의학박사 학위논문

PLZF-양성 선천면역 T 세포가 TGF-β 의존적인 활성화된 조절 T 세포 발달에 미치는 영향

2015 년 2 월

서울대학교 대학원

의학과 면역학 전공

강 병 현

PLZF^{+} innate T cells support TGF-β-dependent generation of activated/memory-like regulatory T cells

Byung Hyun Kang

Supervisor
Professor Kyeong Cheon Jung, M.D., Ph.D.

A Thesis for the Ph.D. Degree in Immunology

December 2014

Graduate School of Immunology
Seoul National University, College of Medicine

PLZF-양성 선천면역 T 세포가 TGF-β 의존적인 활성화된 조절 T 세포 발달에 미치는 영향

지도교수 정 경 천

이 논문을 의학 박사 학위논문으로 제출함

2014 년 12 월

서울 대학교 대학원

의학과 면역학 전공

강 병 현

강병현의 의학박사 학위논문을 인준함

2014 년 12 월

위 원 장 이 왕 재 (인)

부위원장 정 경 천 (인)

위 원 박 성 회 (인)

위 원 이 은 봉 (인)

위 원 김 태 진 (인)

PLZF^{+} innate T cells support TGF-β-dependent generation of activated/memory-like regulatory T cells

by
Byung Hyun Kang

Supervisor

Professor Kyeong Cheon Jung, M.D., Ph.D.

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Immunology) at Seoul National University College of Medicine

December 2014

Approved by Thesis Committee:

Professor _____ Chairman

Professor _____ Vice chairman

Professor _____

Professor _____

Professor _____

ABSTRACT

PLZF-expressing NKT cells and T-T CD4 T cells are unique subsets of innate T cells. Both of them are selected via thymocyte-thymocyte (T-T) interaction and simultaneously produce IL-4 and IFN-γ. Recently, it was reported that these $PLZF^{+}$ cells contributed to the generation of effector/memory-like CD4 and CD8 T cells in the thymus. Based on these previous findings, we investigated the possible role of $PLZF^{+}$ innate T cells in the development and function of $CD4^{+}$ $Foxp^{+}$ regulatory T cells in $CIITA^{Tg}PIV^{-/-}$ mice. In contrast to the counterparts in wild type, $Foxp3^{+}$ regulatory T cells in these mice exhibited effector/memory-like phenotype: up-regulation of surface molecules associated with regulatory functions (CD39 and CD73) and migrations (CCR4), and efficiently suppressed ovalbumin-induced allergic airway inflammation. During the course of intrathymic development, IL-4 produced by thymic $PLZF^{+}$ T cells cooperated with TGF-β for the acquisition of effector/memory phenotype of regulatory T cells. This is also the case in BALB/c mice where $PLZF^{+}$ iNKT cells were the main source of IL-4. Thus, $PLZF^{+}$ innate T cells modulate the thymic development of regulatory T cells as well as effector T cells.

Introduction: $PLZF^+$ iNKT cells and T-T CD4 T cells are unique subsets of innate T cells. Both of them are selected via thymocyte-thymocyte (T-T) interaction and simultaneously produce excessive amounts of IL-4 and IFN-γ. Recently, it was reported that IL-4 produced by $PLZF^+$ cells contributed to the generation of effector/memory-like CD4 and CD8 T cells in the thymus.

Regulatory T cells are subdivided into two categories by the sites of their origin: naturally occurring regulatory T cells and adaptive regulatory T cells, which development is dependent on IL-2 and TGF-β. These adaptive Treg cells show effector/memory phenotype and highly express CD103 molecules. Despite our report that naturally occurring regulatory T cells are well-developed in $CIITA^{Tg}PIV^{-/-}$ mice (T-T mice) where only T-T interaction for positive selection exists, the characteristics of these cells have not been investigated.

Methods: Based on the previous results, we speculated that the thymic development of regulatory T cells is somewhere influenced by IL-4 produced by $PLZF^+$ cells. For this, the phenotype of regulatory T cells of thymus and spleen from $CIITA^{Tg}PIV^{-/-}$ and BALB/c mice were scrutinized. Regulatory T cells isolated from T-T mice were tested for their suppressive capacity in in vitro and in vivo airway inflammation models. Additionally, we tried to find out key factors for their development using various knockout mice, and performed fetal thymic

organ cultures.

Results: Naturally occurring Treg cells developed in CIITA^{Tg}PIV$^{-/-}$ mice showed effector/memory phenotype including upregulation of CD103, activation marker. These cells suppressed Th2-mediated airway inflammation more efficiently than their couterparts from wild-type mice. Upregulation of CD103 was turned out to be dependent on TGF-β signaling, and IL-4 produced by PLZF^{+} cells enhanced the TGF-β-dependent expression of CD103 on Treg cells. This phenomena is physiologically reproducible in wild type BALB/c mice.

Conclusions: Conclusively, this study revealed that PLZF+ T cells have an important role in peripheral immune network by regulating not only the generation of effector CD4 and CD8 T cells, but also the generation of effector/memory-like regulatory T cells in the thymus.

Keywords: PLZF^{+} T cell, Regulatory T cell, TGF-β, IL-4, Effector/memroy phentype

Student Number: 2010-31151

CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

ALK5: Activin-like kinase 5

β2m: Beta-2 microglobulin

BAL: Bronchoalveolar lavage

BM: Bone marrow

BMT: Bone marrow cell transfer

CCR: C-C chemokine receptor

CD: Cluster differentiation

CFSE: Carboxyfluorescein succinimidyl ester

CIITA: Class II transactivator

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

DC: Dendritic cell

DN: Double negative

DP: Double positive

Eomes: Eomesodermin

Foxp3: Forkhead box P3

FTOC: Fetal thymic organ culture

GFP: Green fluorescent protein

GITR: Glucocorticoid induced tumor necrosis factor receptor

GVHD: Graft-versus-host disease

HA: Hemagglutinin

ICOS: Inducible T-cell co-stimulator

IFN-γ: interferon-gamma

IL: Interleukin

iNKT: invariant natural killer T cell

iTreg: induced Treg cells

KI: Knock-in

LAP: Latency-associated protein

Lck: lymphocyte-specific protein tyrosine kinase

MHC: Major histocompatibility complex

nTreg: naturally occurring Treg cells

OVA: Ovalbumin

PLZF: Promyelocytic leukaemia zinc finger protein

SAP: SLAM-associated protein

SLAM: Signaling lymphocyte activation molecule

SP: Single positive

TCR: T cell receptor

T-E: thymocyte-epithelial cells

TEC: Thymic epithelial cell

TGF-beta: Transforming growth factor-beta

Th2: T helper type 2

Treg: Regulatory T cells

T-T: thymocyte-thymocyte

INTRODUCTION

‘Internal image of the External world’ is a phrase to describe what happens in the thymus. The fundamental goal of the immune system is to protect the individual from an anticipated rapidly evolving external foe. To resolve this complicated matter, the immune system employs specialized training systems in the thymus, which are referred to as positive and negative selection. Because the thymus is quarantined, self-antigens rather than exogenous ones are responsible for these processes. A vast array of self-antigens generates mature T cells with extremely diverse T cell receptors (TCRs) for the anticipated encounter with rapidly evolving pathogens. Due to the chance or intense, some pathologically auto-reactive ones survive negative selection. Therefore, the simultaneous generation of regulatory cells to prevent deleterious responses against the self is essential. This strategy is truly powerful in defending the host from external foes and internal enemies and can also result in unmanageable immune problems due to the imbalance between the effector and regulatory cycle.

Several sophisticated regulatory mechanisms are used to maintain immune homeostasis, prevent autoimmunity and moderate inflammation induced by pathogens and environmental insults (1). The negative selection of self-reactive T cells in the thymus leads to the elimination or

inactivation of self-reactive T cell clones and is likely responsible for neutralization of most high-affinity T cells recognizing self-antigens. In the periphery, the steady-state migrating DCs loaded with tissue antigens via the phagocytosis of apoptotic cells in the tissues are transported to the regional lymph node and present self-antigens to T cells, thereby inducing tolerance (2). One of the well-documented regulatory mechanisms involves the action of $Foxp3^+$ regulatory T (Treg) cells, which are now widely regarded as the primary mediators of peripheral tolerance (1).

$CD4^+Foxp3^+$ regulatory T cells (Treg cells) can be divided into two subsets based on their site of origin: naturally occurring Treg (nTreg) cells and induced Treg (iTreg) cells, which acquire Foxp3 expression and suppressor function in the periphery (3). nTreg and iTreg cells differ in their principal antigen specificities and the strength of T-cell receptor (TCR) stimulation needed for their generation (4). Analysis of transgenic mice with both T cell receptors (TCRs) and cognate antigens has illustrated that high-affinity interactions with a single self-peptide induce thymocytes bearing an autoreactive TCR to be selected and become nTregs (5, 6). Consistent with this analysis, autoantigen expression on $AIRE^+$ thymic medullary epithelial cells can induce Treg cell development (7), indicating that the intrathymic generation of Treg cells requires high affinity interactions with cognate self-peptide-MHC

complexes. Their generation is also highly dependent on strong CD28-B7 co-stimulation because the number of nTreg cells was markedly reduced in CD28- or B7-deficient mice (8, 9). In contrast, iTreg cells are generated from peripheral naïve $CD4^+$ T cells ($CD4^+CD25^-$) when they encounter pathogenic antigens or migrate into and are exposed to the gut microenvironment (4). The conversion of naïve $CD4^+$ T cells to $Foxp3^+$ iTreg cells requires relatively weaker, suboptimal TCR stimulation (4) and the inhibitory co-stimulatory molecule CTLA-4 because these iTreg cells do not develop in CTLA4-deficient mice (10). Unlike the crucial role of the CD28-B7 interaction in the generation of nTreg cells (9, 11), iTreg cells do not require this interaction (12).

IL-2 and TGF-β are known to be necessary not only for the generation of nTreg cells and iTreg cells but also for the maintenance and survival of both subsets in the periphery (13-18). Interleukin-6 (IL-6) is a cytokine involved in inflammation and infection responses and also in the regulation of metabolic, regenerative, and neural processes (19). IL-6 combined with endogenous TGF-β was reported to induce a loss of foxp3 expression of nTreg cells and was also found to convert these cells into pathogenic Th17 cells. This finding is in contrast to the long-held belief that Treg cells represent a highly stable lineage. The converted pathogenic Th17 cells subsequently produced pro-inflammatory

cytokines and triggered the development of autoimmunity, indicating the dynamic regulation of Foxp3 expression (20). Unlike nTreg cells, TGF-β-induced iTreg cells were shown to be completely resistant to the Th17-promoting effects of IL-6 (4). This outcome seemed to be due to the markedly reduced IL-6 receptor expression of iTregs compared with nTregs (21).

