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Thymic Low Affinity/Avidity Interaction Selects Natural Th1 Cells

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Identification of intrathymic eomesodermin⁺ (Eomes⁺) CD4 T cells creates a novel idea that there is more than one way for the generation of innate CD4 T cells. Promyelocytic leukemia zinc finger protein⁺ T cells and natural Th17 cells are known to be generated by sensing a high and persistent TCR strength, whereas this is not the case for Eomes⁺ CD4 T cells. These cells go through low-level signal during the entire maturation pathway, which subsequently leads to induction of high susceptibility to cytokine IL-4. This event seems to be a major determinant for the generation of this type of cell. These T cells are functionally equivalent to Th1 cells that are present in the periphery, and this event takes place both in transgenic and in wild-type mice. There is additional evidence that this type of Eomes⁺ innate CD4 T cell is also present in human cord blood. *The Journal of Immunology*, 2015, 194: 5861–5871.

Conventional T cells, the essential component in adaptive immunity, display extreme TCR repertoire when they emigrate out from thymus, and do not exhibit immediate effector function (1). Another distinct lineage of T cells, designated as innate or nonconventional T cells, has been reported (2–9). Compared with the conventional T cells, innate T cells show several distinct features: they have an effector-memory phenotype

in the thymus, a limited TCR repertoire, show rapid production of cytokine upon Ag encounter, and are dependent on signaling lymphocytic activation molecule (SLAM) and SLAM-associated protein (SAP) signaling pathway (1, 4). The best characterized innate T cells are $\gamma\delta$ T cells (5) and CD1d-restricted CD4⁺ NKT cells (8). Others include mucosa-associated invariant T cells (7), H2-M3–restricted CD8 T cells (2), and CD8 $\alpha\alpha$ ⁺ intraepithelial lymphocytes (3). All of these subsets are restricted by nonclassical MHC class Ib molecules, such as CD1d, Qa-1, H2-M3, and MR-1, and consequently have a highly restricted oligoclonal TCR repertoire (10).

More recently, different subsets of innate T cells with diverse TCR repertoire have been reported (11, 12). Promyelocytic leukemia zinc finger protein⁺ (PLZF⁺) CD4 T cells generated by MHC class II–dependent thymocyte–thymocyte (T-T) interaction (T-T CD4 T, also referred to as T-CD4 T) are included in this group (11). We first identified a T cell subset that is restricted by MHC II molecules on the T cells in vitro (13, 14), and subsequently, we and others confirmed this feature in truly in vivo setting (15, 16). Subsequent experiments revealed that some proportion of the T-T CD4 T cells in CIITA^{tg}pIV^{−/−} mice expressed PLZF, which was identified as a transcription factor necessary for the development of invariant NKT (iNKT) cells and for directing the innate characteristics of iNKT cells (17). Very recently, we reported on another subset of CD8 T cells that were developed in the presence of IL-4 produced by PLZF⁺ T-T CD4 T cells, and showed upregulated expression of eomesodermin (Eomes) in CIITA^{tg}pIV^{−/−} mice (12). Both PLZF⁺ T-T CD4 T cells and Eomes⁺ CD8 T cells have innate characteristics and diverse TCR repertoires (18); they are also found in humans (11, 12).

Recent studies have reported that a number of mice deficient in T cell signaling molecules or transcription factors have elevated thymic innate CD8 T cells. These include Kruppel-like factor 2, CREB binding protein, and inhibitor of DNA binding 3 (19–23). All of these mouse models were found to have elevated Eomes⁺ CD8 T cells in the thymus, showing a memory phenotype and rapid production of IFN- γ upon TCR stimulation. Moreover, cytokine IL-4 was the essential factor that allows innate CD8 T cells to develop via upregulation of Eomes expression in CD8 single-positive (SP)

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Abbreviations used in this article: γ_c , γ -chain; CB, cord blood; cTh1, classical Th1; DP, double-positive; Eomes, eomesodermin; Id3, inhibitor of DNA binding 3; iNKT, invariant NKT; MAIT, mucosa-associated invariant T; MFI, median fluorescence intensity; nTh17, natural Th17; PLZF, promyelocytic leukemia zinc finger protein; SAP, SLAM-associated protein; SLAM, signaling lymphocytic activation molecule; SP, single-positive; T-T, thymocyte–thymocyte.

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thymocytes, as was the case for innate CD8 T cells in *CIITA^{tg}pIV^{-/-}* mice (12).

Th1, Th2, and Th17 are the effector subsets of CD4 T cells. Recently, a new population of Th17 cells, natural Th17 (nTh17) cells, was demonstrated to be developed in the thymus (24). That study found that, during thymic ontogeny, nTh17 cells acquired innate characteristics, such as the expression of an activated/memory phenotype before peripheral Ag exposure and rapid cytokine production. Compared with conventional Th17 cells, the nTh17 cells showed a preference for TCR V β 3 and differential signaling requirements for thymic development.

In this study, the novel CD4 T cell population was able to be generated in *CIITA^{tg}* and BALB/c mice, which acquired innate characteristics together with Eomes upregulation. Low-level signal strength delivered inside the cells and subsequent higher susceptibility for IL-4 that was mainly produced by either PLZF⁺ T-T CD4 T cells or iNKT cells seem to be major requirements for the expression of Eomes. Eomes⁺ CD4 SP thymocytes showed a CD24^{lo}CD44^{hi}CD62L^{hi} central memory phenotype and were able to produce IFN- γ and TNF- α rapidly in response to TCR stimulation, indicating innate functional properties. These innate cells had a diverse TCR repertoire without any skewing toward a particular V β usage, compared with other types of innate T cells. In BALB/c mice, a relatively larger number of NKT cells seemed to be responsible for the generation of Eomes⁺ innate CD4 T cells via the production of IL-4. Interestingly, the CD4 T cell population of the similar phenotype was found in the human umbilical cord blood (CB), as was in the case of PLZF⁺ T-T CD4 T cells (11) and innate CD8 T cells (12) of human fetal lymphoid tissues. On a theoretical basis, emergence of this novel type of innate cell seems to provide an additional defense mechanism against exogenous Ag, either viral origin or possibly all the other types of incoming pathogens.

