medium
Anti-CD3/28
non-Tx
Tx
Foxp3
10.3%
0.267%
86.2%
3.17%
12.4%
1.26%
56.7%
29.7%
16.0%
0.398%
80.2%
3.40%
17.6%
2.70%
48.1%
31.6%
Bcl-xl
E
Medium
Anti-CD3/28
non-Tx
Tx
Foxp3
11.6%
0.307%
86.5%
1.52%
15.2%
0.340%
77.6%
6.87%
15.4%
0.679%
81.4%
2.57%
20.2%
0.822%
67.8%
11.2%
Bcl-2

Figure 5

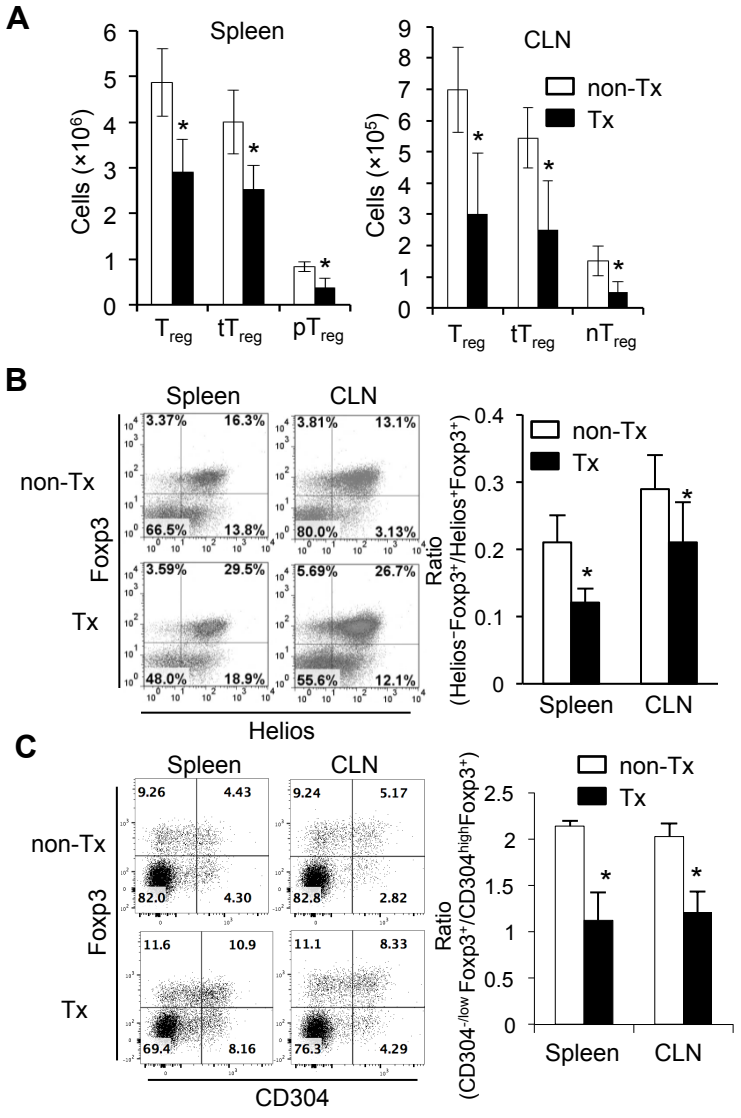

A
Spleen
CLN
non-Tx
Tx
Cells (×10^6)
Cells (×10^5)
T_reg
tT_reg
pT_reg
nT_reg
B
Spleen
CLN
non-Tx
Tx
Foxp3
Helios
3.37%
16.3%
66.5%
13.8%
3.81%
13.1%
80.0%
3.13%
3.59%
29.5%
48.0%
18.9%
5.69%
26.7%
55.6%
12.1%
Ratio
(Helios⁻Foxp3⁺/Helios⁺Foxp3⁺)
Spleen
CLN
C
Spleen
CLN
non-Tx
Tx
Foxp3
CD304
9.26
4.43
82.0
4.30
9.24
5.17
82.8
2.82
11.6
10.9
69.4
8.16
11.1
8.33
76.3
4.29
Ratio
(CD304^-/low Foxp3⁺/CD304^high Foxp3⁺)
Spleen
CLN