CD103 is the alpha chain of integrin αEβ7 and provides tissue retention at sites enriched in E cadherin, particularly at the epithelial lining of the gut, lungs and skin but also at sites of inflammation (22). In the case of $Foxp3^+$ regulatory T cells, CD103 was identified as a marker of a highly potent Treg cell subset isolated from murine secondary lymphoid organs (23-25). $CD103^-Foxp3^+$ Treg cells are known to recirculate through lymphoid tissues, whereas $CD103^+Foxp3^+$ Treg cells display an effector/memory-like phenotype and express multiple adhesion molecules and receptors for inflammatory chemokines (26). When these cells migrated into the inflamed site of an ongoing disease model, they efficiently suppressed immune responses *in situ* (23, 25-27). Although $CD103^+$ activated/memory-like Tregs are predominant during the *in vitro* (28) and *in vivo* (29) generation of iTregs and during the activation of nTregs when they encountered cognate antigens in the periphery (29), a small number of $CD103^+Foxp3^+$ Tregs with an

activated/memory-like phenotype still develop from wild-type thymus. In humans, it is not well characterized whether CD103 is a good surface marker for *in vivo*-activated effector/memory Treg cells. The percentage of $CD103^+$ cells among $Foxp3^+CD4^+$ Treg cells was reported to be relatively low, at approximately 5% in healthy controls (30). However, this percentage was increased significantly in patients with conditions marked by chronic inflammation such as multiple sclerosis where TGF-β secretion is usually up-regulated (31). The identification of the best surface marker with which to distinguish human effector/memory Treg cells is required, and CD103 still remains one of the top candidates.

Unlike mouse thymocytes, human fetal thymocytes express MHC class II molecules on their surface (32), and the possibility that $CD4^+$ T cells are able to be positively selected by MHC class II-dependent thymocyte-thymocyte (T-T) interaction was first suggested by reaggregate fetal thymic organ culture (33). Then, this suggestion was confirmed in transgenic mice in which the expression of a human CIITA transgene was controlled by the Lck promoter, which restricted its expression to developing thymocytes and mature T cells (34-36). Invariant natural killer T (iNKT) cells, another well-documented innate cell type, were known to be positively selected by T-T interaction (restricted to CD1d molecules expressed on thymocytes) and to express PLZF (37). In a similar manner, MHC class II-dependent T-T interaction

allows the generation of PLZF$^+$ T-T CD4$^+$ T cells (34). Most importantly, the existence of human PLZF$^+$ CD4$^+$ T cells was demonstrated in human fetal thymus and spleen, signifying that T-T interaction is a physiological event (34, 38). These human counterparts were 42- to 290-fold more frequently found than CD1d/aGalCer tetramer-positive iNKT cells in human fetal lymphoid organs, and they showed diverse TCRβ chain usage. Although PLZF$^+$ T-T CD4$^+$ T cells are somewhat different from iNKT cells in that they have a diverse TCR repertoire and are restricted by MHC class II molecules, these two types of cells share several functional features, including the expression of PLZF as their master regulator, the rapid production of both IL-4 and IFN-γ upon TCR stimulation, and their sole dependence on the SLAM-SAP signaling pathway during their generation (38-40). Recently, several groups reported the significant role of IL-4 produced by these two types of cells in the generation of activated/memory-like T cells in the thymus, such as eomesodermin-expressing innate CD8$^+$ (41, 42) and CD4$^+$ (43, 44) T cells, indicating that changes in the cytokine milieu are able to alter the properties of developing bystander thymocytes.

In the present study, it was demonstrated that PLZF$^+$ innate T cells are able to augment the generation of CD103$^+$ activated/memory-like nTreg cells in the thymuses of CIITA-transgenic (CIITA^{Tg}) mice and

wild-type BALB/c mice. As a mechanism controlling this event, the acquisition of an activated/memory-like phenotype by nTreg cells was dependent on TGF-β, and IL-4 synergistically enhanced the effect of this cytokine. Interestingly, the major source of IL-4 was $PLZF^{+}$ T-T $CD4^{+}$ T cells in $CIITA^{Tg}$ mice and NKT cells in wild-type BALB/c mice. These findings indicate that $PLZF^{+}$ innate T cells allow both effector and regulatory T cells to be activated in the thymus prior to their exit into the periphery.

MATERIALS AND METHODS

1. Mice

As described previously, CIITA^{Tg} mice were generated in our laboratory (35). In this transgenic mouse, expression of the human CIITA gene is under the control of the proximal Lck (lymphocyte protein tyrosine kinase) promoter, which is first expressed early in thymocyte development at the double negative stage. B7$^{-/-}$, CD28$^{-/-}$, β2m$^{-/-}$, IL-4$^{-/-}$, Plzf$^{lu/lu}$, CD45.1 congenic B6, BALB/c mice and BALB/c.IL-4$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). BALB/c.CD1d$^{-/-}$ mice were provided by Dr. DooHyun Jung (Seoul National University College of Medicine). Mice carrying a deletion of the *Mhc2ta* promoter IV (PIV$^{-/-}$) were kindly gifted from H. Acha-Orbea (University of Lausanne, Lausanne, Switzerland). Positive selection of CD4^{+} T cells is abrogated in this mouse due to the absence of MHCII expression in thymic epithelial cells (74). Backcrossing CIITA^{Tg} mice to PIV$^{-/-}$ mice produced CIITA^{Tg}PIV$^{-/-}$ mice in which positive selection signals can only be provided by MHC class II-expressing thymocytes due to the lack of MHC class II molecules on thymic epithelial cells. Foxp3-IRES-GFP-KI (knock-in) mice were a generous gift from Dr. A. Rudensky (University of Washington, Seattle, WA). Breeding CIITA^{Tg} mice with Plzf$^{lu/lu}$ or IL-4$^{-/-}$ mice resulted in CIITA^{Tg}Plzf$^{lu/lu}$ or

CIITA^{Tg}IL-4$^{-/-}$ mice. CIITA^{Tg}PIV$^{-/-}$ mice were bred to β2m$^{-/-}$ or Foxp3-GFP-KI mice to obtain CIITA^{Tg}PIV$^{-/-}$β2m$^{-/-}$ mice and Foxp3-GFP-KI CIITA^{Tg}PIV$^{-/-}$ mice, respectively. C57BL/6 mice were provided from the Center for Animal Resource Development at the Seoul National University College of Medicine. All animals were bred or maintained under specific pathogen-free conditions in the animal facility at the Center for Animal Resource Development at the Seoul National University College of Medicine. Experiments were performed after receiving approval from the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Resources at Seoul National University.

2. Antibodies and flow cytometric analysis

The following fluorochrome- or biotin-labeled antibodies were purchased from BD Bioscience (San Jose, CA, USA) or eBioscience (San Diego, CA, USA): anti-CD4 (RM4-5), anti-CD8 (GK1.5), anti-CD11c (HL3), anti-CD25 (PC61), anti-TCRβ(OX-39), anti-CD45.1 (H1.2F3), anti-CD45.2 (MEL-14), anti-CD39 (24DMS1), anti-CD44 (IM7), anti-CD54 (3E2), anti-CD62L (UC10-4F10-11), anti-CD73 (TY/23), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-CD103 (M290), anti-CD127 (AF6-88.5), anti-CTLA4 (53-6.7), anti-CD183 (7D4), anti-

CD194 (OX-38), anti-CD195 (OX-18), anti-CD197(4B12), anti-I-Ab(AF6-120.1), anti-Gr-1(RB6-8C5), anti-Siglec F(E50-2440), and anti-Foxp3(FJK-16s) antibodies. PE-conjugated goat polyclonal anti-mouse TGF-beta RII was purchased from R&D Systems (Minneapolis, USA), and phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (D27F4) rabbit mAb was obtained from Cell Signaling Technology (Danvers, Massachusetts, USA). Cell surface staining was performed in fluorescence-activated cell sorting (FACS) buffer (1× PBS with 0.1% BSA and 0.1% sodium azide) with different combinations of antibodies for 30 min at 4°C. Intracellular staining of Foxp3 was performed according to the instructions of the Foxp3 staining kit (eBiosciences, San Diego, CA, USA). Samples were analyzed by BD LSRFortessa^{TM} (Becton-Dickinson, Mountain View, CA, USA), and the acquired data were further processed with FlowJo software (Becton Dickinson, San Jose, CA, USA).

3. *In vitro* T_{reg} suppression assays

To determine the suppressive ability of regulatory T cells, $CD4^+GFP^+$ regulatory T cells were isolated from the spleens of $CD45.1^+$ Foxp3-IRES-GFP-KI mice and $CD45.1^+$ Foxp3-IRES-GFP-KI-$CIITA^{Tg}pIV^{-/-}$ mice using a BD FACSAria^{TM} III Cell Sorter. The responder $CD4^+GFP^-$ T cell population was collected from the spleens of $CD45.2^+$ Foxp3-

IRES-GFP-KI mice with the same procedure described above; cell purity was usually greater than 95%. Sorted $CD4^+$ GFP^- responder T cells (5.0×10^4) were CFSE (molecular probe, C34554)-labeled (0.5 μM) and cultured with Dynabeads® Mouse T-Activator CD3/CD28 (Gibco, 11452) at a 1:1 ratio (beads-to-cells) in a 96-well culture plate. $CD4^+GFP^+$ regulatory T cells were added to culture wells at various ratios (from 1:1, 1:3 and 1:9). The experiments were performed in triplicate. After 72 h of co-culture, cells were harvested and stained with APC-conjugated CD45.1 and then analyzed using BD FACSCalibur™.

4. Bone marrow chimeras

Recipient $CIITA^{Tg}$ $PIV^{-/-}$ mice were exposed to 900 rad total body irradiation from a [^{137}Cs] source in two-split doses given 4 h apart. The mice were rested for 24 h before receiving bone marrow (BM) cells. Total BM cells were extracted from the femurs and tibiae of donor mice, and T cells were depleted using a magnetic sorting method (Miltenyi Biotech, Auburn, CA). Each recipient mouse received 3 x 10^6 of T cell-depleted BM cells in a volume of 300 ml PBS via lateral tail vein injection. The thymus and spleen were analyzed 8 wks later.

5. Fetal Thymic Organ Culture

On embryonic day 15.5 (E15.5), fetal thymuses from C57BL/6 mice

were removed and cultured on polycarbonate filters (pore size, 0.8 mm; Millipore, Medford, MA) in RPMI 1640 medium supplemented with 10% fetal bovine calf serum (HyClone, Logan, UT), 1% penicillin and streptomycin (HyClone), and 50 nM 2-ME (Sigma) in the presence or absence of mouse rIL-4 (10 ng/ml; PeproTech) and/or TGFβ1 (15 ng/ml; Cell Signaling). Where appropriate, 1 μM SB431542 (selective inhibitor of TGF-β Type 1 receptor kinases) or 10 μg/ml neutralizing antibody to TGF-β (1D11; R&D Systems) was added once every 2 days. After 7 d, the thymuses were harvested and single-cell suspensions were prepared and analyzed for the expression of CD4, CD8, Foxp3, and CD103 by flow cytometry.

6. Induction of allergic airway inflammation

C57BL/6 mice were sensitized via i.p. injection with 100 μg of ovalbumin emulsified in 50 μl of PBS containing 2 mg of aluminum hydroxide (Thermo) at days 0 and 7. A total of 2.0 x 10^5 cells of sorted GFP^+ regulatory T cells were transferred to the sensitized mice i.v. a day before the initial intranasal challenge (day 13). The intranasal injection of 50 μg ovalbumin in 50 μl of PBS and the challenge were performed for three consecutive days (from day 14 to day 16). Animals were subjected to isoflurane anesthetization for pain relief. Twenty-four hours

after the last challenge, the mice were sacrificed for further analysis (day 17).