Materials and Methods

Mice

C57BL/6 mice were obtained from the animal facility at the Biomedical Center for Animal Resource Development, Seoul National University College of Medicine. BALB/c mice were purchased from SAMTAKO (Seoul, Korea). The *CIITA^{tg}* and *CIITA^{tg}pIV^{-/-}* mice were previously generated (12). PLZF^{Lu/Lu}, IL-4^{-/-}, B7^{-/-} (CD80/CD86^{-/-}), β 2m^{-/-}, BALB/c.CD1d^{-/-}, and BALB/c.IL-4^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The *CIITA^{tg}* mice were backcrossed to PLZF^{Lu/Lu}, IL-4^{-/-}, or B7^{-/-} mice to generate *CIITA^{tg}PLZF^{Lu/Lu}* or *CIITA^{tg}PLZF^{Lu/Lu}IL-4^{-/-}*, or *CIITA^{tg}B7^{-/-}* mice, respectively. *CIITA^{tg}pIV^{-/-}* β 2m^{-/-} mice were bred by crossing *CIITA^{tg}pIV^{-/-}* to β 2m^{-/-} mice. BALB/c.Zap70^{m1Saka/m1Saka} (SKG) mice were kindly provided by S. Sakaguchi, and these mice were also backcrossed to *CIITA^{tg}* mice for six generations to obtain *CIITA^{tg}Zap70^{m1Saka/+}* mice. All mice were raised in a specific pathogen-free environment in the animal facility at the Biomedical Center for Animal Resource Development, Seoul National University College of Medicine (Seoul, Korea). Experiments were performed after receiving approval from the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Resources, Seoul National University.

Flow-cytometric analysis

Thymocytes or splenocytes were prepared from mice, and cells (1×10^6) were stained with various combinations of the following Abs purchased from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), R&D Systems (Minneapolis, MN), Santa Cruz Biotechnologies (Dallas, TX), Biolegend (San Diego, CA), Miltenyi Biotec (Auburn, CA), and DiNonA (Seoul, Korea): anti-mouse CD4 (RM4.5), CD5 (53-7.3), CD8 α (53-6.7), CD24 (M1/69), CD62L (MEL-14), CD44 (IM7), CD124 (IL-4R α) (mIL4R-M1), CD183 (CXCR3; CXCR3-173), TCR β (H57-597), TNF- α (TN3-19.12), IFN- γ (XMG1.2), IL-4 (11B11), IL-5 (JES1-39D10), IL-13 (eBio13A), IL-17 (TC11-18H10), GATA3 (LS50-823), T-bet (O4-46), Helios (22F6), PLZF (D-9), Eomes (Dan1mag), Bcl-2 (3F11) and PE Hamster IgG Isotype Control (A19-3), pSTAT-6 (pY641; J71-773.58.11)

and Mouse IgG1 (MOPC-21), anti-mouse V β 2 (B20.6), V β 3 (KJ25), V β 4 (KT4), V β 5.1&5.2 (MR9-4), V β 6 (RR4-7), V β 7 (TR310), V β 8 (F23.1), V β 10 (B21.5), V β 11 (RR3-15), and V β 13 (MR12-3). Allophycocyanin-conjugated PBS57/mCD1d tetramers were kindly gifted from the National Institutes of Health tetramer core facility. Human samples were stained with the following Abs: anti-human CD3 (UCHT1), CD4 (RPA-T4 or SK3), CD31 (M89D3), CD45RA (HI100), CD45RO (UCHL1), CD122 (Mik-b3), CCR7 (3D12), PTK7 (188B), IFN- γ (45.15), IL-4 (4D9), and Eomes (WD1928). PE-conjugated PBS57/hCD1d tetramers were also kindly gifted from the National Institutes of Health tetramer core facility. All flow-cytometry analyses were performed on an LSRII or LSR-Fortessa (Becton Dickinson, Mountain View, CA). A total of $\geq 10,000$ events were acquired, and the obtained data were processed with FlowJo software (Tree Star, Ashland, OR).

Intracellular staining

For intracellular staining of PLZF and Eomes, fresh cell suspensions of thymocytes were first stained for surface markers, followed by fixation, permeabilization, and intracellular staining using the Foxp3 staining buffer set (eBioscience). For cytokine staining, cells purified using MACS (Miltenyi Biotec, Auburn, CA) were treated with 50 ng/ml PMA and 1.5 mM ionomycin (Sigma, St. Louis, MO) for 5 h or were cultured in anti-CD3 (1.0 μ g/ml; 2C11)-coated 96-well plate with soluble anti-CD28 (1.0 μ g/ml; 37.51) Abs for 20 h. A total of 10 μ g/ml brefeldin A (Sigma) was added during the last 3 h of PMA and ionomycin stimulation or the last 5 h of anti-CD3/CD28 stimulation. After the reaction, the stimulated cells were harvested and immediately fixed and permeabilized using the Foxp3 staining buffer set (eBioscience). Abs against Eomes and the indicated cytokine and surface molecules were treated for staining. For p-STAT6 staining, freshly isolated thymocytes were allowed to rest in PBS at 37°C for 2 h. The cells were subsequently treated with murine IL-4 (Peprotech, Rocky Hill, NJ) for 10 min at 37°C, followed by fixation with BD Phosflow Lyse/Fix Buffer (BD Bioscience) and treatment with ice-cold Perm Buffer III (BD Bioscience). Then the cells were stained with anti-CD4, anti-CD8 α , anti-Eomes, and anti-pSTAT6 Abs.

Preparation of CD4 SP thymocytes and MLRs

CD4 SP thymocytes from C57BL/6, B7^{-/-}, *CIITA^{tg}*, and *CIITA^{tg}B7^{-/-}* mice were purified by magnetic cell sorting using MACS with anti-CD4 and -CD8 microbeads. For further FACS sorting, the isolated CD4 SP thymocytes of *CIITA^{tg}* and *CIITA^{tg}B7^{-/-}* mice were immediately subjected to surface staining with Abs against CD3, CD4, CD62L, and CXCR3, and divided into CD3⁺CD4⁺CD62L⁺CXCR3⁺ and the rest of CD3⁺CD4⁺ thymocytes using FACS sorter; the expression of Eomes in the former and latter cell populations was confirmed. The purified CD4 SP thymocytes (1.0×10^5) were labeled with CellTrace Violet (Life Technologies, Carlsbad, CA) and then were stimulated with irradiated (2500 cGy) T-depleted CD45.1 C57BL/6 splenocytes (3.0×10^5). After 5 d of culture, the harvested cells from the MLR were stained with Abs against mouse CD3, CD4, and CD45.2, and analyzed by LSRII (BD).

