Thymic Stromal Lymphopoietin-Activated Plasmacytoid Dendritic Cells Induce the Generation of FOXP3+ Regulatory T Cells in Human Thymus

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Abstract

Human thymus contains major dendritic cell (DC) subsets, myeloid DCs (mDCs), and plasmacytoid DCs (pDCs). We previously showed that mDCs, educated by thymic stromal lymphopoietin (TSLP) produced by the epithelial cells of the Hassall’s corpuscles, induced differentiation of CD4+CD25− thymocytes into Forkhead Box P3+ (FOXP3+) regulatory T cells (T_R) within the medulla of human thymus. In this study, we show that pDCs expressed the TSLP receptor and IL-7 receptor a complexes upon activation and became responsive to TSLP. TSLP-activated human pDCs secrete macrophage-derived chemokine CCL-22 and thymus- and activation-regulated chemokine CCL-17 but not Th1- or Th2-polarizing cytokines. TSLP-activated pDCs induced the generation of FOXP3+ T_R from CD4+CD8− CD25− thymocytes, which could be strongly inhibited by Th1-polarizing cytokine IL-12 or Th2-polarizing cytokine IL-4. Interestingly, the FOXP3+ T_R induced by the TSLP-pDCs expressed more IL-10 but less TGF-b than that induced by the TSLP-mDCs. These data suggest that TSLP expressed by thymic epithelial cells can activate mDCs and pDCs to positively select the FOXP3+ T_R with different cytokine production potential in human thymus. The inability of TSLP to induce DC maturation without producing Th1- or Th2-polarizing cytokines may provide a thymic niche for T_R development.

Nondeletional tolerance leading to the generation of CD4+ CD25+FOXP3+ regulatory T cells (T_R) within the thymus represents a key mechanism to establish immunologic self-tolerance (1–6). How some of the medium- to high-affinity self-reactive T_R precursors...
escape negative selection and what type of APCs positively selects them to become $T_R$ within the thymus are unsolved questions.

Pioneering studies using three different models suggested that thymic epithelial cells are critical for the induction of nondeletional tolerance or the generation of $T_R$. The first model was based on xeno- or allotransplantation of embryotic thymic rudiments of chick, quail, or mice into a developing embryo before the colonization of hematopoietic progenitors (7–9). These embryonic thymic rudiments at this stage were called precolonized embryotic thymic rudiments and were believed to give rise to thymic epithelial cells but not hematopoietic cells. Therefore, it has been generally accepted that the resulting chimeric thymus contains epithelial cells of donor origin and hematopoietic cells of host origin. The results of these experiments suggested that thymic epithelial cells, but not hematopoietic cells, are responsible for the induction of central tolerance, either by deletion or by a nondeletional mechanism involving the induction of $T_R$ (1, 10, 11). A major question is whether the precolonized thymic rudiments contain hematopoietic progenitors that will give rise to thymic dendritic cells (DCs) in the medulla. Because the window during which precolonized thymic rudiments can be isolated without colonization of the hematopoietic stem cells is very narrow and variable in all three species, it is likely that the hematopoietic progenitors within these thymic rudiments were contaminated in these early studies. In particular, donor DCs might have been mistakenly classified as reticular cells or stromal cells in these earlier studies.

The second model involved the transfer of thymic epithelial cells or thymic stroma cells derived from thymic grafts treated with irradiation or with deoxyguanosine that kills the cycling hematopoietic cells within the thymus (12). However, it is unclear whether such treatment would have efficiently eliminated all the noncycling thymic DCs in the medulla.

The third model involved targeted expression of MHC class II (MHC-II) molecules on thymic cortical epithelial cells in class II-deficient mice and showed that the CD4$^+$CD25$^+$ $T_R$ are selected by thymic cortical epithelial cells (5, 13, 14). The questions posed were: how did the CD4$^+$CD25$^+$ $T_R$ that were selected by cortical epithelial cells escape the negative selection mediated by medullary DCs, and if cortical epithelial cells selected the CD4$^+$CD25$^+$ $T_R$ at the CD4$^+$CD8$^+$ double-positive stage, why were so few T cells expressing Forkhead Box P3$^+$ (FOXP3$^+$)$T_R$ found in the cortex (15, 16)?

We have recently shown that a subset of myeloid DCs (mDCs) educated by thymic stromal lymphopoietin (TSLP) produced by epithelial cells of the Hassall’s corpuscles induced differentiation of CD4$^+$CD25$^-$ thymocytes into FOXP3$^+$ $T_R$ within the medulla of human thymus (16). The ability of TSLP-activated mDCs to induce $T_R$ appeared to be linked with their unique features, including: 1) expression of high MHC-II and costimulatory molecules (CD80, CD86); 2) sparse production of proinflammatory cytokines IL-1, IL-6, and IL-12, which inhibit $T_R$ development; and 3) prolonged formation of stable conjugate with T cells that provide sustained TCR signaling and costimulation (17). We believe that DCs have several advantages over epithelial cells as the APCs for selecting $T_R$ in thymus: 1) DCs can transport peripheral Ags into the thymus; 2) DCs have the ability to cross-present self-Ags, including epithelial cell-derived Ags; and 3) TSLP-matured DCs can provide better quality of costimulation to prevent the developing T cells’ pro-pensity for apoptosis when they are signaled through medium- to high-affinity self-reactive TCR.