7. Isolation of Bronchoalveolar lavage fluid and Lung cells

To obtain bronchoalveolar lavage (BAL) cells, the trachea was cannulated and the lungs were lavaged with 3 ml of PBS. Total and differential cell counts of the BAL were determined by hemocytometer and cytospin preparation stained with Hemacolor® for microscopy (Merck). After collecting BAL cells, the lungs were immediately removed, minced, and placed in 4 ml of complete RPMI 1640 containing 1 U/ml collagenase D and 1 mg/ml DNase I (Roche). The tissue was then incubated at 37°C for 1 h with shaking at 200 rpm. After the incubation, cells were pelleted and treated with 2 ml of ACK lysis buffer to remove RBCs. The remaining tissue was forced through a 70-μm cell strainer, washed thoroughly with complete RPMI 1640, and then counted via the trypan blue exclusion assay. Further analysis was then performed. In some instances, after collecting BAL cells, lungs were infused with 10% neutral buffered formalin to approximate the normal state of inflation, after which they were harvested. The tissues were preserved in formalin for more than 24 h and then subjected to routine paraffin processing and hematoxylin and eosin staining.

8. Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad Software). Bar graphs denoting the percentage of each cell type represent the mean value ± SEM, and the data were compared using an unpaired *t* test.

RESULTS

Foxp3$^+$CD4$^+$ nTregs are efficiently selected by MHC class II molecules on thymocytes.

It has been previously demonstrated that CD4$^+$Foxp3$^+$ Treg cells developed in CIITA^{Tg}CIITA$^{-/-}$ mice where MHC class II molecules were expressed exclusively in thymocytes and mature T cells. Therefore, CD4$^+$ T cells were positively selected only by their interaction with MHC class II molecules expressed on other double positive (DP) thymocytes (35, 36). In these mice, however, thymic negative selection by medullary thymic epithelial cells and dendritic cells was impaired because these cells did not express MHC class II molecules. Therefore, in the present study, it was first assessed whether Treg cells still develop in the thymus in CIITA^{Tg}PIV$^{-/-}$ mice in which MHC class II expression is defected only in cortical thymic epithelial cells and therefore CD4$^+$ T cells are able to undergo negative selection in the thymic medulla (34, 38). Consistent with previous findings in CIITA^{Tg}CIITA$^{-/-}$ mice (35, 36), comparable percentages of Foxp3$^+$ cells were found in both CD4 single positive (SP) thymocytes and splenic CD4$^+$ T cell populations of CIITA^{Tg}PIV$^{-/-}$ mice (Figure 1A and B). Compared to Treg cells of wild-type B6 mice, CD4$^+$Foxp3$^+$ T cells of CIITA^{Tg}PIV$^{-/-}$ mice exhibited comparable suppressive activity during activation and proliferation of T cells *in vitro*

(Figure 1C).

Next, the generation of Treg cells was investigated in CIITA^{Tg}PIV$^{-/-}$ β2m$^{-/-}$ mice and PIV$^{-/-}$ mice because it was previously reported that CD4^{+}Foxp3^{+} T cells could be positively selected in an MHC class I-dependent manner in MHC class II-deficient mice (45). Unlike CIITA^{Tg}PIV$^{-/-}$ mice, only a very small number of CD4^{+}Fox3^{+} cells was detected in the thymus of PIV$^{-/-}$ mice (Figure 1A and B). Moreover, Treg cell generation in CIITA^{Tg}PIV$^{-/-}$ mice was not seriously affected by the absence of MHC class I expression (Figure 1A and B). Taken together, these data confirm that the majority of Treg cells that developed in CIITA^{Tg}PIV$^{-/-}$ mice were selected by MHC class II molecules, rather than MHC class I molecules.

It is well-documented that B7-CD28 costimulation is required for the generation and maintenance of Treg cells (9, 11, 46). To determine whether Treg cells generated via T-T interaction are also dependent on this costimulation, bone marrow (BM) chimeras were established. BM cells isolated from CD45.2^{+} CIITA^{Tg} or CD45.2^{+} CIITA^{Tg}CD28$^{-/-}$ mice were transferred to lethally irradiated CD45.1^{+} CIITA^{Tg}PIV$^{-/-}$ mice (referred to as T-T and T-T CD28$^{-/-}$, respectively). Flow cytometric analysis at 8 weeks after bone marrow transfer (BMT) revealed that a substantial fraction of CD4^{+}Foxp3^{+} T cells was detected in the thymuses and spleens of recipients of CIITA^{Tg} BM cells, whereas Treg cells were

almost completely absent in mice that received CIITA^{Tg}CD28$^{-/-}$ BM cells (Figure 2A and B). These findings indicate that the development of Treg cells in CIITA^{Tg}PIV$^{-/-}$ mice was dependent on CD28 costimulation.

In the mouse thymus, the natural ligand of CD28, B7, is expressed only in medullary epithelial cells and dendritic cells (DCs) (47, 48). However, dependency on CD28-B7 interaction during the development of T-T Tregs led us to investigate whether B7-1 and/or B7-2 molecules were expressed in CIITA^{Tg}pIV$^{-/-}$ thymocytes. As expected, DCs expressed high levels of B7-1 and B7-2 molecules, whereas expression was almost completely absent in wild-type thymocytes (Figure 2C). However, thymocytes in CIITA^{Tg}pIV$^{-/-}$ mice expressed a detectable level of B7-1 and B7-2 on their surface, unlike wild-type thymocytes.

Foxp3^{+} regulatory T cells that developed in CIITA^{Tg}PIV$^{-/-}$ mice have an effector/memory phenotype.

Next, the expression patterns of surface molecules on Treg cells in the thymus were compared for wild-type and CIITA^{Tg}PIV$^{-/-}$ mice. The analysis was specifically focused on the expression of CD103, a well-known marker used to discriminate activated/memory-like subsets of Treg cells (23, 25, 26). The fraction of CD103^{+} cells among CD4^{+}Foxp3^{+} T cells was approximately 2.5-fold higher in the thymus and spleen of CIITA^{Tg}PIV$^{-/-}$ mice than that in wild-type mice (Figure 3A and B). This

result was also found in CIITA^{Tg}PIV$^{-/-}$β2m$^{-/-}$ mice, confirming that the MHC class II-dependent T-T interaction is responsible for the development of activated/memory-like CD103^{+} Treg cells. Consistent with the previous reports (26, 45), CD103^{+} Treg cells in wild-type mice had an activated/memory-like phenotype, as shown by the up-regulation of CD44, CD54, CD127 and ICOS and the down-regulation of CD62L (Figure 3C). In contrast, most of Treg cells in the thymus and spleen of CIITA^{Tg}PIV$^{-/-}$ mice exhibited an activated/memory-like phenotype regardless of CD103 expression.

Then, the expression levels of inhibitory surface molecules and tissue-homing chemokine receptors were investigated in Treg cells of wild-type B6 and CIITA^{Tg}PIV$^{-/-}$ mice. As expected, the expression pattern of these molecules overlapped with that of activated/memory markers. In wild-type B6 mice, CD103^{+} Treg cells showed a higher expression level of inhibitory surface molecules (CD39, CD73 and latency-associated protein (LAP)) (Figure 4A) and tissue-homing chemokine receptors (CXCR3 and CCR4) (Figure 4B), compared to that of CD103^{-} Treg cells; both CD103^{+} and CD103^{-} Treg cells of CIITA^{Tg}PIV$^{-/-}$ mice highly expressed these molecules at a level similar to that of wild-type CD103^{+} Treg cells (Figure 4A and B). However, the expression levels of GITR and the CCR7 lymphoid homing receptor were lower in Treg cells of CIITA^{Tg}PIV$^{-/-}$ mice than in those of wild-type

mice, regardless of CD103 expression (Figure 4A and B).

Regulatory T cells of CIITA^{Tg}PIV$^{-/-}$ mice exhibited enhanced suppressive activity during airway inflammation.

It has been shown that Treg cells accumulate and attenuate the inflammatory response in the lungs of ovalbumin-induced allergic model mice in a CCR4-dependent manner (49, 50). Based on this finding, the *in vivo* function of Treg cells was assessed in an ovalbumin-induced airway hypersensitivity model. C57BL/6 mice were first sensitized by the intraperitoneal injection of OVA emulsified in an aluminum-based adjuvant at days 0 and 7. Then, 2.0 x 10^5 Treg cells isolated from Foxp3-GFP knock-in B6 or CIITA^{Tg}PIV$^{-/-}$ mice were transferred to the sensitized mice 1 day before the first intranasal ovalbumin challenge. After 3 days of consecutive intranasal ovalbumin challenge, the severity of airway inflammation was assessed 24 hours after the last ovalbumin challenge (on day 17). Differential BAL cell counts revealed an intense influx of immune cells including eosinophils and neutrophils, macrophages and lymphocytes into the airways of mice exposed to ovalbumin compared to intact mice (Figure 5A). FACS analysis and lung histology of the mice challenged with ovalbumin again confirmed the severely induced inflammatory status. Specifically, the challenged mice had a 6-fold higher number of infiltrated eosinophils (CD11c$^-$ Siglec-F^{hi}

Gr-1^{int} MHCII^{-} cells) and intense parenchymal inflammation characterized by diffuse cell infiltrates (Figure 5B and 5D). Interestingly, the transfer of Treg cells from Foxp3-GFP knock-in CIITA^{Tg}PIV$^{-/-}$ mice (TT Treg), but not from Foxp3-GFP knock-in B6 mice (TE Treg), significantly decreased the cellular infiltration of eosinophils and neutrophils both in BAL and lung (Figure 5A and 5B). Analysis of lung histology also supported this result (Figure 5D). However, the transfer of Treg cells from Foxp3-GFP knock-in CIITA^{Tg}PIV$^{-/-}$ mice did not reduce the number of infiltrating macrophages and lymphocytes into the airways, suggesting that there are specific inhibitory effects of these populations on the infiltration of eosinophils (Figure 5A). Consistent with the reduced number of eosinophils in the airways and lung, the percentage of lung CD4^{+} T cells producing IL-5 was also lower in mice that received Treg cells from Foxp3-GFP knock-in CIITA^{Tg}PIV$^{-/-}$ mice when compared to mice that received Treg cells from Foxp3-GFP knock-in B6 mice or none (Figure 5C). These data indicate that Treg cells developed in CIITA^{Tg}PIV$^{-/-}$ mice have more potent suppressive activity on Th2-mediated allergic airway inflammation compared to wild-type Treg cells.

TGF-β signaling is responsible for the generation of activated/memory-like regulatory T cells in the thymus.