Th1 cell differentiation

Naive CD4 T cells were isolated from single-cell suspension of wild-type spleen using the CD4⁺CD62L⁺ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Purified T cells (2.5×10^5) were incubated with Dynabeads Mouse T-Activator CD3/CD28 (1:1 ratio; Invitrogen, Carlsbad, CA) in 96-well round-bottom plates and differentiated for 5 d under the Th1 conditions: IL-12 (2 ng/ml), IL-2 (50 U/ml), and anti-IL-4 Ab (5 μ g/ml).

Immunohistochemistry

Tissue samples from each mouse were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained using a Leica BOND-MAX automated immunostainer (Leica Microsystems, Wetzlar, Germany). Ag retrieval was performed through incubation in 6 mM citrate buffer at 99°C for 20 min, and nonspecific staining was prevented by treating tissue sections with rabbit serum (1% in PBS) for 30 min. Anti-mouse Eomes (rabbit polyclonal; 1:100 dilution: ab23345; Abcam, Cambridge, U.K.) and anti-mouse PLZF (mouse monoclonal IgG1 1:50 dilution: sc-28319; Santa Cruz) were applied to each section for 30 min and Ab was visualized using a VECTASTAIN Elite ABC kit (PK6101; Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin. Microscopic observations were performed with ECLIPSE 80i Bright-Field Microscope Set (Nikon).

Affymetrix gene array

Total RNA from the indicated samples was isolated using RNeasy Micro Kit (Qiagen). The prepared RNA was subjected to microarray and hybridized

according to standard Affymetrix protocols (Affymetrix GeneChip Mouse Gene 2.0 ST Array: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67158>, accession no. GSE67158). Arrays were scanned on the Affymetrix GeneChip scanner. Microarray images were visually inspected for quality, and the probes with low signal intensity and excessively noisy background were removed before further analysis using the Affymetrix Microarray Analysis Suite v5.0. The signal values were determined using the GeneChip Operating System 1.2 (Affymetrix). Hierarchical clustering of the differentially expressed RNAs was performed in the PermutMatrix software (25). In those analyses, we used the Complete Linkage method and the Euclidean distance calculation method.

Fetal thymic organ culture

On embryonic day 15.5, fetal thymuses from BALB/c mice were removed and cultured on polycarbonate filters (pore size, 0.8 μ m; Millipore, Bedford, 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 murine rIL-4 (10–100 ng/ml; Peprotech). After 7-d culture, the thymuses were analyzed for the expression of CD3, CD4, CD8, and Eomes by flow cytometry.

Human adult and umbilical CB

PBMCs were isolated from healthy adult human volunteers. Human umbilical CB cells were collected during normal full-term deliveries from Jang's Women's Hospital. All samples were obtained with written, informed consent in accordance with the guidelines set forth by the Institutional Review Board of the Clinical Research Institute, Seoul National University Hospital. Mononuclear cells were isolated from whole blood using Ficoll-Hypaque density gradient centrifugation (GE Healthcare).

Statistical analysis

All data were analyzed using GraphPad Prism software (version 5; GraphPad Software, La Jolla, CA). Bar graphs showing the percentages or absolute numbers of each cell, or median fluorescence intensity (MFI) of each molecule, represent the mean values \pm SEM. The data were compared using an unpaired *t* test, and a *p* value <0.05 was considered significant.

Results

Identification of innate CD4 T cells expressing Eomes in mouse thymus

In CIITA^{tg} mice, where MHC class II molecules are designed to be expressed on T cells, we previously reported on a subset of CD4 SP thymocytes that belongs to the category of innate CD4 T cells and uniquely express transcription factor PLZF (11). These cells prime very nice cytokine production, such as high amounts of IFN- γ and IL-4 in response to PMA and ionomycin stimulation. Subsequently, we found out another type of intrathymic T cell that expresses high levels of Eomes. In these mice, there are 10–20% Eomes⁺ CD4 T cells (Fig. 1A). Expression of Eomes and PLZF transcriptional factors was rather unique in that the expression of two molecules was mutually exclusive. These cells are mature (CD24^{low}Qa2^{high}), show a central memory CD4 T cell phenotype, and have high levels of CD44 and CD62L (Fig. 1B). This was not the case in wild-type C57BL/6 mice where expression of Eomes⁺ was barely seen (Fig. 1A).

Subsequent analysis revealed that these cells behave very much like Th1 cells, producing large amounts of IFN- γ and TNF- α in response to PMA/ionomycin (Fig. 1C) and CD3/CD28 stimulation (Supplemental Fig. 1A), but negligible amounts of IL-4 (Fig. 1C). Instead of producing T-bet, now these cells express large amount of Eomes. On the contrary, GATA3, a transcriptional factor for Th2 type cells, is not expressed in these cells (Fig. 1C). Intrathymically developed Eomes⁺ CD4 T cells have extremely diverse TCR repertoire, similar to conventional CD4 T cells (Fig. 1D). To further dissect the nature of the cells, we compared the gene expression pattern of Eomes⁺ CD4 T cells with that of classical Th1 (cTh1) cells. For cTh1 cells, naive CD4 T cells from the spleen of wild-type mice were cultured in Th1-polarizing conditions for 5 d. Naturally occurring Eomes⁺ CD4 T cells (CXCR3⁺CD62L^{hi}CD4

SP) were obtained from the thymus of CIITA^{tg} mice by FACS sorter, and most of the sorted cells were confirmed to express Eomes (Supplemental Fig. 1B). CD4⁺CD62L^{hi} naive T cells from wild type spleens and CXCR3⁺CD62L^{hi}CD4 SP cells from CIITA^{tg} mice were used as control, respectively. Compared with control cells, both cTh1 and nTh1 cells showed upregulation of Ccr5, Ccl3, Il12rb2, Ifng, Il18rap, Ifngr1, and Il2rb, and down-regulation of Il4, Il21, Egr2, and inhibitor of DNA binding 3 (Supplemental Fig. 1C–E). In terms of transcription factors, these two types of cells were somewhat different in that Eomes⁺ CD4 T cells literally showed an increased expression level of Eomes and Runx3, whereas cTh1 cells expressed Tbx21 and Runx3. In addition, unlike cTh1 cells, Eomes⁺ CD4 T cells upregulated molecules associated with the trafficking and adhesion of T cells (Cxcr3, Cd44, and Xcl1) without showing activated/effector phenotype (Il2ra, Il10, and Csf2), suggesting that these Eomes⁺ CD4 T cells are more like memory cells. Despite their different developmental pathway, Eomes⁺ CD4 T cells share functional features with cTh1 cells. Based on these data, we infer that there is another type of, as yet unidentified, intrathymic maturation pathway.