Human thymus contains at least two major subsets of DCs, including conventional mDCs and plasmacytoid DCs (pDCs) (18, 19). We have previously shown that TSLP selectively activates ex vivo-derived peripheral blood mDCs but not other blood cell types (20). Recently, we found that although resting pDCs do not express the TSLP receptor (TSLPR),
they do rapidly express mRNA encoding for TSLPR and IL-7 receptor α (IL-7Rα) following activation, suggesting that pDCs may acquire the ability to respond to TSLP following activation.

In our current study, we showed that pDCs rapidly expressed surface TSLPR and IL-7Rα complexes and became responsive to TSLP activation. TSLP-activated human pDCs expressed higher levels of CD80 and CD86 and secreted thymus and activation-regulated chemokine CCL-17 and macrophage-derived chemokine CCL-22 but not Th1- or Th2-polarizing cytokines. TSLP-activated pDCs could efficiently induce the generation of FOXP3+ Treg from the CD4+CD8–CD25– thymocytes, which could be strongly inhibited by Th1-polarizing cytokine IL-12 or Th2-polarizing cytokine IL-4. Interestingly, the FOXP3+ Treg induced by the TSLP-activated pDCs appeared to produce more IL-10 and to express less TGF-β than that induced by the TSLP-activated mDCs. Our study suggests that there are multiple types of APCs such as TSLP-activated mDCs and TSLP-activated pDCs in thymus that are responsible for the selection of functional distinct subsets of FOXP3+ Treg. The inability of TSLP to induce DC maturation without producing Th1- or Th2-polarizing cytokines may provide a suitable thymic niche for Treg development.

Materials and Methods

Thymic and peripheral DC purification and culture
This study was approved by the Institutional Review Board for Human Research at The University of Texas M. D. Anderson Cancer Center, Houston, TX. Thymuses from human newborns and children 0 d to 2 y old were obtained from the Texas Children’s Hospital, Houston, Texas. Adult blood buffy coats from healthy donors were obtained from the Gulf Coast Regional Blood Center, Houston, TX. For isolation of thymic pDCs, thymuses were cut into small pieces and digested as previously described (18). After separation of the mononuclear cells by Ficoll centrifugation, blood DC Ag4+ (BDCA-4+) cells were obtained from thymus or PBMCs using a Microbeads-BDCA-4 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched cells were stained with FITC-conjugated anti-CD3, CD14, CD16, CD56, CD19, CD20, PE-conjugated anti-CD11c, allophycocyanin-conjugated anti-CD123, and allophycocyanin-Cy7–conjugated anti-CD4 Abs (BD Biosciences, San Jose, CA), and then lineage−CD11c−CD4+CD123+ cells were purified using FACS on an FACS Aria (BD Biosciences) to reach >99% purity, as described (21). Isolated pDCs were cultured for 48 h with various stimuli with or without TSLP (50 ng/ml; recombinant protein was prepared in house using an adenovirus vector system) as described (17). After 48 h of culture, we determined the cell-surface marker characteristics of activated pDCs also as described (17).

Isolation of thymic T cells
For isolation of T cell lineage thymocytes, thymuses were digested, and mononuclear cells were separated by Ficoll centrifugation as described (17). Briefly, T cell lineage thymocytes were obtained by negative deletion using a mixture of mouse mAbs against the lineage markers CD11c, CD14, CD15, CD20, CD56, and CD235a (produced in-house). This was followed by incubation of these thymocytes with goat anti-mouse IgG-coated magnetic beads (M-450; Dynal, Oslo, Norway) and microbeads goat anti-mouse IgG (Miltenyi Biotec). Enriched cells were stained with FITC-conjugated anti-CD11c, CD14, CD16, CD19, CD20, CD56, and BDCA-2, PE-conjugated anti-CD8, PE-Cy7–conjugated anti-CD25, and allophycocyanin-Cy7 conjugated anti-CD4 Abs (BD Biosciences), and then lineage−CD4+CD8–CD25− cells were purified using FACS on an FACS Aria (BD Biosciences) to reach >99% purity.
DCs and T cell coculture

After 2 d of culture, pDCs were collected and washed three times to remove any cytokines. Viable DCs were counted by trypan blue exclusion of dead cells. The remaining cells were cocultured with $2 \times 10^4$ freshly isolated allogeneic thymocytes or peripheral blood-naive CD4$^+$ T cells in round-bottomed 96-well culture plates in IMDM containing 2% human AB serum and 10 ng/ml of IL-7. Cultures were done in triplicate at a 1:2 or 1:1 ratio of DCs:T cells. Neutralizing anti–HLA-DR (BD Biosciences), anti-CD80, anti-CD86, and anti–IL-2 (R&D Systems, Minneapolis, MN) mAbs were used in culture at a concentration of 30 μg/ml. The labeling of T cells with CFSE (Molecular Probes, Carlsbad, CA) or PKH26 (Sigma-Aldrich, St. Louis, MO) was performed as described (17). In some experiments, isolated T cells were cultured with plate-bound anti-CD3 Ab (OKT-3; 2 μg/ml), soluble anti-CD28 (28.2; 1 μg/ml), 20 U/ml IL-2, and 10 ng/ml IL-7 with or without 10 μg/ml TGF-β (R&D Systems).