What is the cellular mechanism driving the increased generation of

activated/memory-like Treg cells in CIITA^{Tg}PIV$^{-/-}$ mice? TGF-β was reported to be a major factor inducing CD103 expression on CD4^{+} T cells (51-55), and the concomitant expression of CD103 during the course of the TGF-β-induced conversion of naïve CD4^{+} T cells into iTreg cells was supportive (29). It was investigated to see whether TGF-β stimulation is able to induce CD103 expression during the development of Treg cells by performing fetal thymic organ culture (Figure 6A). After 7 days of *ex vivo* culture, Foxp3^{+}CD4^{+} Treg cells were detected in cultured thymic tissue, and the addition of TGF-β1 did not alter the development of Foxp3^{+} cells but rather markedly increased the CD103-positive fraction in the Foxp3^{+} Treg population. Moreover, this TGF-β-induced effect was almost completely inhibited by a specific inhibitor of ALK5 (SB-431542) and a TGFβ neutralizing antibody (1D11). Then, BM chimeras were established to confirm whether TGF-β signaling is responsible for the increased CD103^{+} Treg cells observed in CIITA^{Tg}PIV$^{-/-}$ mice. Based on the fact that Smad proteins are major mediators of intracellular TGF-β signaling (56), mixed BM cells from CD45.1/CD45.2^{+} CIITA^{Tg} mice and CD45.2^{+} wild-type or Smad4$^{-/-}$ mice were transferred to irradiated CD45.1^{+} CIITA^{Tg}PIV$^{-/-}$ mice in which WT or Smad4$^{-/-}$ thymocytes are able to be positively selected by MHC class II-expressing DP thymocytes, respectively. Flow cytometric analysis was done 8 weeks after BMT. To

validate the effect of TGF-β on the development of $Foxp3^+$ T-T $CD4^+$ T cells, the ratio of $CD45.22^+$ cells to $CD45.11^+$ cells among $Foxp3^+CD4$ SP thymocytes was compared between WT-BM and $Smad4^{-/-}$-BM chimeras (Figure 6B). Consistent with the previous report that TGF-β is not crucial for the development of nTreg cells (57), no significant differences were observed in the generation of $Foxp3^+$ cells from $Smad4^{-/-}$-BM compared with WT-BM cells. On the other hand, the frequency of $CD103^+$ Treg cells was markedly reduced in $Smad4^{-/-}$ Treg cells compared to $CD103^+$ Treg cells developed from $CIITA^{Tg}$ BM, whereas there was no obvious difference in the frequency of $CD103^+$ cells between Treg cells derived from wild-type and $CIITA^{Tg}$ BM cells (Figure 6C). These data indicate that TGF-β is responsible for the induction of CD103 molecules on Treg cells developed in the thymuses of $CIITA^{Tg}PIV^{-/-}$ mice.

IL-4 produced by thymic $PLZF^+$ innate T cells enhances the generation of TGF-β-driven effector/memory regulatory T cells.

Previous studies have demonstrated that $PLZF^+$ T cells induce the generation of activated/memory-like $CD8^+$ (41, 42) and $CD4^+$ (43, 44) T cells in the thymuses of $CIITA^{Tg}$ and $CIITA^{Tg}PIV^{-/-}$ mice, and *itk*$^{-/-}$ mice, respectively, in an IL-4-dependent manner. Based on these findings, it

was investigated whether a similar mechanism operated in the development of activated/memory-like Treg cells. For this investigation, Treg cells in the thymuses of CIITA^{Tg} mice were compared to those of CIITA^{Tg}IL-4$^{-/-}$ and CIITA^{Tg}Plzf$^{lu/lu}$ mice. In CIITA^{Tg}IL-4$^{-/-}$ mice, the frequency of CD103^{+} Treg cells was decreased to the level found in wild-type or IL-4$^{-/-}$ mice, and a similar tendency was found in CIITA^{Tg}Plzf$^{lu/lu}$ mice in which PLZF expression was fairly undetectable (42). These data suggest that the generation of CD103^{+} Treg cells was influenced by IL-4 (Figure 7A). However, FTOC in the presence of IL-4, TGF-β or IL-4 plus TGF-β revealed that IL-4 alone was not able to increase the expression of CD103 on CD4^{+}Foxp3^{+} T cells, but rather, it reinforces the effect of TGF-β (Figure 7B). Moreover, Treg cells cultured in the presence of IL-4 and TGF-β expressed a higher level of CD54, an activated/memory cell marker, than the level found in culture conditions in the presence of IL-4 or TGF-β alone (Figure 7C).

IL-4 produced by PLZF^{+} iNKT cells enhances the generation of CD103^{+} Foxp3^{+} regulatory T cells in BALB/c mice.

In the present study, it was demonstrated that more Treg cells from CIITA^{Tg} and CIITA^{Tg}PIV$^{-/-}$ mice than those of wild-type mice acquired an activated/memory-like phenotype during their developmental process,

and the acquisition was dependent on two cytokines: IL-4 (produced by $PLZF^+$ T-T $CD4^+$ T cells) and TGF-β. This result raised the question of whether the activated/memory-like phenotype is one of the characteristics of Treg cells selected by MHC class II-dependent T-T interaction, or whether Treg cells selected by thymic epithelial cells are able to acquire an activated/memory-like phenotype in the presence of IL-4 and TGF-β. To address this issue, the phenotype of Treg cells developed in BALB/c mice in which there was a much higher number of $PLZF^+$ NKT cells in the thymus than that observed in B6 mice (58, 59) were compared to Treg cells developed in BALB/c.$CD1d^{-/-}$ and BALB/c.$IL\text{-}4^{-/-}$ mice. Notably, a higher percentage of Treg cells both in the thymus and spleen from wild-type BALB/c mice were of an activated/memory-like phenotype than were those of BALB/c.$CD1d^{-/-}$ and BALB/c.$IL\text{-}4^{-/-}$ mice (Figure 8). This finding strongly suggests that even Treg cells selected by conventional thymocyte-thymic epithelial cell interaction are able to acquire an activated/memory-like phenotype in IL-4-rich conditions as long as there are sufficient numbers of $PLZF^+$ innate T cells such as T-T $CD4^+$ T cells and NKT cells.

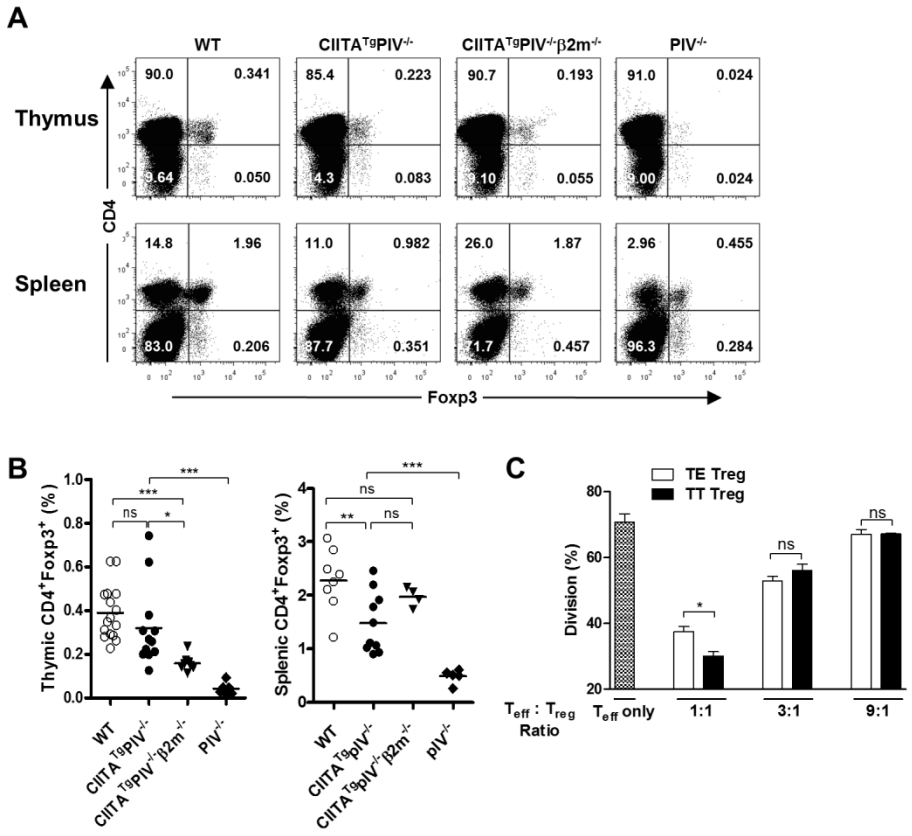

Figure 1. **Functional CD4$^+$Foxp3$^+$ T cells are generated in the thymus of CIITA^{Tg}PIV$^{-/-}$ mice via MHC class II-dependent T-T interaction**.

(A, B) Flow cytometric analysis of thymocytes and splenocytes from 8-week-old wild-type (WT), CIITA^{Tg}PIV$^{-/-}$, CIITA^{Tg}PIV$^{-/-}$β2m$^{-/-}$ and PIV$^{-/-}$ mice was performed to assess CD4 and Foxp3 expression. Representative dot plots (A) and summarized results (B) from seven (CIITA^{Tg}pIV$^{-/-}$) or eight (wild-type) mice are shown. The numbers indicate the percentage of cells in each quadrant. (C) CFSE-labeled

$CD4^+GFP^-$ T cells isolated from Foxp3-GFP-KI mice were mixed with $CD4^+GFP^+$ T cells from CD45.1 Foxp3-GFP-KI or CD45.1 Foxp3-GFP-KI-$CIITA^{Tg}PIV^{-/-}$ mice and were stimulated with anti-CD3/CD28 microbeads for 3 days. Proliferation was assessed by measuring the dilution of CFSE in $CD45.1^-$ cells by flow cytometry. A representative result from three independent experiments is shown. n.s.: not significant; $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$.

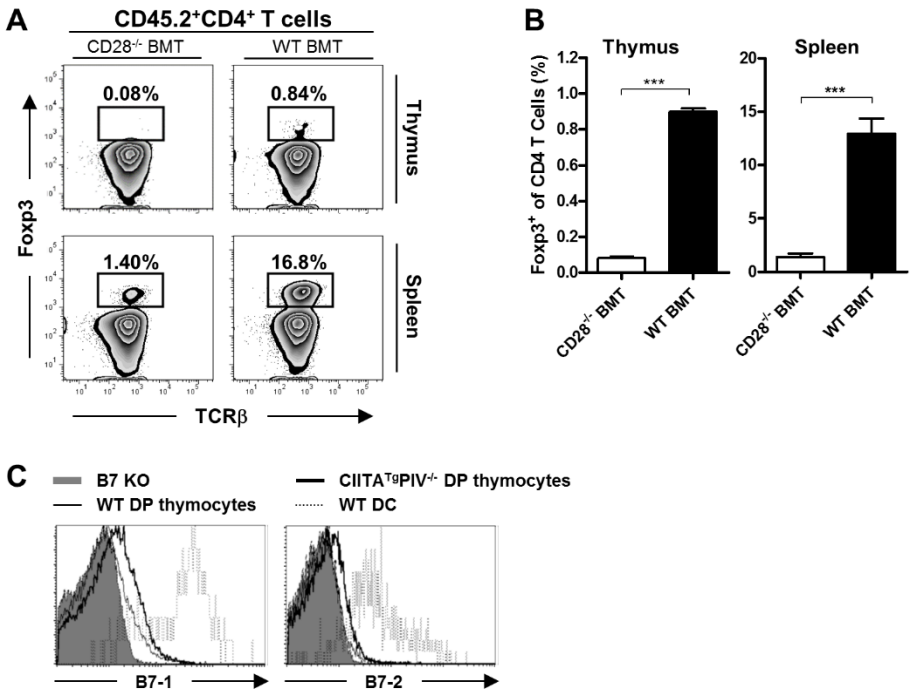

Figure 2. **Generation of Foxp3^{+} regulatory T cells in CIITA^{Tg}PIV$^{-/-}$ mice is dependent on CD28-B7 interaction**.