PLZF⁺ T cells are responsible for the expression of Eomes in CD4 SP thymocytes

Previous observations of PLZF⁺ T-T CD4 T cells facilitating the development of Eomes⁺ memory-like CD8 T cells in the thymus of CIITA^{tg}pIV^{−/−} mice (12) brought us to wonder about whether similar events take place in CD4 T cells. This, in fact, turned out to be the case. We phenotypically dissected CIITA^{tg}PLZF^{Lu/Lu} thymi in terms of the relationship between PLZF and Eomes expression (Fig. 2A). Eomes⁺ CD4 T cells were almost completely absent from this mouse, indicating that PLZF expression is a critical requirement for the generation of these cells. We were able to get large numbers of Eomes⁺ cells (on average, 8–10%) in CIITA^{tg} PLZF^{Lu/+} and much more in CIITA^{tg} mice (on average, 17–20%). These data dramatically illustrate the fact that almost the same type of maturing process also takes place for the development of Eomes⁺ CD8 T cells.

Next, we asked whether same things work out to be true in wild-type mice. It has been well-known that the number of PLZF⁺ NKT cells varies, depending on the inbred mouse strain. One representative example of this is that BALB/c strains have relatively high numbers of PLZF⁺ NKT cells. In the C57BL/6 strain, however, PLZF⁺ NKT cells are not as abundant as those in BALB/c (26, 27). Present data are consistent with previous observations (Fig. 2B). Substantial numbers of Eomes⁺ CD4 T cells were present in BALB/c mice, whereas presence of these cells was not the feature in the C57BL/6 strain. In CD1d-deficient BALB/c mice in which PLZF⁺ NKT cells were either barely detectable or almost completely absent, Eomes⁺ cells again were not present, suggesting that the presence of PLZF⁺ cells seems to be a real basis for generation of Eomes⁺ innate CD4 T cells. As to the TCR repertoire of Eomes⁺ CD4 T cells, these cells were almost comparable with that of conventional T cells, with flow-cytometric analysis, using a large panel of V β mAbs, whereas NKT cells were restricted to V β 2, V β 7, and V β 8 (Fig. 2C). Eomes⁺ CD4 T cells of CIITA^{tg} and wild-type BALB/c mice shared some features, such as membrane expression of CD24, CD44, and CD62L, and cytokine production profile (Fig. 2D).

IL-4-dependent expression of Eomes in CD4 SP thymocytes

We tested whether Eomes⁺ innate CD4 T cells could be induced in much the same way with what happened in Eomes⁺ CD8 T cells, where IL-4 is a basic requirement for generation of this type of cells (12, 23, 28). Analysis of individual T cell subsets in the