T cell proliferation and expansion assay

After 7 d of culture, cells were collected and resuspended in an EDTA-containing medium to dissociate the clusters. Viable cells were counted by trypan blue exclusion of dead cells. The remaining cells were stained with PE-conjugate anti-CD25, and Alexa Fluor 647-conjugated anti-FOXP3 Abs (259D or 236A/E7) (Biolegend, San Diego, CA, and eBioscience, San Diego, CA) and analyzed with a LSR-II (BD Biosciences). Dead cells were excluded on the basis of side- and forward-scatter characteristics, and viable CD4$^+$CD25$^+$ and FOXP3$^+$ T cell numbers were calculated as: the percentage of cells in the cell type 3 the number of viable cells.

Suppression assay

After 7 d of culture with TSLP-DCs, CD4$^+$ thymocytes were stained with PE-conjugated anti-CD25 (Miltenyi Biotec), and then CD25$^{\text{high}}$ cells and CD25$^{\text{dim}}$ cells, which divided >5 times, were isolated by cell sorting. The mixture of these sorted cells and peripheral CD4$^+$CD25$^-$ T cells at a 1:1 ratio or multiple ratios were stimulated with immobilized anti-CD3 (OKT-3, 2 μg/ml) and anti-CD28 Abs (CD28.2, 1 μg/ml) for 4 d. Cellular proliferation was assessed by [3H]thymidine incorporation, as described in Ref. 17. Peripheral CD4$^+$CD25$^-$ T cells were also labeled with CFSE and cultured with sorted CD25$^{\text{high}}$ cells and CD25$^{\text{dim}}$ cells at a 1:1 ratio in the presence of immobilized anti-CD3 (OKT-3, 2 μg/ml) and soluble anti-CD28 Abs (CD28.2, 0.5 μg/ml). After 4 d of cultures, cell division of CFSE-labeled CD4$^+$CD25$^-$ T cells was evaluated by flow cytometry.

DC and T cell cytokine production

DC culture supernatants were collected at 24 h, and chemokine and cytokine production was assessed as described Ref. 17. CD4$^+$CD25$^-$ T cells from adult peripheral blood, fetal, or neonatal thymuses were cultured for 7 d with TSLP-DCs and IL-7 or with immobilized anti-CD3, soluble anti-CD28 Abs, IL-2, and IL-7. T cells were then washed and restimulated with 50 ng/ml PMA and 2 μg/ml ionomycin for 6 h (Sigma-Aldrich). A total of 10 μg/ml of brefeldin A (Sigma-Aldrich) was added during the last 2 h. The T cells were stained with PE-conjugated anti–IL-10 (BD Biosciences) and Alexa Fluor 647-conjugated anti-FOXP3 (259D or 236A/E7) Abs using CALTAG FIX and PERM kit (Invitrogen, Carlsbad, CA) and the FOXP3 staining kit (eBioscience). The T cells were also stimulated with plate-bound 2 μg/ml of anti-CD3 and 1 μg/ml of soluble anti-CD28 Abs at a concentration of $10^6$ cells/ml for 24 h. The level of IL-10 in the supernatants was measured by ELISA (R&D Systems). For detection of membrane-bound latency associated peptide (LAP) (TGF-β1) on the surface, cells were stained with LAP (TGF-β1) Ab (27232, R&D Systems), followed by staining with PE-conjugated anti-mouse IgG1 Abs (BD Biosciences).
Intracellular FOXP3 staining

After 7 d of culture with DCs, T cells were stained with PE-conjugated anti-CD25 and then fixed with Fixation/Permeabilization working solution (eBioscience) for 1 h. After washing, the cells were treated with Permeabilization Buffer (eBioscience) in the presence of Alexa Fluor 647-conjugated anti-FOXP3 Ab (259D or 236A/E7) for 30 min.

Immunohistochemistry

For immunofluorescence staining, the slides were incubated with biotinylated mouse anti-CD123 mAb (BD Biosciences) at room temperature for 1 h. After washing, anti-CD123 mAb-dependent tissue deposition of biotin was increased using a tyramide signal biotin amplification system and then was visualized by Alexa Fluor 549-conjugated streptavidin (Molecular Probes). The slides were then stained with FITC-conjugated anti-FOXP3 mAb (eBioscience) for 1 h. Images were acquired by using an inverted microscope, BX41 (Olympus, Tokyo, Japan). Final image processing was performed by using Photoshop (Adobe, San Jose, CA).

Results

TSLP costimulates pDCs

We have previously shown that TSLP could strongly activate mDCs to express costimulatory molecules CD80 and CD86 and to produce Th2 chemokines CCL-17 and CCL-22 without producing Th1-polarizing cytokine IL-12 (20). Through gene expression analyses, we recently found that pDCs rapidly express mRNA encoding for TSLPR and IL-7Rα following activation through TLR-7 or TLR-9 (data not shown). This stimulated us to further investigate whether pDCs have the ability to express surface TSLPR at the protein level and to become functionally responsive to TSLP. We found that whereas pDCs constitutively expressed surface IL-7Rα, pDCs expressed surface TSLPR following activation by TLR7-ligand R848 and TLR9-ligand CpG-B (Fig. 1A). IL-3 plus CD40L, CpG-C, HSV, and influenza A moderately upregulated the expression of TSLPR by pDCs. We found that TSLP strongly upregulated surface expression of CD80 and CD86 on pDCs activated by CpG-B or R848 (Fig. 1B) and promoted pDCs to produce CCL-17 and CCL-22 without inducing or effecting IFN-α or IL-12 production in culture (Fig. 1C). These data suggest that, unlike CD40L or ligands for different TLRs, TSLP represents a very unique stimulus that induces activation of both mDCs and pDCs to upregulate costimulatory molecules without producing either Th1-polarizing cytokines or proinflammatory cytokines.