Lethally irradiated CD45.1^{+} recipient CIITA^{Tg}PIV$^{-/-}$ mice were reconstituted with T cell-depleted bone marrow (BM) cells from wild-type or CD28$^{-/-}$ mice, which were then analyzed 8 weeks after the BM transfer. (A) A representative dot plot of CD45.2^{+} CD4^{+} T cells both from thymus (upper panel) and spleen (lower panel) of CD28$^{-/-}$ BMT (n=4) and WT BMT (n=4). (B) The cumulative frequency of Foxp3^{+} cells from the CD4^{+} T cell population is shown. *** $P < 0.0001$. (C) The B7 expression level in DP thymocytes from CIITA^{Tg}pIV$^{-/-}$ mice (thick black line) was compared to the expression on indicated cells from wild-type

(WT) or $CD80^{-/-}CD86^{-/-}$ (B7 KO) mice. Representative flow cytometry data from two independent experiments are shown.

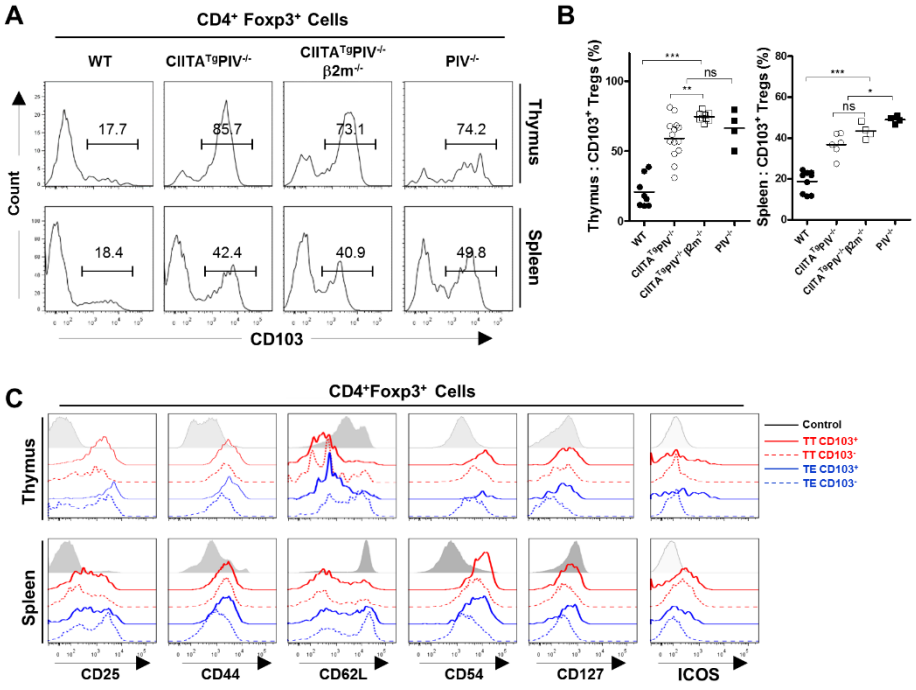

Figure 3. **Foxp3$^+$ regulatory T cells in CIITA^{Tg}PIV$^{-/-}$ mice show an activated/memory-like phenotype**.

CD4$^+$ Foxp3$^+$ T cells from the thymus and spleen of wild-type, CIITA^{Tg}PIV$^{-/-}$, CIITA^{Tg}PIV$^{-/-}$β2m$^{-/-}$, and PIV$^{-/-}$ mice were analyzed for their expression pattern of effector/memory markers. (A) Representative flow cytometry data of CD103 expression in thymic and splenic CD4$^+$Foxp3$^+$ T cells. The numbers in the histogram indicate the percentage of CD103$^+$ cells. (B) Summary of CD103$^+$ cell percentages among CD4$^+$ Foxp3$^+$ T cells. The bar indicates group means. n.s.: not significant; *P < 0.05; **P < 0.001; ***P < 0.0001. (C) Surface expression of activated/memory-like markers (CD25, CD44, CD62L,

CD54, CD127 and ICOS) on $CD103^+$ and $CD103^-$ $CD4^+$ $Foxp3^+$ T cells from wild-type and $CIITA^{Tg}PIV^{-/-}$ mice. $CD4^+$ $Foxp3^-$ T cells from wild-type mice were used as a control. Data are representative of three independent experiments.

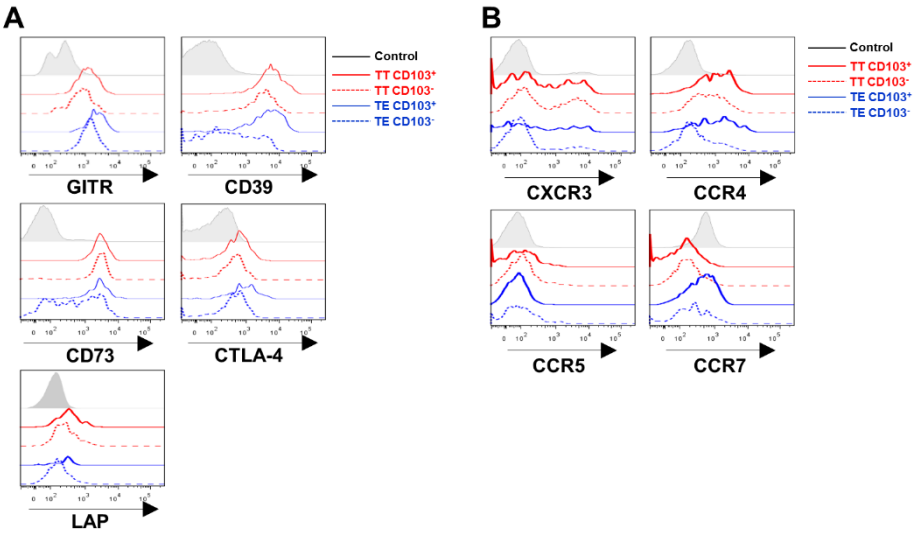

Figure 4. **Regulatory T cells from CIITA^{Tg}PIV$^{-/-}$ mice up-regulated the expression of molecules associated with regulatory function and tissue migration**.

Flow cytometry data of the surface expression of (A) regulatory molecules: GITR, CD39, CD73, CTLA-4, and LAP. Expression of (B) effector chemokine receptors: CXCR3, CCR4, CCR5 and CCR7 on CD103^{+} and CD103^{-} Foxp3^{+} CD4 SP thymocytes from wild-type and CIITA^{Tg}PIV$^{-/-}$ mice. Foxp3^{-} CD4 SP thymocytes from wild-type mice were used as a control.

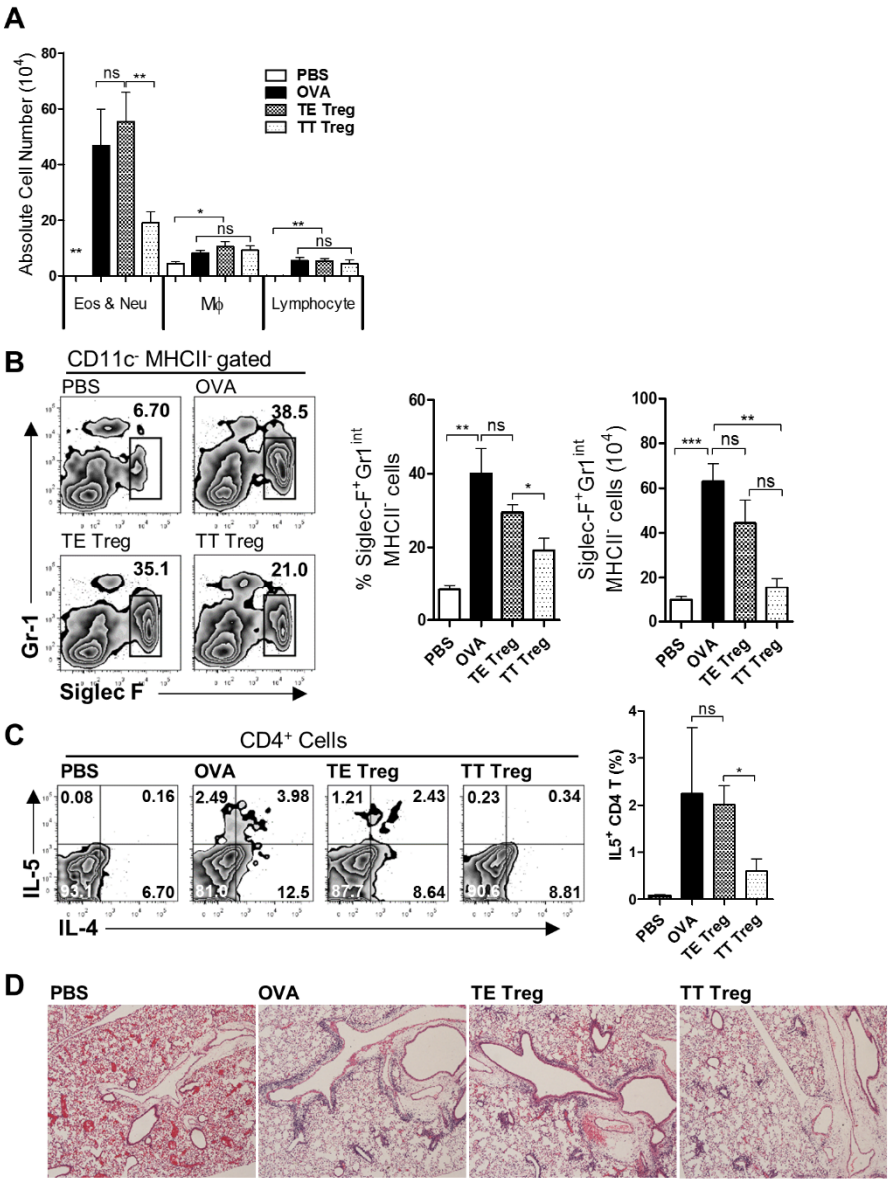
A
Absolute Cell Number (10^4)
PBS
OVA
TE Treg
TT Treg
Eos & Neu
Mϕ
Lymphocyte
B
CD11c⁻ MHCII⁻ gated
PBS
OVA
TE Treg
TT Treg
6.70
38.5
35.1
21.0
Gr-1
Siglec F
% Siglec-F⁺Gr1^{int} MHCII⁻ cells
Siglec-F⁺Gr1^{int} MHCII⁻ cells (10^4)
C
CD4⁺ Cells
IL-5
IL-4
0.08
0.16
6.70
2.49
3.98
12.5
1.21
2.43
8.64
0.23
0.34
8.81
IL5⁺ CD4 T (%)
D

Figure 5. **Airway inflammation is efficiently suppressed by Tregs from CIITA^{Tg}PIV$^{-/-}$ mice**.

The ovalbumin-sensitized C57BL/6 mice at days 0 and 7 received 2.0 x 10^5 CD4$^+$ GFP$^+$ T cells isolated from CD45.1$^+$Foxp3-GFP-KI or CD45.1$^+$Foxp3-GFP-KI CIITA^{Tg}PIV$^{-/-}$ mice by FACSAria cell sorter 1 day before three consecutive intranasal administrations of ovalbumin (at days 14, 15 and 16). These mice were sacrificed on day 17. (A) Differential counts of cytospin preparations from BAL cells. (B) Representative flow cytometry data of lung eosinophils (I-A^{b-}CD11c$^-$ Siglec F^{hi}Gr-1^{int}) (left panel). Cells harvested from lungs were stained for I-A^b, CD11c, Siglec F, and Gr-1 and gated in I-A^{b-}CD11c$^-$ cells. Numbers in boxes indicate the percentage of the cells. The cumulative data for the percentage (middle panel) and absolute number (right panel) of eosinophils of the lungs are displayed. The bar indicates the mean value ± SEM. (C) Representative pattern of lung CD4$^+$ T cells expressing IL-5 and IL-4 and their cumulative percentages are shown. Cells recovered from the lung were cultured for 5 days with ovalbumin (100 μg/ml) and re-stimulated with PMA and ionomycin in the presence of Brefeldin A for the last 6 hours. Samples were harvested and stained for TCRβ, CD4, IL-5 and IL-4 and gated in CD4$^+$TCRβ$^+$ cells. Numbers in quadrants represent the percentage of the cells. (D) Representative lung histology stained with hematoxylin/eosin for analysis of cellular

inflammation (original magnification x40). n.s.: not significant; *p < 0.05; **p < 0.001.