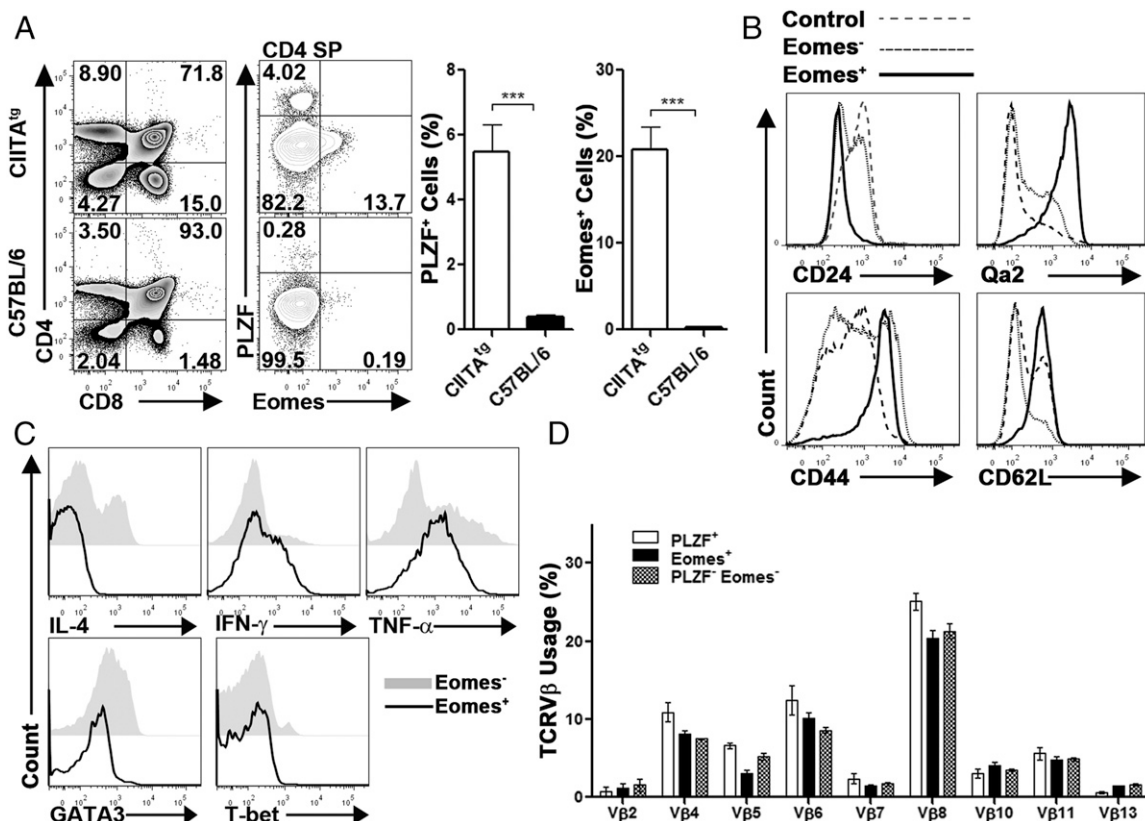


FIGURE 1. Development of Eomes⁺ CD4 T cells in CIITA^{tg} mice. **(A)** Representative CD4 and CD8 profiles of total thymocytes (left panels), and expression of Eomes and PLZF in CD4 SP thymocytes (middle panels) from indicated mice. Numbers in quadrants indicate cell percentages. Cumulative data ($n = 10$ in each, 8–12 wk old) of PLZF⁺ or Eomes-expressing cell frequency in CD4 SP thymocytes were summarized in the right panel; bars indicate the mean \pm SEM. *** $p < 0.001$. **(B)** Representative expression pattern of maturation (CD24 and Qa2) and memory (CD44 and CD62L) markers on Eomes⁺ (solid line) and Eomes⁻ (dashed line) CD4 SP thymocytes of CIITA^{tg} mice. Data are representative of three independent experiments. CD4 SP thymocytes from wild-type thymus were used as control cells (long dashed line). **(C)** Phenotype of the Eomes⁺ CD4 SP thymocytes. Sorted CD24^{lo} CD4 SP thymocytes from CIITA^{tg} mice were stimulated with PMA and ionomycin for 5 h, followed by intracellular cytokine staining of Eomes⁺ or Eomes⁻ cells, and representative data of one of three experiments are presented in the upper panels. Lower panels, Expression pattern of transcription factors, GATA3 and T-bet, of Eomes⁺ and Eomes⁻ CD4 SP thymocytes from CIITA^{tg} mice is depicted. Solid lines represent Eomes⁺ populations; gray shaded histograms represent Eomes⁻ populations. **(D)** The Vβ-chain usage of PLZF⁺, Eomes⁺, and double-negative (PLZF⁻ Eomes⁻) CD4 SP thymocytes from CIITA^{tg} mice. Bars indicate the mean \pm SEM.

thymus of CIITA^{tg} IL-4^{-/-} and BALB/c.IL-4^{-/-} mice showed substantially fewer Eomes⁺ CD4 T cells (Fig. 3A, 3B). This IL-4 dependency of the generation of Eomes⁺ cells was also confirmed by BALB/c fetal thymic organ culture in the presence of IL-4 in a dose-dependent manner (Fig. 3C). To assess the issue whether the IL-4 susceptibility varied between Eomes⁺ and Eomes⁻ thymocytes, we analyzed the expression levels of IL-4Rα and phosphorylation status of Stat6 with flow-cytometric analysis. The Eomes⁺ CD4 subset was the cells that have the highest IL-4Rα expression (Fig. 3D) and Stat6 phosphorylation levels (Fig. 3F). Another interesting point that we emphasize is that there was a dramatic upregulation of the antiapoptotic molecule, Bcl-2 (Fig. 3E). These data suggest that something is set in motion for the survival of this type of cell during the maturation process.

Initiation of Eomes expression at the postselection stage of double-positive thymocytes

We particularly focused on the expression pattern of Eomes through the entire developmental process of CD4 T cells. In both CIITA^{tg}pIV^{-/-} and BALB/c mice, Eomes expression was clearly visible from TCRβ^{hi} postselection double-positive (DP) thymocytes (Fig. 4A), and their expression remained high until full maturation into CD4 SP thymocytes (Fig. 4B, Supplemental Fig. 2). However, this early expression of Eomes was not observed in IL-4- or CD1d-deficient BALB/c mice (Supplemental

Fig. 2). To rule out the possibility that the CD4 T cells sometimes come from the interaction between MHC class I/peptides and TCR rather than from the MHC class II-restricted pathway, we used CIITA^{tg}pIV^{-/-} β2m^{-/-} mice in which we were able to turn off the MHC class I-restricted pathway. When again gated on TCRβ^{hi}, we were able to get three different subsets of cells—DP (CD4⁺CD8⁺), CD4^{hi}CD8^{int}, and CD4 SP—in a row (Fig. 4B). The point we wanted to make is that Eomes expression was clearly visible in both CD4^{hi}CD8^{int} and CD4 SP thymocytes, whereas it was barely detected in TCRβ^{hi}-DP thymocytes. Another point is that these TCRβ^{hi}-DP thymocytes seem to have higher chances for cell death, as confirmed by Annexin V staining and Bcl-2 expression (Fig. 4B). Immunohistochemistry done in CIITA^{tg}pIV^{-/-} β2m^{-/-} mouse thymus suggests that Eomes seems to start to be expressed mainly in the thymic cortex and subsequently migrates into thymic medulla where full maturation of CD4 T cells take place, and the generation of Eomes⁺ CD4 T cells stems from the ligation between a MHC class II/peptide and the appropriate TCR (Fig. 4C).

Low avidity interaction between MHC class II/peptide and TCR with appropriate specificity is the basis for the generation of Eomes⁺ CD4 T cells

To ascertain what kind of mechanism controls the IL-4 susceptibility of T cells, we wondered the possible role of signal