TSLP-conditioned CpG-B preactivated pDCs induce generation of FOXP3+ T\(_R\)

In the human system, we have recently shown that TSLP is expressed by epithelial cells of the Hassall’s corpuscles in the thymic medulla, and TSLP-conditioned mDCs could induce the differentiation of CD4+CD25− thymocytes into CD4+CD25+FOXP3+ T\(_R\) cells (16). Because human thymus contains both mDC and pDC, we next investigated whether TSLP-activated human pDCs could induce the differentiation of CD4+CD25− thymocytes into CD4+CD25+FOXP3+ T\(_R\) cells. CD4+CD25− FOXP3+ thymocytes isolated from human thymus were labeled with CFSE and cultured for 7 d with allogeneic pDCs precultured for 2 d with CpG-B in the presence or absence of TSLP. The sorted CD4+CD25− thymocytes contained <0.2% of FOXP3+ cells as assessed by staining with anti-human FOXP3 mAbs 259D (Fig. 2A) and 236A/E7 (data not shown). We found that TSLP-conditioned CpG-B preactivated pDCs (TSLP-pDCs) induced a vigorous expansion of CD4+ thymocytes (Fig. 2B, 2D, 2F) and the generation of CD4+CD25+ FOXP3+ cells (Fig. 2B, 2E, 2G), whereas pDCs activated by CpG-B alone induced less expansion and few generation of FOXP3+ cells. The number of the FOXP3+ cells induced by TSLP-pDCs followed the number of
TSLP-pDCs added in the cultures (Fig. 2G). In contrast, whereas CD4^+CD25^− thymocytes differentiated into FOXP3^+ cells by stimulating with anti-CD3, anti-CD28, IL-2, and TGF-β-like peripheral naive CD4^+ T cells (22), stimulation of CD4^+CD25^− thymocytes with anti-CD3 and IL-2 or anti-CD3, anti-CD28, and IL-2 induced vigorous expansion but not the differentiation of CD4^+CD25^− cells into FOXP3^+ cells (Fig. 2C). These data suggest that the stimulation by anti-CD3, anti-CD28, and IL-2 is not sufficient to induce FOXP3^+ cells from human CD4^+CD25^− thymocytes.

We then examined whether TSLP-pDCs induced the differentiation of CD4^+CD25^−FOXP3^− cells into CD4^+CD25^+FOXP3^+ cells or the expansion of a small number of CD4^+CD25^+FOXP3^− T cells in sorted CD4^+CD25^− thymocytes. To determine this, we performed cell-mixed culture experiments with CFSE-labeled CD4^+CD25^+ T cells and PKH26-labeled CD4^+CD25^− cells (16). Sorted CD4^+CD25^+ cells were labeled with CFSE and mixed with PKH26-labeled CD4^+CD25^− thymocytes at a 1:9 ratio. This ratio was chosen because the original CD4^+ thymocyte population contains CD25^+ and CD25^− cells at 1:9 ratios (Fig. 2A) (16). These mixed cells were then cultured together with TSLP-pDCs, and the proliferative response of the two populations was compared (Fig. 3A, 3B). We found that thymic CD4^+CD25^+ T cells in mixed culture expanded ~4-fold, which is a similar extent as the expansion of CD4^+CD25^− cells. Thus, it seems unlikely that the contaminated CD4^+CD25^+FOXP3^+ T cells, which is <0.2% in CD4^+CD25^− cells, can reach to 4.8%. Based on the cell number, contaminated CD4^+CD25^+FOXP3^+ cells have to expand >30–200-fold to reach 4.8% of cultured cells. Therefore, these data indicate that TSLP-pDCs preferentially induce CD4^+CD25^− thymocytes to differentiate into CD4^+CD25^+FOXP3^+ T cells.

The generation of CD4^+CD25^+FOXP3^+ T cells induced by TSLP-pDCs was completely blocked by the addition of Abs to MHC-II (HLA-DR), CD80/CD86, or IL-2 (Fig. 4A, 4B), suggesting the critical role of the interaction of TCR and Ag/MHC-II complex, CD28 signaling through CD80/CD86, and IL-2 in the generation and expansion of CD4^+CD25^+FOXP3^+ T cells in thymus, as previously observed in both humans and mice (23–26).

We next examined the phenotype, cytokine profile, and suppressive activity of FOXP3^+ cells generated from CD4^+CD25^−FOXP3^− thymocytes by TSLP-pDCs. CD4^+CD25^−FOXP3^− thymocytes were labeled with CFSE and cultured with TSLP-pDCs. After 7 d of culture, we evaluated the surface phenotype of FOXP3^+ or FOXP3^− cells in the same culture, which went through similar rounds of cell division after culture with pDCs. As shown in Fig. 5A, FOXP3^+ cells expressed much higher levels of CD25, CTLA-4, and ICOS than FOXP3^− cells and a similar level of glucocorticoid-induced TNF receptor to that expressed by FOXP3^− cells. Furthermore, FOXP3^+ cells induced by TSLP-pDCs did not produce IL-2 and IFN-γ, whereas FOXP3^− cells in the same culture produced significant levels of IL-2 and IFN-γ (Fig. 5B).