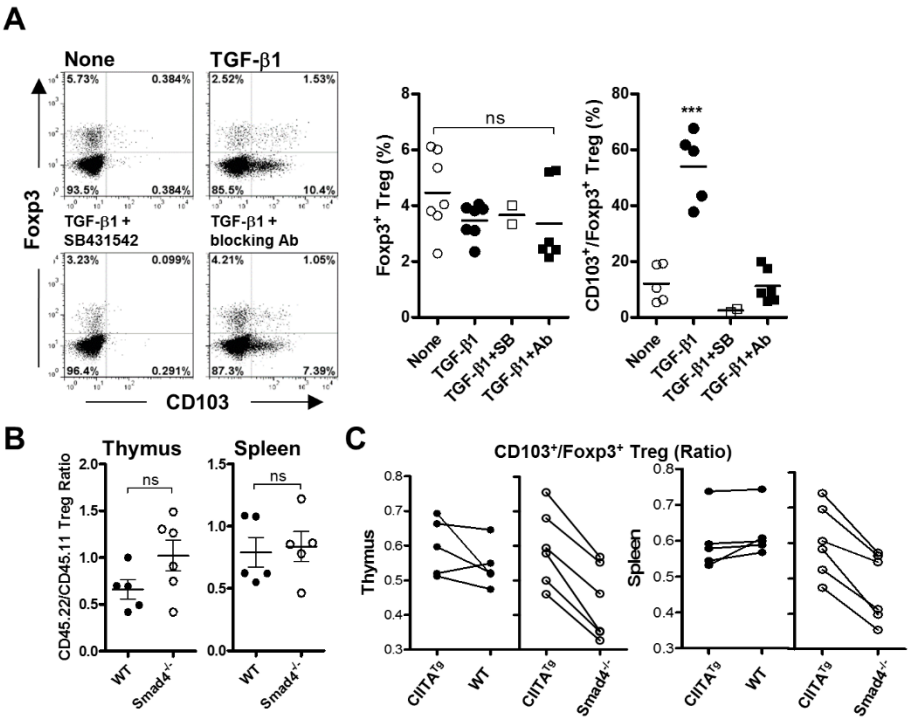

Figure 6. **TGF-β signaling is responsible for the generation of activated/memory-like regulatory T cells in the thymus**.

(A) Fetal thymuses of E15.5 B6 mice were cultured in the presence or absence of exogenous mouse IL-4, mouse TGF-β1 or both for 7 days. Two to three fetal thymic lobes were used for each experimental condition, and pooled cells were analyzed by flow cytometry. A representative profile of CD4 SP thymocytes from five independent experiments (left panel) and their cumulative summary (right panel) are shown. The bar indicates the mean value. n.s.: not significant; ***$P <$ 0.0001. (B) T cell-depleted bone marrow cells from CD45.11^{+} CIITA^{Tg}

mice were mixed with wild-type or Smad4$^{-/-}$ mice at a 1:1 ratio. The mixed BM cells were transferred to lethally irradiated (900 rads) CD45.12^{+} CIITA^{Tg}PIV$^{-/-}$ recipients via i.v. injection. Six to seven weeks after transfer, the thymus and spleen were extracted and analyzed by flow cytometry: CD45.11-positive thymocytes were considered to be generated from CIITA^{Tg} BM; CD45.22-positive thymocytes from wild-type or Smad4$^{-/-}$ BM; and CD45.12-double positive cells were excluded. The ratio of CD45.22 Foxp3^{+} CD4^{+} T cells to CD45.11 Foxp3^{+} CD4^{+} T cells in WT or Smad4$^{-/-}$ BMT chimeras was calculated and summarized: filled circle (●, CIITA^{Tg} + WT → CIITA^{Tg}PIV$^{-/-}$, n=5) and white circle (○, CIITA^{Tg} + Smad4$^{-/-}$ → CIITA^{Tg}PIV$^{-/-}$, n=6). n.s.: not significant. (C) The ratio of CD103^{+} cells among CD4^{+}Foxp3^{+} cells was calculated independently in each population and then compared. The dots (which represent the calculated ratio) connected by a line show data obtained from one individual.

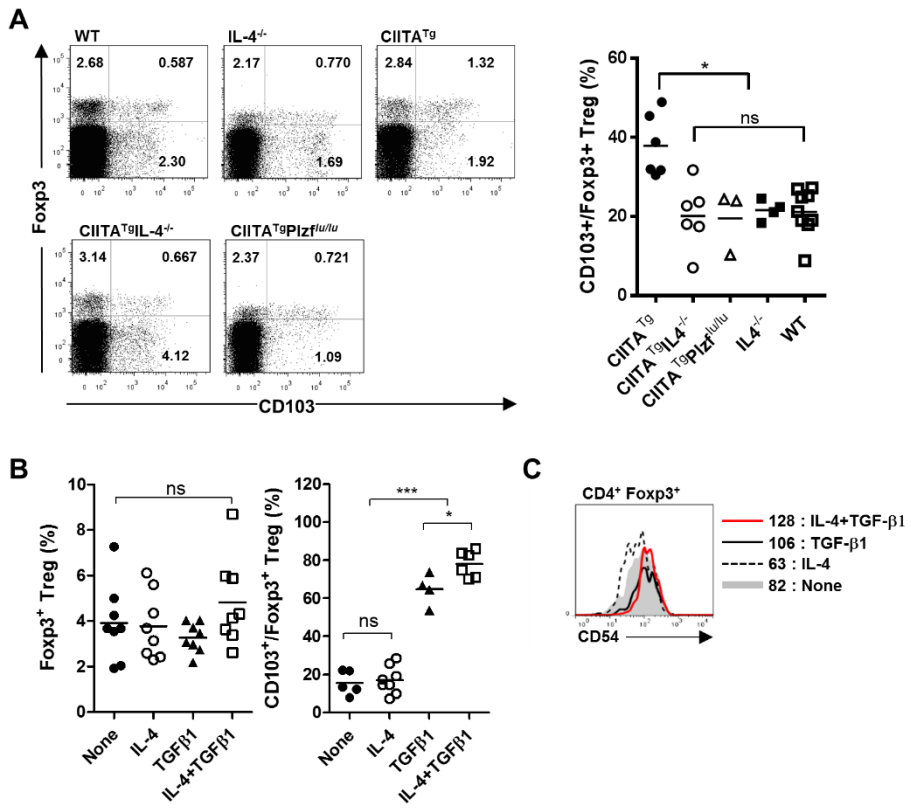

Figure 7. IL-4 produced by thymic PLZF^{+} innate T cells enhances the generation of TGF-β-driven activated/memory-like regulatory T cells.

(A) Representative profile of Foxp3 and CD103 of CD4 SP thymocytes from C57BL/6, IL-4$^{-/-}$, CIITA^{Tg}, CIITA^{Tg}IL-4$^{-/-}$ and CIITA^{Tg}Plzf$^{lu/lu}$ mice (left panel), and the cumulative summary of the percentage of CD103^{+} cells among CD4^{+}Foxp3^{+} cells (right panel). The numbers in quadrants indicate the percentage of the cells. (B) FTOC was performed in the presence or absence of mouse IL-4, mouse TGF-β1 or both for 7 days.

Samples were analyzed by flow cytometry, and the percentage of $Foxp3^+$ cell and the calculated frequency of $CD103^+$ cells among $CD4^+Foxp3^+$ thymocytes are displayed as a cumulative summary. The bars in the summary indicate the mean value. (C) The expression level of the representative activation molecule, CD54, was measured by flow cytometry in $CD4^+Foxp3^+$ T cells developed in each condition of FTOC. Numbers indicate the median fluorescence intensity (MFI). n.s.: not significant; $*P < 0.05$; $***P < 0.0001$.

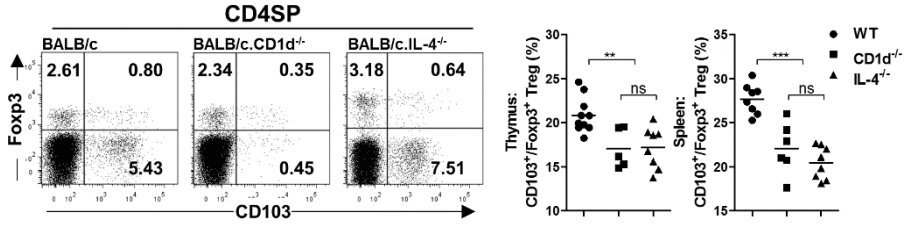

Figure 8. NKT cells enhance the generation of CD103$^+$ Foxp3$^+$ regulatory T cells in wild-type BALB/c mice.

Representative data of the expression pattern of Foxp3 and CD103 in CD4 SP thymocytes from BALB/c, BALB/c.CD1d$^{-/-}$ and BALB/c.IL-4$^{-/-}$ thymuses (left). The numbers in boxes indicate the percentage of the cells. The percentage of CD103$^+$ of CD4$^+$Foxp3$^+$ cells was calculated both from the thymus and spleen, and their cumulative data are summarized (right). n.s.: not significant; $^{**}P < 0.001$; $^{***}P < 0.0001$.

DISCUSSION

This study revealed a novel role of IL-4 produced by $PLZF^+$ innate T cells in the intrathymic generation of activated/memory-like $Foxp3^+$ Treg cells. In general, naturally occurring $Foxp3^+$ Treg cells acquire an activated/memory-like phenotype mostly when they encounter a specific antigen in the periphery (4, 27). However, Treg cells from $CIITA^{Tg}PIV^{-/-}$ mice already exhibit an activated/memory-like phenotype when in the thymus. Specifically, these cells demonstrate a higher level of CD44, CD54, CD103, CD127 and ICOS and a lower level of CD62L compared to Treg cells found in the wild-type control. By using $CIITA^{Tg}$ mice in a PLZF- and IL-4-deficient background, it was demonstrated that the intrathymic acquisition of the activated/memory-like phenotype was affected by the expression of PLZF and the presence of IL-4. These findings indicated that IL-4 produced by $PLZF^+$ T-T $CD4^+$ T cells played a crucial role in the generation of activated/memory-like Treg cells in the thymuses of $CIITA^{Tg}PIV^{-/-}$ mice. The IL-4-dependent acquisition of an activated/memory-phenotype by Treg cells was also found in wild-type BABL/c mice. In these mice, CD1d- or IL-4 deficiency also lowered the expression of the activated/memory-like phenotype in intrathymic Treg cells, indicating that the generation of activated/memory-like Treg cells in BALB/c thymuses was also dependent on IL-4 produced by iNKT

cells.