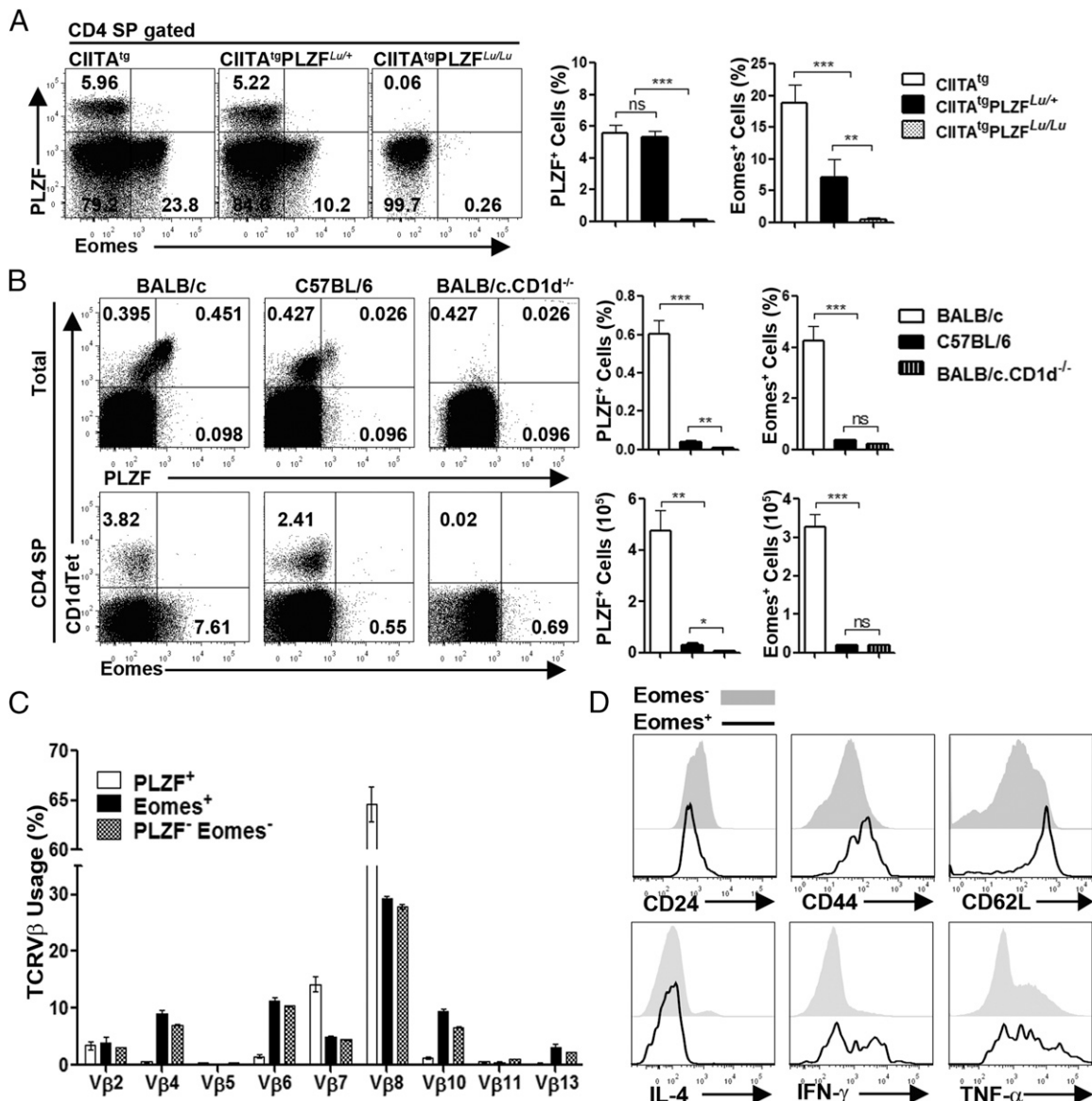


FIGURE 2. PLZF expression is essential for the development of Eomes⁺ CD4 SP thymocytes. **(A)** Expression of Eomes and PLZF in CD4 SP thymocytes from CIITA^{tg}, CIITA^{tg} PLZF^{Lu/+}, and CIITA^{tg} PLZF^{Lu/Lu} ($n = 3\sim 5$, 8–12 wk old). Representative data from four experiments (left panel) and cumulative data (right panel) are shown; numbers in quadrants indicate cell percentages, and bars indicate the mean \pm SEM. **(B)** PLZF⁺ iNKT cells in total thymocytes (left top panel), and Eomes⁺ cells of CD4 SP thymocytes (left bottom panel) from BALB/c, C57BL/6, and BALB/c.CD1d^{-/-} mice (6–8 wk old). Representative flow-cytometry data (left panel) and cumulative data (right panel) are shown. **(C)** The V β -chain usage of PLZF⁺, Eomes⁺, and double-negative (PLZF⁻ Eomes⁻) CD4 SP thymocytes from BALB/c mice. Bars indicate the mean \pm SEM. **(D)** Surface expression of CD24, CD44, and CD62L (upper panel), and intracellular cytokine production (lower panel) in Eomes⁺ (solid line) and Eomes⁻ (gray shaded) CD4 SP thymocytes from BALB/c mice. For intracellular staining of cytokine production, CD24^{lo} CD4 SP thymocytes from BALB/c mice were magnetically sorted and stimulated with PMA and ionomycin for 5 h ex vivo. Data are representative of one of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant.

strength that is delivered to cells during TCR-MHC/peptide interaction. To test this idea, we checked expression levels of CD5 (29), Helios (30), and Nur77 (31), all of which directly reflect total avidity during T cell selection. Importantly, there was a clear hierarchy in TCR signal strength that is delivered to T cells in the three different subsets (PLZF⁺, PLZF⁻ Eomes⁻, and Eomes⁺) of CD4 SP thymocytes. In CIITA^{tg} mice, Eomes⁺ T cells had the lowest CD5, Helios, and Nur77 expression, whereas PLZF⁺ and PLZF⁻ Eomes⁻ CD4 T cells had much higher levels of expression (Fig. 5A, 5B, 5C). This turned out to be exactly the same case in BALB/c mice, showing the lowest level of expression in CD5, Helios, and Nur77. Thus, these data strongly suggest that Eomes⁺ CD4 SP thymocytes are prefer-

entially selected via relatively weak affinity/avidity TCR interaction.

To further investigate whether the reduced TCR signal strength directly enhanced the expression of Eomes in CD4 SP thymocytes, we backcrossed CIITA^{tg} mice six times into *Zap70* spontaneous mutant (SKG; *Zap70*^{m1Saka}) mice, which are known to have a spontaneous mutation on the *ZAP70* gene. It is almost impossible to dissect the phenotypic feature of T cells with homozygote mutant thymi (CIITA^{tg}*Zap70*^{m1Saka/m1Saka}) due to defective positive selection (data not shown). For this reason, we dissected the phenotypic feature in heterozygous (CIITA^{tg}*Zap70*^{m1Saka/+}) mice. Despite the fact that the number of PLZF⁺ cells in the CD4 SP population is not significantly different between CIITA^{tg} and