Figure 6

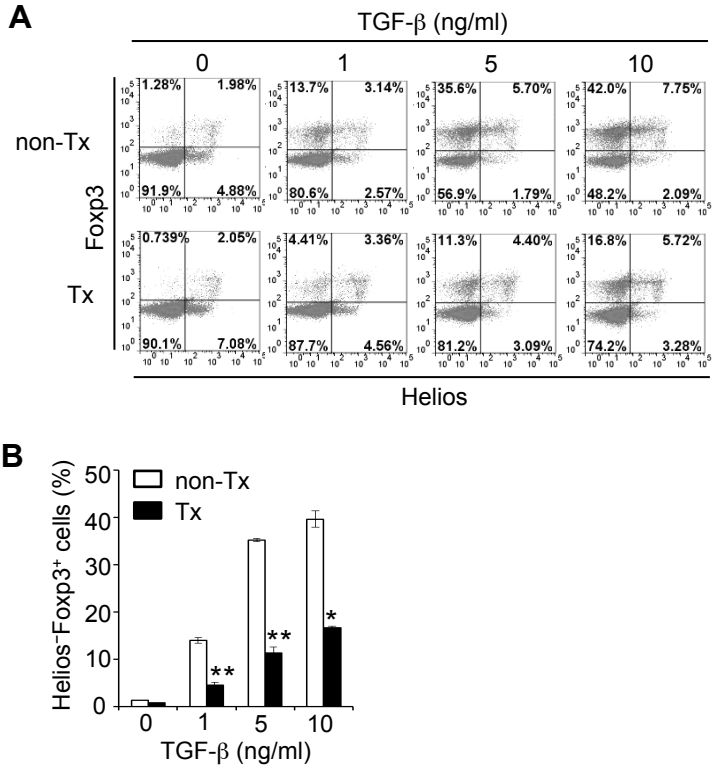

A
TGF-β (ng/ml)
0
1
5
10
non-Tx
Tx
Foxp3
1.28%
1.98%
91.9%
4.88%
13.7%
3.14%
80.6%
2.57%
35.6%
5.70%
56.9%
1.79%
42.0%
7.75%
48.2%
2.09%
0.739%
2.05%
90.1%
7.08%
4.41%
3.36%
87.7%
4.56%
11.3%
4.40%
81.2%
3.09%
16.8%
5.72%
74.2%
3.28%
Helios
B
non-Tx
Tx
Helios⁻Foxp3⁺ cells (%)
50
40
30
20
10
0
**
**
*
0
1
5
10
TGF-β (ng/ml)

Figure 7

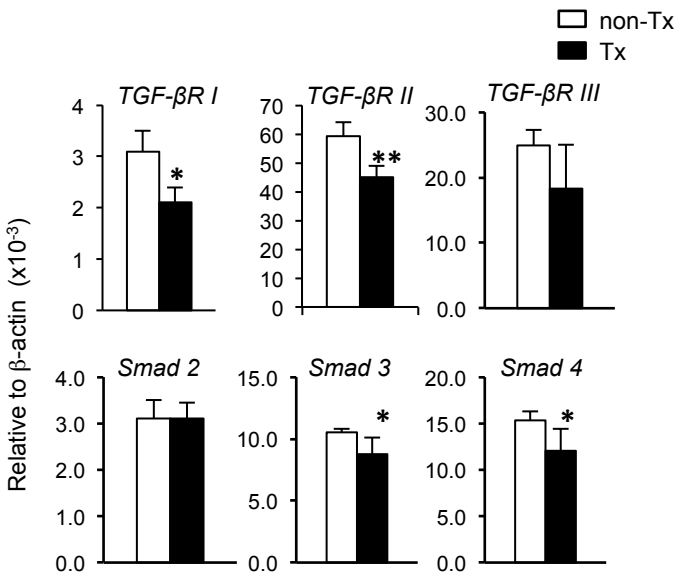
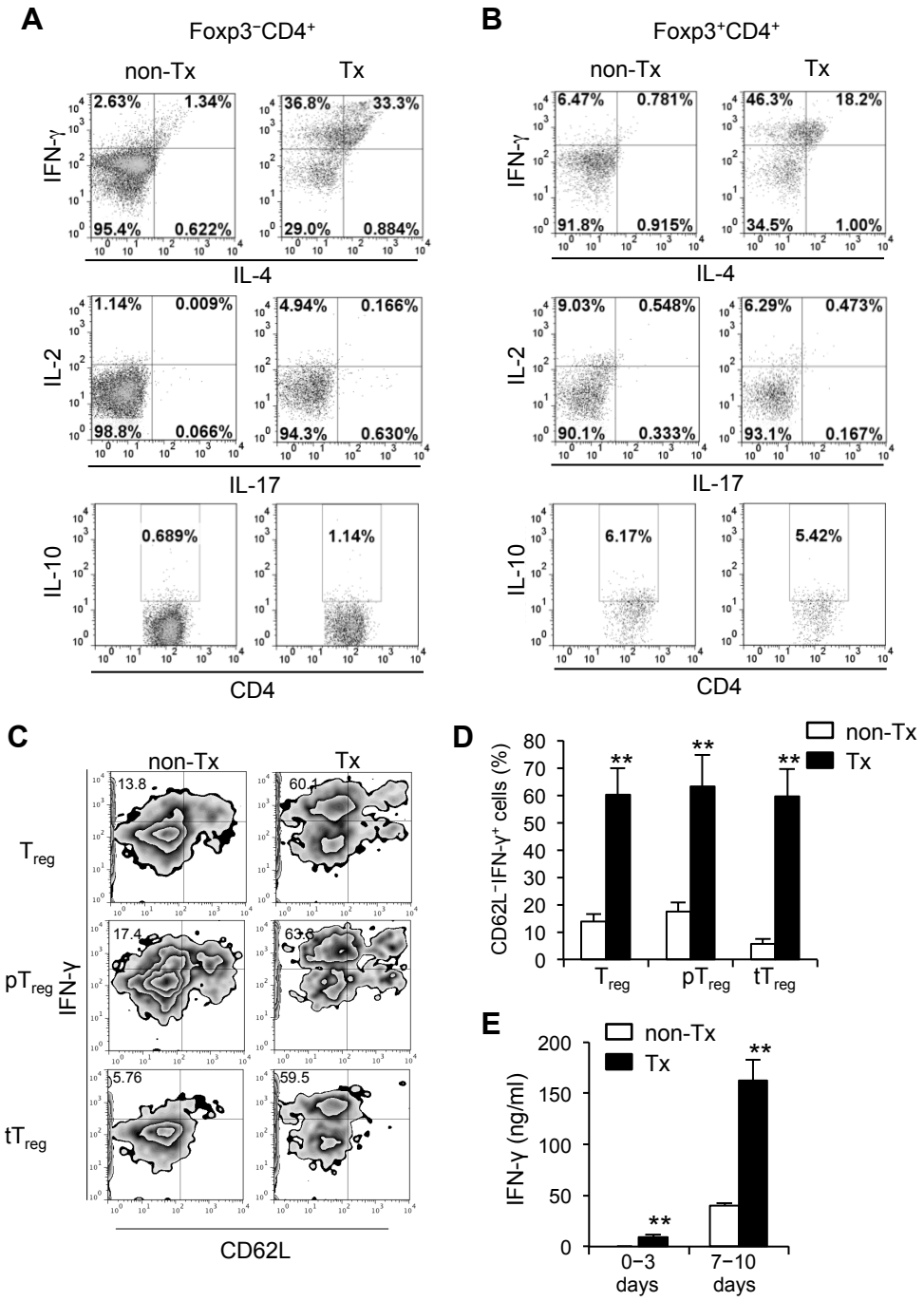
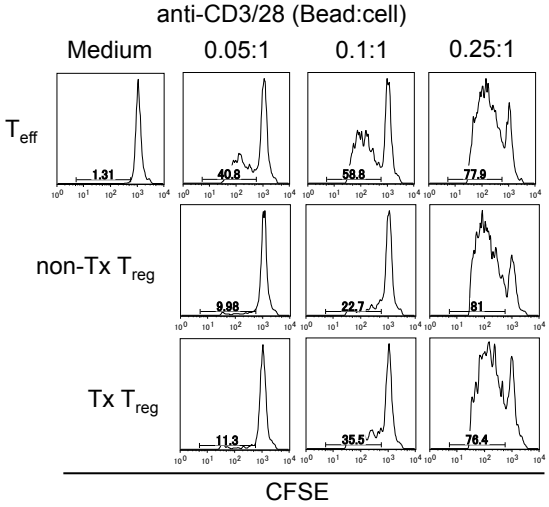