Several groups have previously reported the effects of IL-4 on the development and function of naturally occurring $Foxp3^+$ Treg cells and induced $Foxp3^+$ Treg cells (60-63). IL-4 partly prevents the apoptosis of nTreg cells, resulting in increased survival, and also preserves their suppressive ability by maintaining the mRNA levels of Foxp3 and CD25 (60). In the case of iTreg cell generation, the role of IL-4 is somewhat controversial. One group reported that IL-4 and IL-13 play an important role in the extrathymic generation of $Foxp3^+CD25^+$ Treg cells via an antigen-dependent mechanism (61), whereas other groups have reported that IL-4 inhibits the TGF-β-mediated generation of Treg cells (62, 63). In the present study, it was demonstrated by using IL-4- or Smad4-deficient mice that the intrathymic acquisition of an activated/memory-like phenotype by Treg cells was dependent on both IL-4 and TGF-β signaling without affecting the generation of Treg cells. However, TGF-β seemed to be the main cytokine responsible for this Treg cell phenotype because in FTOC, TGF-β alone was able to induce the expression of activated/memory markers such as CD103 on the Treg cells. IL-4 alone did not have this effect and instead synergistically enhanced the TGF-β-dependent development of activated/memory-like Treg cells. As mentioned earlier (41, 42, 59), the major source of IL-4 in the thymus

was PLZF^{+} innate T cells: T-T CD4^{+} T cells in CIITA^{Tg} mice and iNKT cells in BALB/c mice.

We used CD103 as a major marker of activated/memory-like Treg cells. Murine Treg cells can be divided into two subsets based on CD103 expression (23, 25, 26). CD103^{-} Treg cells resemble naïve T cells and recirculate through lymph nodes. In contrast, CD103^{+} Treg cells had features typical of activated/memory-like cells. Regarding suppressive activity, CD103^{+} activated/memory-like Treg cells were initially reported to be more potent suppressors, both *in vitro* and *in vivo*, than CD103^{-} Treg cells (26). However, in several subsequent studies, CD103^{+} Treg cells showed no difference in the suppression of effector T cell proliferation and cytokine production *in vitro* compared with the CD103^{-} subset, when an anti-CD3 antibody was used as a polyclonal stimulator (45, 64). Treg cells from CIITA^{Tg}PIV$^{-/-}$ mice attenuated the severity of ovalbumin-induced airway inflammation more efficiently compared with Treg cells of wild-type mice. However, an *in vitro* T cell suppression assay failed to demonstrate the superior activity of Treg cells isolated from CIITA^{Tg}PIV$^{-/-}$ mice. During the inflammatory process, the migratory capacity of Treg cells is one of the major factors determining the *in vivo* suppressive effect of Treg cells (49, 64). In a skin inflammation model, only CD103^{+} Treg cells showed an efficient migration into the inflamed skin (26), and the *in vivo* suppressor function

of tumor-derived effector/memory-like Treg cells was dependent on CCR5 expression (64). Similar to these types of $CD103^+$ Treg cells, activated/memory T cells of $CIITA^{Tg}PIV^{-/-}$ mice also showed higher level expression of tissue-homing chemokine receptors, such as CXCR3 and CCR4, than those of wild-type mice. Although adaptively transferred Treg cells of $CIITA^{Tg}PIV^{-/-}$ mice were not detected in the allergy-induced lung by flow cytometry, based on the previous report that CCR4 is critically involved in the attenuation of allergic airway inflammation (49), the efficient *in vivo* suppressive function of Treg cells from $CIITA^{Tg}PIV^{-/-}$ mice might be associated with enhanced migration capacity into inflamed tissue.

The notion that natural regulatory T cells originate from thymocytes expressing auto-reactive TCRs was demonstrated in 6.5 TCR transgenic mice, which were engineered to express a particular self-peptide (influenza hemagglutinin, HA) or its derivatives under the control of the I-Eα promoter (7). Using $Nur77^{GFP}$ mice, it was subsequently confirmed that a high-affinity interaction is directly responsible for the development of natural $Foxp3^+$ regulatory T cells (6). Consistent with the previous reports, $CD4^+Foxp3^+$ T cells from $CIITA^{Tg}$ and $CIITA^{Tg}PIV^{-/-}$ mice showed a much higher level of CD5, a negative regulator of the TCR signal strength, than was observed in $CD4^+Foxp3^-$ T cells from the same mice.

A novel function of $PLZF^+$ innate T cells during the process of Treg cell development was initially investigated in $CIITA^{Tg}$ mice and then confirmed in wild-type BALB/c mice. However, other mechanisms that regulate the generation of activated/memory-like T cells likely exist. First, a considerable fraction of $CD103^+$ Treg cells was still present in the thymuses of PLZF- or IL-4 deficient mice. In $CIITA^{Tg}PIV^{-/-}$ mice, the $CD103^-$ Treg cell subset also exhibited activated/memory-like phenotype, unlike $CD103^-$ Treg cells in wild-type mice, suggesting that the MHC class II-dependent T-T interaction itself might contribute to the expression of the activated/memory-like phenotype in intrathymic Treg cells. In addition, MHC class I-restricted activated/memory-like $CD4^+Foxp3^+$ Treg cells had been previously identified (45). This finding raises the possibility that Treg cells in $CIITA^{Tg}PIV^{-/-}$ mice might be selected by MHC class I molecules. However, the results from $CIITA^{Tg}PIV^{-/-}$ mice back-crossed into a β2m-deficient background confirmed that most of the Treg cells in $CIITA^{Tg}PIV^{-/-}$ mice were selected by MHC class II molecules expressed on thymocytes.

Along with the TCR-MHC interaction, the positive selection of Tregs requires simultaneous co-stimulatory signals through CD28 (9, 11). As shown in a mixed BMT chimeric mouse system, the development of T-T Tregs was also dependent on CD28 signaling (Figure 2). CD28 is the main co-stimulatory molecule on T cells, and its ligands B7-1 (CD80)

and B7-2 (CD86) were expressed primarily on B cells, DCs and medullary TECs; other cells (including T cells) have low B7 expression (8, 65). Thus, CD28 dependency during the development of T-T Tregs led us to investigate whether B7 molecules were expressed in thymocytes. Compared with thymic DCs, wild-type thymocytes expressed only a very low level of B7-1 and did not express B7-2. However, B7 expression was slightly higher in the CIITA-transgenic thymocytes of $CIITA^{Tg}pIV^{-/-}$ mice. It remains unclear whether T cells were able to receive sufficient signals for Treg maturation with this level of B7 on DP thymocytes or whether they required CD28 stimulation by B7 on thymic epithelial cells in $CIITA^{Tg}pIV^{-/-}$ mice.

$PLZF^{+}$ innate T cells have received considerable attention owing to intrathymic selection by T-T interaction, pleiotropic production of cytokines and their role in the intrathymic generation of $Eomes^{+}$ effector/memory type $CD8^{+}$ T cells (41, 42, 59) and $CD4^{+}$ T cells (43, 44). In this study, we demonstrated that $PLZF^{+}$ innate T cells also allowed Treg cells to be activated in the thymus prior to their exit into the periphery.

REFERENCES

1. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat. Rev. Immunol.* 2008;8(7):523-32.
2. Hilkens CM, Isaacs JD, Thomson AW. Development of dendritic cell-based immunotherapy for autoimmunity. *Int. Rev. Immunol.* 2010;29(2):156-83.
3. Gottschalk RA, Corse E, Allison JP. TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. *J. Exp. Med.* 2010;207(8):1701-11.
4. Horwitz DA, Zheng SG, Gray JD. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol.* 2008;29(9):429-35.
5. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2001;2(4):301-6.
6. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* 2011;208(6):1279-89.
7. Apostolou I, Sarukhan A, Klein L, von Boehmer H. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 2002;3(8):756-63.
8. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat. Rev. Immunol.* 2002;2(2):116-26.
9. Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat. Immunol.* 2005;6(2):152-62.
10. Zheng SG, Wang JH, Stohl W, Kim KS, Gray JD, Horwitz DA. TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive

CD4+CD25+ regulatory cells. *J. Immunol.* 2006;176(6):3321-9.

11. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4(+)CD25(+) immunoregulatory T cells that control autoimmune diabetes. *Immunity*. 2000;12(4):431-40.
12. You S, Thieblemont N, Alyanakian MA, Bach JF, Chatenoud L. Transforming growth factor-beta and T-cell-mediated immunoregulation in the control of autoimmune diabetes. *Immunol. Rev.* 2006;212:185-202.
13. Bayer AL, Yu AX, Malek TR. Function of the IL-2R for thymic and peripheral CD4(+)CD25(+) Foxp3(+) T regulatory cells. *J. Immunol.* 2007;178(7):4062-71.
14. Burchill MA, Yang JY, Vogtenhuber C, Blazar BR, Farrar MA. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3(+) regulatory T cells. *J. Immunol.* 2007;178(1):280-90.
15. Lio CW, Hsieh CS. A two-step process for thymic regulatory T cell development. *Immunity*. 2008;28(1):100-11.
16. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat. Immunol.* 2008;9(6):632-40.
17. Bird L. Regulatory T Cells Nurtured by Tgf Beta. *Nat. Rev. Immunol.* 2010;10(7):466.
18. Ouyang W, Beckett O, Ma Q, Li MO. Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development. *Immunity*. 2010;32(5):642-53.
19. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim. Biophys. Acta.* 2011;1813(5):878-88.

20. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* 2009;10(9):1000-7.

21. Zheng SG, Wang J, Horwitz DA. Cutting edge: Foxp3+CD4+CD25+ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6. *J. Immunol.* 2008;180(11):7112-6.

22. Karecla PI, Bowden SJ, Green SJ, Kilshaw PJ. Recognition of E-cadherin on epithelial cells by the mucosal T cell integrin alpha M290 beta 7 (alpha E beta 7). *Eur. J. Immunol.* 1995;25(3):852-6.

23. Lehmann J, Huehn J, de la Rosa M, Maszyna F, Kretschmer U, Krenn V, et al. Expression of the integrin alpha Ebeta 7 identifies unique subsets of CD25+ as well as CD25- regulatory T cells. *Proc. Natl. Acad. Sci. U.S.A.* 2002;99(20):13031-6.

24. McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, et al. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity*. 2002;16(2):311-23.

25. Banz A, Peixoto A, Pontoux C, Cordier C, Rocha B, Papiernik M. A unique subpopulation of CD4+ regulatory T cells controls wasting disease, IL-10 secretion and T cell homeostasis. *Eur. J. Immunol.* 2003;33(9):2419-28.

26. Huehn J, Siegmund K, Lehmann JC, Siewert C, Haubold U, Feuerer M, et al. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. *J. Exp. Med.* 2004;199(3):303-13.

27. Zhao D, Zhang C, Yi T, Lin CL, Todorov I, Kandeel F, et al. In vivo-activated CD103+CD4+ regulatory T cells ameliorate ongoing chronic graft-versus-host disease. *Blood.* 2008;112(5):2129-38.