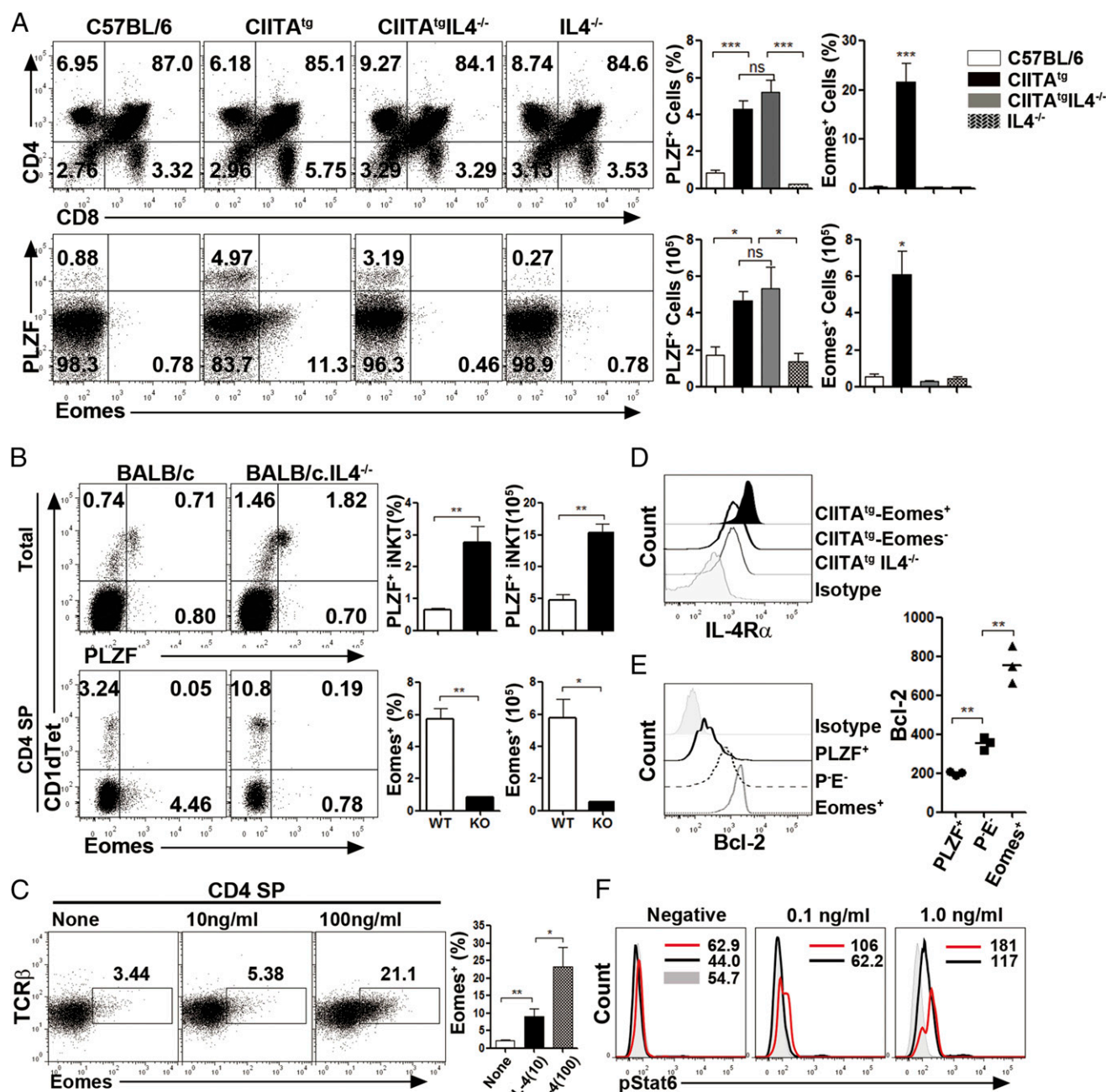


FIGURE 3. IL-4 produced by PLZF⁺ cells is critical for Eomes expression in CD4 SP thymocytes. **(A)** CD4/CD8 ratio of total thymocytes (*top panel*) and expression of PLZF and Eomes in CD4 SP thymocytes (*bottom panel*) from C57BL/6, CIITA^{tg}, CIITA^{tg}IL4^{-/-}, and IL4^{-/-} mice; representative data from three independent experiments. Numbers in quadrants indicate cell percentages. *Right panel*, Summarized data ($n = 3$ in each, 8–12 wk old) of PLZF⁺ or Eomes-expressing cell frequency among CD4 SP population and their absolute number are shown; bars indicate the mean \pm SEM. **(B)** PLZF⁺ iNKT cells in total thymocytes (*left top panel*), and Eomes⁺ cells in CD4 SP thymocytes (*left bottom panel*) from BALB/c and BALB/c.IL4^{-/-} mice ($n > 3$, 6–8 wk old). Representative flow-cytometry data (*left panel*) and cumulative data (*right panel*) are shown. **(C)** Induction of Eomes expression by exogenous IL-4. Fetal thymus of BALB/c mice were cultured in the presence (10 or 100 ng/ml) or absence (None) of IL-4 for 7 d. Two to three fetal thymic lobes were used for each condition, and pooled cells were analyzed. Representative expression pattern of TCRβ and Eomes of CD4 SP cells (*left panel*) and their cumulative data ($n > 2$, *right panel*) were displayed. **(D)** IL-4Rα expression on Eomes⁺ and Eomes⁻ CD4 SP thymocytes from CIITA^{tg} mice. CD4 SP thymocytes from CIITA^{tg}IL4^{-/-} were used as a negative control. Data are representative of three experiments. **(E)** Intracellular p-Stat6 in Eomes⁺ and Eomes⁻ CD4 SP thymocytes from CIITA^{tg} mice in response to exogenous IL-4 at the indicated concentrations. The numbers on the graph indicate MFI of pStat6. Data are representative of one of two experiments. **(F)** Expression of Bcl-2 in three subsets of CD4 SP thymocytes from CIITA^{tg} mice (*left panel*) and their cumulative data of MFI (*right panel*). Each symbol represents an individual mouse, and small horizontal lines indicate the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant.