We next examined whether CD4^+CD25^+FOXP3^+ T cells generated by TSLP-pDCs exhibited suppressive function. After 7 d of culture, we sorted both CD25^high and CD25^dim cells, which went through >5 times of division. More than 60% of the sorted CD25^high cells and <5% of CD25^dim cells expressed FOXP3 (Fig. 6A). These cells were cultured with CD4^+CD25^− T cells isolated from peripheral blood in the presence of immobilized anti-CD3 and soluble anti-CD28. We found that CD4^+CD25^highFOXP3^+ cells induced by TSLP-pDCs were anergic and displayed the ability to strongly inhibit the proliferation of CD4^+CD25^− T cells (Fig. 6B, 6D). In contrast, CD4^+CD25^dimFOXP3^− cells were neither anergic nor suppressive (Fig. 6C, 6D). They even promoted the expansion of responder CD4^+CD25^− T cells rather than inhibited their proliferation. We confirmed these results by CFSE-labeling...
experiments. The proliferation of CFSE-labeled responder CD4^+CD25^- T cells was significantly inhibited by CD25^+FOXP3^+ cells but not CD4^+CD25^{dim}FOXP3^- cells (Fig. 6E).

These findings show that newly generated CD4^+CD25^+FOXP3^+ T cells by TSLP-pDCs possess some of the key features of naturally occurring T_R developed in mice and human thymus.

**FOXP3^+ T_R induced by TSLP-pDCs or by TSLP-mDCs express different levels of IL-10 and LAP (TGF-β1)**

We have recently found that human thymus contains two distinct FOXP3^+ T_R subsets defined by surface expression of the costimulatory molecule ICOS (27). Although the ICOS^+FOXP3^+ T_R produced high levels of IL-10 that suppressed DC function and expressed low levels of LAP (TGF-β1) that directly suppressed T cell proliferation, the ICOS^-FOXP3^+ T_R expressed high levels of LAP (TGF-β1) but produced low levels of IL-10, suggesting that there may be different populations of APCs selecting the FOXP3^+ T_R. Thus, we next questioned whether there are phenotypic differences between TSLP-pDC–induced and TSLP-mDC–induced T_R. Interestingly, the FOXP3^+ T_R generated by TSLP-pDCs expressed lower LAP (TGF-β1) (Fig. 7A) and produced higher IL-10 levels (Fig. 7B, 7C) than did the FOXP3^+ T_R induced by TSLP-mDCs, suggesting that TSLP-pDCs and TSLP-mDCs may play different roles in T_R development.

**Th1/Th2 cytokines inhibit the ability of TSLP-pDCs to induce the generation of FOXP3^+ T_R from CD4^+CD25^- thymocytes**

Unlike CD40L or ligands for different TLRs that all induce mDCs to produce Th1-polarizing cytokine IL-12, TSLP does not induce mDCs or pDCs to produce IL-12. We hypothesized that the absence of Th1-polarizing cytokines provided by TSLP-activated DCs may provide a permissive environment that allows the development of T_R. To test this hypothesis, CFSE-labeled CD4^+CD25^-FOXP3^- thymocytes were cultured for 7 d with TSLP-pDCs or TSLP-mDCs in the presence or absence of IL-4, IFN-γ, IL-12, TNF-α, or TGF-β. IL-12 and IL-4 strongly inhibited the differentiation of CD4^+CD25^- thymocytes into CD4^+CD25^+FOXP3^+ cells (Fig. 8A, 8B). However, IFN-γ and TNF-α did not inhibit the generation of FOXP3^+ cells. Interestingly, TGF-β, which is known to induce FOXP3 in peripheral CD4^+ T cells activated by DCs (Fig. 8C, 8D) (28) or by anti-CD3 and anti-CD28 (22, 29, 30) and seems to be important to convert peripheral T cells to FOXP3^+ T cells and to maintain the expression of FOXP3 in peripheral T_R, significantly inhibited the generation of FOXP3^+ thymocytes induced by TSLP-pDCs. These data suggest that both Th1-polarizing cytokine IL-12 and Th2-polarizing cytokine IL-4 can inhibit the ability of TSLP-pDCs to induce the generation of FOXP3^+ T_R from thymocytes in culture and that the expression of FOXP3 in thymus and in periphery may be regulated by different molecular mechanisms.

**TSLPR^+ pDCs are present in human thymus and colocalize with FOXP3^+ T_R**

We next investigated whether human thymic pDCs expressed TSLPR and searched for evidence of in vivo interaction between pDCs and FOXP3^+ T_R in situ. pDCs were enriched from total thymocytes by anti-BDCA4 magnetic bead sorting. The pDCs were further defined by a phenotype of lin^−CD4^+CD123^+ using flow cytometry. These cells showed the typical phenotype of peripheral pDCs, including expression of BDCA-2, BDCA-4, CD45RA, and IL-7Rα (Fig. 9A). Freshly isolated thymic pDCs also had the ability to produce IFN-α but not IL-12 and increased the expression of HLA-DR, CD80, and CD86 in response to HSV or CpGs (data not shown). As shown in Fig. 9B, significant numbers of thymic pDCs (5–20%) expressed TSLPR without any stimulation. Importantly, these
TSLPR⁺ pDCs expressed higher levels of CD80, CD86, CD40, and HLA-DR than TSLP⁻ pDCs. These results clearly indicate that TSLPR is expressed on pDCs residing in human thymus.