non-Tx
Tx
TGF-βR I
TGF-βR II
TGF-βR III
Smad 2
Smad 3
Smad 4
Relative to β-actin (x10-3)
*
**

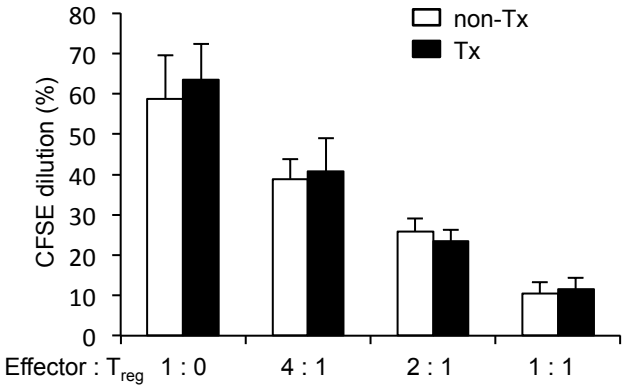
Figure 8


A
Foxp3⁻CD4⁺
non-Tx
Tx
IFN-γ
IL-4
2.63%
1.34%
95.4%
0.622%
36.8%
33.3%
29.0%
0.884%
IL-2
IL-17
1.14%
0.009%
98.8%
0.066%
4.94%
0.166%
94.3%
0.630%
IL-10
CD4
0.689%
1.14%
B
Foxp3⁺CD4⁺
6.47%
0.781%
91.8%
0.915%
46.3%
18.2%
34.5%
1.00%
9.03%
0.548%
90.1%
0.333%
6.29%
0.473%
93.1%
0.167%
6.17%
5.42%
C
Treg
pTreg
tTreg
13.8
60.1
17.4
63.6
5.76
59.5
CD62L
D
CD62L⁻IFN-γ⁺ cells (%)
non-Tx
Tx
**
Treg
pTreg
tTreg
E
IFN-γ (ng/ml)
0–3 days
7–10 days

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3