28. Rao PE, Petrone AL, Ponath PD. Differentiation and expansion of T cells with regulatory function from human peripheral lymphocytes by stimulation in the presence of TGF-{beta}. *J. Immunol.* 2005;174(3):1446-55.
29. Siewert C, Lauer U, Cording S, Bopp T, Schmitt E, Hamann A, et al. Experience-driven development: effector/memory-like alphaE+Foxp3+ regulatory T cells originate from both naive T cells and naturally occurring naive-like regulatory T cells. *J. Immunol.* 2008;180(1):146-55.
30. Zeng DF, Zhao DC, Nakamura R. Is CD103 a good surface marker for in vivo-activated effector/memory Treg cells in patients with chronic inflammation? Unknown yet Response. *Blood.* 2009;113(9):2120-.
31. Venken K, Hellings N, Thewissen M, Somers V, Hensen K, Rummens JL, et al. Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology*. 2008;123(1):79-89.
32. Park SH, Bae YM, Kim TJ, Ha IS, Kim S, Chi JG, et al. HLA-DR expression in human fetal thymocytes. *Hum. Immunol.* 1992;33(4):294-8.
33. Choi EY, Park WS, Jung KC, Chung DH, Bae YM, Kim TJ, et al. Thymocytes positively select thymocytes in human system. *Hum. Immunol.* 1997;54(1):15-20.
34. Lee YJ, Jeon YK, Kang BH, Chung DH, Park CG, Shin HY, et al. Generation of PLZF+ CD4+ T cells via MHC class II-dependent thymocyte-thymocyte interaction is a physiological process in humans. *J. Exp. Med.* 2010;207(1):237-46.
35. Choi EY, Jung KC, Park HJ, Chung DH, Song JS, Yang SD, et al. Thymocyte-thymocyte interaction for efficient positive selection and maturation of CD4 T cells. *Immunity*. 2005;23(4):387-96.

36. Li W, Kim MG, Gourley TS, McCarthy BP, Sant'Angelo DB, Chang CH. An alternate pathway for CD4 T cell development: thymocyte-expressed MHC class II selects a distinct T cell population. *Immunity*. 2005;23(4):375-86.

37. Treiner E, Lantz O. CD1d- and MR1-restricted invariant T cells: of mice and men. *Curr. Opin. Immunol.* 2006;18(5):519-26.

38. Lee YJ, Jung KC, Park SH. MHC class II-dependent T-T interactions create a diverse, functional and immunoregulatory reaction circle. *Immunol. Cell Biol.* 2009;87(1):65-71.

39. Li W, Sofi MH, Rietdijk S, Wang N, Terhorst C, Chang CH. The SLAM-Associated protein signaling pathway is required for development of CD4(+) T cells selected by homotypic thymocyte interaction. *Immunity*. 2007;27(5):763-74.

40. Alonzo ES, Sant'Angelo DB. Development of PLZF-expressing innate T cells. *Curr. Opin. Immunol.* 2011;23(2):220-7.

41. Weinreich MA, Odumade OA, Jameson SC, Hogquist KA. T cells expressing the transcription factor PLZF regulate the development of memory-like CD8+ T cells. *Nat. Immunol.* 2010;11(8):709-16.

42. Min HS, Lee YJ, Jeon YK, Kim EJ, Kang BH, Jung KC, et al. MHC Class II-Restricted Interaction between Thymocytes Plays an Essential Role in the Production of Innate CD8(+) T Cells. *J. Immunol.* 2011;186(10):5749-57.

43. Prince AL, Kraus Z, Carty SA, Ng C, Yin CC, Jordan MS, et al. Development of Innate CD4+ and CD8+ T Cells in Itk-Deficient Mice Is Regulated by Distinct Pathways. *J. Immunol.* 2014;193(2):688-99.

44. Prince AL, Watkin LB, Yin CC, Selin LK, Kang J, Schwartzberg PL, et al. Innate PLZF+CD4+ alphabeta T Cells Develop and Expand in the Absence of Itk. *J. Immunol.* 2014;193(2):673-87.

45. Stephens GL, Andersson J, Shevach EM. Distinct subsets of FoxP3(+) regulatory

T cells participate in the control of immune responses. *J. Immunol.* 2007;178(11):6901-11.

46. Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, et al. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J. Immunol.* 2003;171(7):3348-52.
47. Nelson AJ, Hosier S, Brady W, Linsley PS, Farr AG. Medullary thymic epithelium expresses a ligand for CTLA4 in situ and in vitro. *J. Immunol.* 1993;151(5):2453-61.
48. Reiser H, Schneeberger EE. The Costimulatory Molecule B7 Is Expressed in the Medullary Region of the Murine Thymus. *Immunology*. 1994;81(4):532-7.
49. Faustino L, da Fonseca DM, Takenaka MC, Mirotti L, Florsheim EB, Guereschi MG, et al. Regulatory T cells migrate to airways via CCR4 and attenuate the severity of airway allergic inflammation. *J. Immunol.* 2013;190(6):2614-21.
50. Saito K, Torii M, Ma N, Tsuchiya T, Wang L, Hori T, et al. Differential regulatory function of resting and preactivated allergen-specific CD4+ CD25+ regulatory T cells in Th2-type airway inflammation. *J. Immunol.* 2008;181(10):6889-97.
51. Kilshaw PJ, Murant SJ. Expression and Regulation of Beta-7(Beta-P) Integrins on Mouse Lymphocytes - Relevance to the Mucosal Immune-System. *Eur. J. Immunol.* 1991;21(10):2591-7.
52. Hadley GA, Rostapshova EA, Gomolka DM, Taylor BM, Bartlett ST, Drachenberg CI, et al. Regulation of the epithelial cell-specific integrin, CD103, by human CD8+ cytolytic T lymphocytes. *Transplantation.* 1999;67(11):1418-25.
53. Robertson H, Wong WK, Talbot D, Burt AD, Kirby JA. Tubulitis after renal transplantation: demonstration of an association between CD103+ T cells, transforming growth factor beta1 expression and rejection grade. *Transplantation.* 2001;71(2):306-13.

54. Wang D, Yuan R, Feng Y, El-Asady R, Farber DL, Gress RE, et al. Regulation of CD103 expression by CD8+ T cells responding to renal allografts. *J. Immunol.* 2004;172(1):214-21.

55. El-Asady R, Yuan R, Liu K, Wang D, Gress RE, Lucas PJ, et al. TGF-{beta}-dependent CD103 expression by CD8(+) T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. *J. Exp. Med.* 2005;201(10):1647-57.

56. Li MO, Flavell RA. TGF-beta: A master of all T cell trades. *Cell*. 2008;134(3):392-404.

57. Li MO, Sanjabi S, Flavell RA. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity*. 2006;25(3):455-71.

58. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat. Immunol.* 2013;14(11):1146-54.

59. Lai D, Zhu J, Wang T, Hu-Li J, Terabe M, Berzofsky JA, et al. KLF13 sustains thymic memory-like CD8(+) T cells in BALB/c mice by regulating IL-4-generating invariant natural killer T cells. *J. Exp. Med.* 2011;208(5):1093-103.

60. Maerten P, Shen C, Bullens DM, Van Assche G, Van Gool S, Geboes K, et al. Effects of interleukin 4 on CD25+CD4+ regulatory T cell function. *J. Autoimmun.* 2005;25(2):112-20.

61. Skapenko A, Kalden JR, Lipsky PE, Schulze-Koops H. The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors. *J. Immunol.* 2005;175(9):6107-16.

62. Wei J, Duramad O, Perng OA, Reiner SL, Liu YJ, Qin FX. Antagonistic nature of

T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. *Proc. Natl. Acad. Sci. U.S.A.* 2007;104(46):18169-74.

63. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 inhibits TGF-beta-induced Foxp3(+) T cells and, together with TGF-beta, generates IL-9(+) IL-10(+) Foxp3(-) effector T cells. *Nat. Immunol.* 2008;9(12):1347-55.
64. Chang LY, Lin YC, Kang CW, Hsu CY, Chu YY, Huang CT, et al. The indispensable role of CCR5 for in vivo suppressor function of tumor-derived CD103+ effector/memory regulatory T cells. *J. Immunol.* 2012;189(2):567-74.
65. Hathcock KS, Laszlo G, Pucillo C, Linsley P, Hodes RJ. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J. Exp. Med.* 1994;180(2):631-40.

국문 초록

서론: PLZF 를 발현하는 NKT 세포와 T-T CD4 양성 T 세포는 선천 T 세포들 중에서도 특이한 아형의 세포들이다. 두 세포군 모두 발달하는 동안 다른 흉선세포와의 상호작용 (thymocyte-thymocyte interaction)을 통해 선택되며, 항원의 자극에 의해 IL-4 와 IFN-γ를 동시에 낼 수 있는 능력을 가지고 있다. 최근 흉선 내에서 PLZF 양성 T 세포가 effector/메모리 (Eomesodermin 양성) 형태의 CD4 양성 T 세포와 CD8 양성 T 세포의 생성에 필수적이며 이러한 작용은 PLZF 양성 세포가 분비하는 다량의 IL-4 에 의한 것임이 여러 면역학자들에 의해 보고된 바 있다.

Foxp3 양성 조절 T 세포는 흉선에서 만들어지는 자연적 조절 T 세포와 말초 naive T 세포로부터 항원자극에 의해 유도되는 적응 조절세포로 나눌 수 있고, 그 형성에 있어서 IL-2 와 TGF-β 싸이토카인이 매우 중요하게 작용한다. 적응 조절 T 세포는 effector/메모리의 표현형을 가지고 있으며, CD103 단백질을 높게 발현하고 있다. 자연 조절 T 세포가 T-T 상호작용만 존재하는 형질전환마우스 ($CIITA^{Tg}CIITA^{-/-}$) 에서도 잘 발달한다는 것을 본 연구팀이 보고한 바 있지만, 그 성격에 대해 명확하게 보고된 바가 없다.

방법: 본 연구실에서는 선행연구 결과들을 바탕으로 PLZF 양성 세포가 다량으로 분비하는 IL-4 가 자연 조절 T 세포의 생성에 큰 영향을

줄 것으로 기대하고 CIITA^{Tg}PIV$^{-/-}$ 마우스와 일반 BALB/c 마우스의 흉선과 비장에서 조절 T 세포의 표현형을 관찰하였다. CIITA^{Tg}PIV$^{-/-}$ 마우스에서 생성되는 조절 T 세포의 조절능력을 알아보기 위하여 비장에서 조절 T 세포들을 분리 하였고, 실험관내 (*In vitro*) 시험과 천식을 유도한 마우스 모델을 이용한 생체 내 (*In vivo*) 시험 및 관찰을 병행하였다. 또한, 발달과정에서 중요한 인자를 찾기 위하여 여러 가지 Knock-out 마우스를 이용하였고, IL-4 와 TGF-β1 을 첨가하여 FTOC 를 시행하였다.

결과: CIITA^{Tg}PIV$^{-/-}$ 마우스에서 만들어지는 자연 조절 T 세포들은 effector/메모리의 표현형을 나타내며 그 표식인 CD103 를 높게 발현하고 있었다. Th2 면역 반응에 의해 유도되는 천식을 일반 마우스들에서 생성된 조절 T 세포들보다 더 효율적으로 줄였다. CD103 의 증가 현상은 TGF-β 의존적이었고, PLZF 양성 T 세포에서 분비되는 IL-4 는 이러한 TGF-β 의존적 증가 현상을 더욱 강화 하였다. BALB/c 마우스에서도 이러한 IL-4 에 의한 CD103 강화 현상이 관찰 되었다.

결론: 결론적으로 본 연구를 통해 PLZF 양성 세포가 effector T 세포 형성과 effector/메모리 형태의 자연 조절 T 세포형성을 동시에 조절 하여 말초 면역체계 형성에 큰 영향을 미치고 있음을 밝혔다.

주요어: PLZF 양성 T 세포, 조절 면역 T 세포, TGF-β, IL-4, Effector/메모리

학 번 : 2010-31151