To provide further anatomical evidence that pDCs are involved in FOXP3⁺ Tᵦ development in thymus, we investigated the localization of thymic pDCs and FOXP3⁺ Tᵦ in human thymus by microscopic analysis using specific Abs to pDCs (anti-CD123 mAb) and to FOXP3⁺ Tᵦ (anti-FOXP3 mAb). Both CD123⁺ pDCs and FOXP3⁺ Tᵦ were found in thymic medulla, and some pDCs were in close contact with FOXP3⁺ cells (Fig. 9C). These findings suggest that thymic pDCs as well as mDCs instructed by TSLP produced by Hassall’s corpuscles may have a critical role in inducing the generation and expansion of CD4⁺CD25⁻FOXP3⁺ Tᵦ through direct interaction in thymus.

Discussion

Human thymus contains two major DC subsets, mDCs and pDCs (18, 19). We have previously shown that mDCs from human peripheral blood and thymus expressed the TSLPR/IL-7Ra complex and could be activated by TSLP (20). We presented experimental evidence suggesting that thymic mDCs educated by TSLP produced by the epithelial cells of the Hassall’s corpuscles may induce the differentiation of CD4⁺CD25⁻ thymocytes into FOXP3⁺ Tᵦ within the medulla in human thymus (16).

In this study, we found that the subpopulation of pDCs isolated from human thymus expressed TSLPR at steady state. Furthermore, this pDC subpopulation is located close to and associated with FOXP3⁺ cells in the thymic medulla. We also found that pDCs in peripheral blood express both TSLPR and IL-7Ra after activation through TLRs. TSLP activated pDCs to express higher levels of costimulatory molecule and to produce chemokines CCL-17 and CCL-22, which are important in guiding the traffic of developing immature thymic T cells into the medulla (31–33). We showed that TSLP-activated pDCs induced the expansion and differentiation of CD4⁺CD8⁻CD25⁻ FOXP3⁻ Tᵦ into CD4⁺CD25⁺FOXP3⁺ Tᵦ. The Tᵦ generated by TSLP-pDCs possess a typical feature of naturally occurring Tᵦ as previously reported in mice and humans (3, 34).

Interestingly, the FOXP3⁺ Tᵦ induced by TSLP-pDCs showed distinct cytokine expression potential compared with the FOXP3⁺ Tᵦ induced by TSLP-mDCs. The TSLP-pDC—induced Tᵦ were IL-10₁₀⁻¹ₐ⁺ GFB₁⁺ and the TSLP-mDC—induced Tᵦ were IL-10₁₀⁻¹ₐ⁻ GFB₁⁺ after activation. These data are consistent with our recent observation that human thymus, peripheral blood, and secondary lymphoid tissues contain two subsets of CD25₁₀⁻¹ₐ⁺FOXP3⁺ Tᵦ according to ICOS expression (27). Whereas the ICOS⁺ Tᵦ subset had the potential to express high IL-10 and less TGF-β, the ICOS⁻ Tᵦ subset expressed high TGF-β but lower IL-10. These results suggest that pDCs and mDCs in thymus not only play a critical role in the selection of Tᵦ but may also imprint the two cell subsets of Tᵦ to produce different suppressive cytokines, IL-10 and TGF-β, in response to self-Ag in the periphery.

Previous studies have shown that circulating immature DCs may migrate into the thymus and induce nondeletional tolerance (35–37). A more recent study by Proietto et al. (38) has demonstrated that the circulating peripheral Sirpa⁺CD8₁₀⁻¹ₐ⁻ DC subset migrates into the thymus and selects the FOXP3⁺ Tᵦ. Another interesting study provided direct evidence that pDCs are able to acquire and process allogeneic cell-derived Ags and to cross-present the alloantigen to induce FOXP3⁺ Tᵦ cells in periphery (39). In our study, only ~4–20% of pDCs in the thymus expressed TSLPR. This together with the observation that the peripheral blood pDCs expressed TSLPR only following activation through TLR-7/TLR-9 suggests that peripheral pDCs that carry the peripheral Ags may migrate into the thymus upon
activation and participate in the selection of T<sub>R</sub> together with TSLP-mDCs (16) and medullar epithelial cells (40).

Differentiation of T cells into distinct cell linages is controlled by several key transcription factors that have pivotal roles in cell fate choice during the early stage of lymphoid cell development. These lineage specification factors not only promote a particular T cell fate but also are responsible for repressing alternative differentiation pathways. In our experiment, IL-12 and IL-4 actively repressed the adoption of CD4<sup>+</sup> thymocytes’ fate to be T<sub>R</sub>. It seems that transcription factors induced by these cytokines, which are critical to promote Th1 or Th2 differentiation, override FOXP3-dependent development of T<sub>R</sub>. Our current study suggests that primary CD4<sup>+</sup> thymocytes have less ability to produce cytokines, including IL-4 and IFN-γ, by stimulation with DCs or anti-CD3 plus anti-CD28 than do peripheral naive CD4<sup>+</sup> T cells (S. Hanabuchi and Y.J. Liu, unpublished observation). Furthermore, both TSLP-pDCs and TSLP-mDCs produce little Th1-polarizing (IL-12) or Th2-polarizing (IL-4) cytokines that inhibit T<sub>R</sub> development. These features of DCs and thymocytes are likely to be essential for the differentiation of FOXP3<sup>+</sup> T<sub>R</sub> in the thymic microenvironment. Although the existence of a unique niche in thymus has been proposed, its exact nature has remained unclear (40, 41). Decoding the molecular signature of DCs, including surface molecules and soluble factors such as cytokines that may positively or negatively regulate thymic selection, should be very important to accomplish the differentiation of T<sub>R</sub> in thymus. Thus, we speculate that T<sub>R</sub> differentiation in thymus is regulated in a very subtle way by DCs, providing a suitable niche that allows T<sub>R</sub> to develop.

In conclusion, this study provides experimental evidence showing that activated pDCs rapidly express TSLP-R and IL-7Ra and that TSLP could strongly activate human pDCs to enhance costimulatory molecules and to initiate production of CCL-17 and CCL-22. Furthermore, TSLP-activated pDCs could efficiently induce the generation and expansion of FOXP3<sup>+</sup> T<sub>R</sub>. Interestingly, the FOXP3<sup>+</sup> T<sub>R</sub> induced by TSLP-pDCs produced more IL-10 and less TGF-β than that of the FOXP3<sup>+</sup> T<sub>R</sub> induced by TSLP-mDCs. These data suggest that two subsets of T<sub>R</sub> with different cytokine production potential can be selected by TSLP-activated mDC or pDCs, respectively, in thymus. The inability of TSLP to induce DC maturation without producing Th1- or Th2-polarizing cytokines may provide a thymic niche for T<sub>R</sub> development.

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**Abbreviations used in this paper**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BDCA</td>
<td>blood dendritic cell Ag</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>FOXP3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Forkhead Box P3&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-7Ra</td>
<td>IL-7 receptor α</td>
</tr>
<tr>
<td>LAP</td>
<td>latency associated peptide</td>
</tr>
<tr>
<td>mDC</td>
<td>myeloid dendritic cell</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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MHC-II  MHC class II
pDC  plasmacytoid dendritic cell
T_R  regulatory T cell
TSLP  thymic stromal lymphopoietin
TSLPR  thymic stromal lymphopoietin receptor

References


FIGURE 1. TSLP expressed on pDCs enhances the expression of costimulatory molecules CD80 and CD86 and initiates the production of chemokines CCL-17 and CCL-22. A, Expression of TSLPR and IL-7Rα by pDCs stimulated with each stimuli for 36 h was determined by flow cytometry. Open histograms represent isotype control, and filled histograms represent staining of TSLPR and IL-7Rα on pDCs. B, mDCs were stimulated with TSLP or polyinosinic-polycytidylic acid, or cultured in medium only. pDCs were stimulated with CpG-B or R848 in the presence or absence of TSLP. After 48 h of culture, CD80 and CD86 expression of mDCs and pDCs were determined by flow cytometry. Open histograms represent isotype control, and filled histograms represent CD80 or CD86. The number in the histograms indicates the mean fluorescence intensity (ΔMFI). All data are representative of three experiments. C, The production of CCL-17, CCL-22, IFN-α, and IL-12p70 by mDCs and pDCs cultured with each stimuli for 48 h was determined by ELISA.
FIGURE 2.
TSLP-pDCs induce CD4^+CD25^-FOXP3^- thymocytes to differentiate into CD4^+CD25^+FOXP3^+ T cells. A, Flow cytometric analysis of unsorted T cell lineage thymocytes and FOXP3 expression of sorted CD4^+CD25^- thymocytes before culturing with DCs. B, FOXP3 expression of CFSE-labeled CD4^+CD25^-FOXP3^- thymocytes was analyzed after culturing with allogeneic medium-mDCs, TSLP-mDCs, CpG-B–pDCs, or CpG-B plus TSLP-pDCs for 7 d at 1:2 ratio of DCs:thymocytes. The number in dot plots indicates the percentage of CD4^+FOXP3^+ cells. C, FOXP3 expression of CFSE-labeled CD4^+CD25^-FOXP3^- thymocytes after culturing with anti-CD3 and IL-2, anti-CD3, anti-CD28, and IL-2, or anti-CD3, anti-CD28, IL-2, and TGF-β for 7 d. The number in dot plots indicates the percentage of CD4^+FOXP3^+ cells. D and E, CD4^+CD25^-FOXP3^- thymocytes were cultured for 7 d with CpG-B–pDCs or CpG-B plus TSLP-pDCs at a 1:1 ratio of DCs:thymocytes. Filled circles indicate the total number of CD4^+ cells per well (D) and the number of CD4^+CD25^+FOXP3^+ cells per well (E) in five individual experiments. Horizontal bars indicate the mean. F and G, CD4^+CD25^-FOXP3^- thymocytes were cultured for 7 d at various DC:thymocyte ratios with CpG-B–pDCs (open circles) or CpG-B plus TSLP-pDCs (closed circles), and the total number of CD4^+ cells per well (F) and the number of CD4^+CD25^+FOXP3^+ cells per well (G) were assessed in five individual experiments.
FIGURE 3.

TSLP-pDCs induce the generation of FOXP3^+ T_R from CD4^+CD25^-FOXP3^- thymocytes but not from CD4^+CD25^+FOXP3^+ cells contaminated in sorted CD4^+CD25^- thymocytes.

A, Flowcytometric analysis of FOXP3 expression on sorted CD4^+CD25^+ and CD4^+CD25^- thymocytes before culture. The number in dot plots indicates the percentage of FOXP3^+ cells. B, CFSE-labeled CD4^+CD25^+ thymocytes, PKH26-labeled CD4^+CD25^- thymocytes, and the mixture of two populations were co-cultured with TSLP-pDCs at 1:9 ratio of CD25^+:CD25^- cells. After 5 d culture, FOXP3 expression on each population was analyzed by flow cytometry. The number in dot plots indicates the percentage of PKH26-labeled FOXP3^+ cells (gate II) (upper panel). The fold expansion of PKH26-labeled FOXP3^+ cells (gate II) and CFSE-labeled FOXP3^+ cells (gate III) is shown in the bar graph (lower panel). All data shown are representative of four experiments.
FIGURE 4.
Signalizing through TCR, CD80, and IL-2 are critical for the development of CD4+CD25+FOXP3+ thymocytes induced by TSLP-pDCs. CFSE-labeled CD4+CD25−FOXP3− thymocytes were cultured with TSLP-pDCs at a 1:2 ratio of DCs:thymocytes in the presence of neutralizing anti–HLA-DR, anti-CD80 plus anti-CD86, anti–IL-2 Abs, or isotype control. A, On day 7, CD4+ cells were assessed for FOXP3 expression by flow cytometry. B, The number of CD4+FOXP3+ cells is shown in the bar graph.
FIGURE 5.
Phenotype of CD4^+CD25^+FOXP3^+ cells induced by TSLP-pDCs. CFSE-labeled CD4^+CD25^- thymocytes were cultured with TSLP-pDCs for 7 d. A, Expression of CD25, CTLA-4, ICOS, and glucocorticoid-induced TNF receptor on FOXP3^+ and FOXP3^- cells in the same culture were analyzed by flow cytometry. The number in the histograms indicates the ΔMFI. B, Cultured cells were stimulated with PMA and ionomycin, and expression of intracellular IL-2 and IFN-γ at the single-cell level was analyzed by flow cytometry.
FIGURE 6.
Suppressive function of CD4^+CD25^{high}FOXP3^+ cells induced by TSLP-pDCs. A, CFSE-labeled CD4^+CD25^- thymocytes were cultured with TSLP-pDCs for 7 d. FOXP3 expression on CD4^+CD25^{high} cells and CD4^+CD25^{dim} cells, which went through >5 times of division, were analyzed after sort. The number in dot plots indicates the percentage of FOXP3^+ cell. B and C, Suppressive function of TSLP-pDC–induced CD25^{high}FOXP3^+ cells (B) and CD25^{dim}FOXP3^- cells (C). Sorted CD4^+CD25^{high} cells and CD4^+CD25^{dim} cells were cultured with or without CD4^+CD25^- T cells isolated from peripheral blood in the presence of immobilized anti-CD3 and soluble anti-CD28 Abs for 4 d. Cell proliferation was measured by [^3]H]thymidine incorporation. All data shown are representative of four experiments. D, CD4^+ CD25^+T_{R} isolated from peripheral blood, TSLP-pDC–induced CD4^+CD25^{high} cells, or CD4^+CD25^{dim} cells were cultured with CD4^+CD25^- T cells at various ratios in the presence of immobilized anti-CD3 and soluble anti-CD28 Abs for 4 d. Cell proliferation was measured by [^3]H]thymidine incorporation. The data shown are representative of six experiments. E, CFSE-labeled peripheral CD4^+CD25^- T cells were cultured with CD4^+CD25^{high} cells and CD4^+CD25^{dim} cells in the presence of immobilized anti-CD3 and soluble anti-CD28 Abs. After 4 d of culture, division of CFSE-labeled responder CD4^+CD25^- T cells was analyzed by flow cytometry.
FIGURE 7.
FOXP3+ T_R induced by TSLP-pDCs and TSLP-mDCs show different cytokine profiles. CFSE-labeled CD4+CD25−FOXP3− thymocytes were cultured for 7 d. A, Surface expression of LAT (TGF-β1) on FOXP3+ cells or FOXP3− cells, which both went through similar rounds of cell division after culture with DCs, was evaluated by flow cytometry. Open histograms represent isotype control, and filled histograms represent LAP (TGF-β1). The number in the histograms indicates the ΔMFI. All data are representative of six experiments. B, The cells were stimulated with PMA and ionomycin for 6 h, and then IL-10 expression at the single-cell level was assessed by flow cytometry. C, The cells were also stimulated with immobilized anti-CD3 and soluble anti-CD28 Abs for 24 h, and IL-10 in the culture supernatant was assessed by ELISA.
FIGURE 8.
Th1/Th2 cytokines inhibit the generation of FOXP3+ Tregs by TSLP-pDCs. A and B, CFSE-labeled CD4+CD25−FOXP3− thymocytes were cultured for 7 d with TSLP-pDCs or TSLP-mDCs at a 1:4 ratio of DCs:thymocytes in the presence or absence of IL-4, IFN-γ, IL-12, TNF-α, and TGF-β. FOXP3 expression (A) and the number of CD4+FOXP3+ cells (B) were assessed. C and D, CFSE-labeled peripheral naive CD4+ T cells were cultured for 7 d with TSLP-pDCs at a 1:4 ratio of DCs:T cells in the presence or absence of TGF-β, and FOXP3 expression (C) and the number of CD4+ FOXP3+ cells (D) were assessed.
FIGURE 9.
TSLPR⁺ pDCs are present in human thymus and colocalize with FOXP3⁺ Treg. pDCs were enriched from total thymocytes by anti-BDCA4 magnetic-bead sorting. pDCs were further defined by a phenotype of lineage⁻CD4⁺CD123⁺ by flow cytometry. A, pDCs were assessed for expression of BDCA-2, BDCA-4, CD45RA, and IL-7-Ra by flow cytometry. B, Enriched pDCs were further stained with anti-TSLPR mAb together with anti-CD80, anti-CD86, anti-CD40, or anti-HLA-DR mAb. C, Double immunofluorescence staining of FOXP3 (green) and CD123 (red) was performed on human thymus (original magnification ×40).