CIITA^{tg}Zap70^{m1Saka/+} mice (Fig. 5D), a higher number of Eomes⁺ CD4 T cells were generated in the CIITA^{tg}Zap70^{m1Saka/+} thymus as compared with that of the CIITA^{tg} thymus. This was also true in

mice with defective expression of costimulatory molecules, B7-1 and B7-2, in that much higher number of Eomes⁺ cells were generated in B7-deficient mice (Fig. 5E). These data re-emphasize

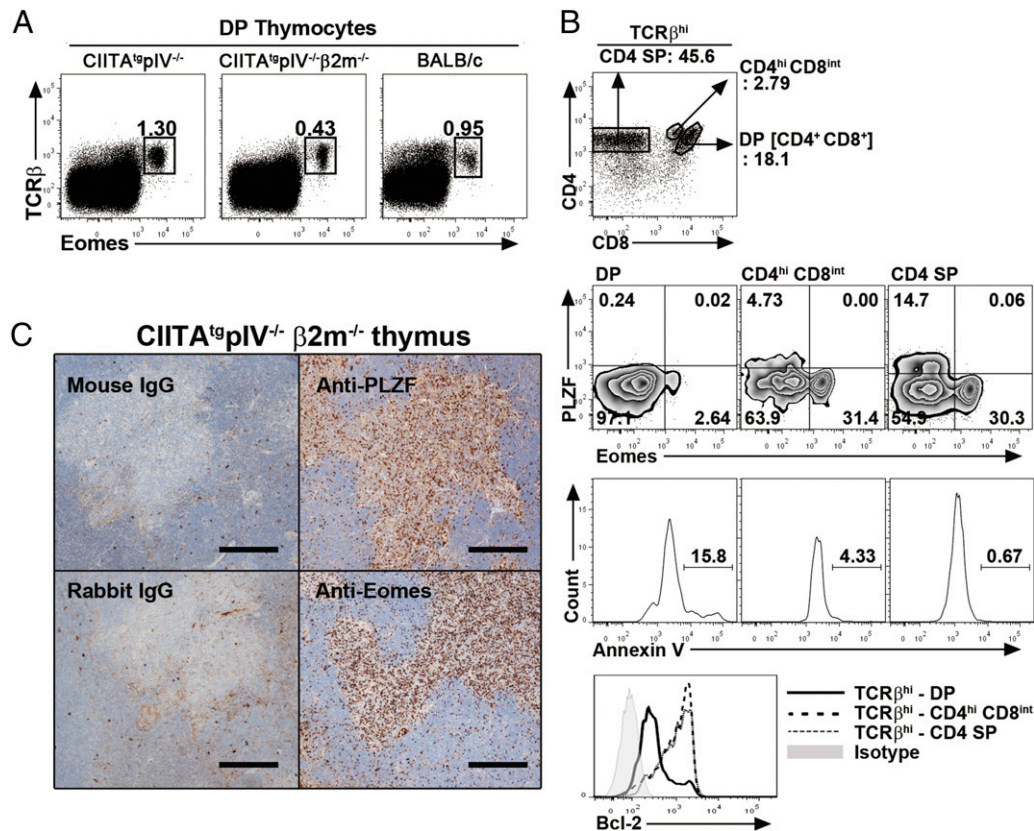


FIGURE 4. Eomes⁺ cells initially appear at the postselection stage of DP thymocytes. **(A)** Surface expression of TCR β and intracellular expression of Eomes in CD4⁺CD8⁺ (DP) thymocytes from CIITA^{tg}pIV^{-/-}, CIITA^{tg}pIV^{-/-}β2m^{-/-}, and BALB/c mice. Numbers in plots indicate the percentage of the Eomes⁺TCR β ^{hi} cells. Representative data of one of three independent experiments were displayed. **(B)** CD4/CD8 profile of thymocytes expressing a high level of TCR β from total thymocytes of CIITA^{tg}pIV^{-/-}β2m^{-/-} mice (*top panel*). Numbers in plots indicate the percentage of the cells. The expression of PLZF, Eomes (*upper middle panel*), and Annexin V (*lower middle panel*) in three subsets of TCR β ^{hi} thymocytes. Expression levels of Bcl-2 in the three subsets were also compared (*bottom panel*). Data are representative of one of two experiments. Numbers in quadrants of dot plots indicate cell percentages. Numbers in histograms indicate the percentage of Annexin V⁺ apoptotic cells. **(C)** Immunohistochemical localization of PLZF- or Eomes-expressing cells in the thymus of CIITA^{tg}pIV^{-/-}β2m^{-/-} mouse. Expression of PLZF (*top right panel*) and Eomes (*bottom right panel*) is predominantly found in the medulla and occasionally seen in the cortex. As negative control, tissues were stained with normal mouse (*top left panel*) or rabbit (*bottom left panel*) IgG. Original magnification $\times 100$. Scale bars, 200 μ m.

the fact that reduced avidity/affinity during the interaction between MHC/peptide and TCR allows CD4 T cells to drive expression of Eomes in response to IL-4.

Presence of CD4 T cells with the Th1-like phenotype in human CB cells

We have already reported that there exist PLZF-expressing T-T CD4 T cells and Eomes-expressing CD8 T cells in human fetal lymphoid organs and umbilical CB cells (11, 12). To extend our study, we tried to identify whether Eomes⁺CD4 T cells with innate phenotype are present in humans. Because it was much more difficult to get human fetal tissues, we were able to investigate the presence of these cells only in CB cells. Eomes⁺CD3⁺CD4 T cells were present in CB (0.2–2.6%; Fig. 6A), and most of them were CD45RA^{hi} and CD45RO^{low}. Interestingly, these cells were of CD122^{hi} and CCR7^{hi} and showed a higher expression of PTK7 and CD31, surface markers that are highly expressed on recent thymic emigrants, than CD45RA^{hi}CD4 T cells of adult human PBMCs, suggesting that Eomes⁺CD3⁺CD4⁺ T cells have not experienced the Ag recognition (Fig. 6B). Moreover, these cells were negative for human CD1d-PBS57 tetramer staining, indicating that they are a different cell population from human NKT cells (Fig. 6C). Unlike CB-Eomes⁺ CD4 T cells, the CB-Eomes⁺ CD4 T cells produced greater amounts of IFN- γ within a few hours in response to PMA/ionomycin without production of

IL-4 (Fig. 6D). This innate property is consistent with that of the Eomes⁺ nTh1 cells found in CIITA^{tg} and BALB/c mice.

Discussion

This study demonstrated that Eomes⁺ Th1 cells are able to be developed intrathymically. Remarkable specialization that this mechanism has is that low level of signals delivered to post-selection thymocytes play a major role in determining the cell fates. A series of events underlies the generation of this type of innate T cell; low affinity/avidity interaction between the TCR and MHC peptide as an initial event increased susceptibility to common γ -chain (γ_c) cytokines (in this case, IL-4), and finally, concomitant expression of Eomes and dramatic upregulation of Bcl-2 for their survival. This mechanism appears to work contrary to other types of innate cells, such as iNKT cells (31), nTh17 cells (24), and PLZF⁺ T-T CD4 T cells (32, 33), which receive high levels of signals from the interaction between the MHC/peptide and the appropriate TCR. This was also true in wild type mice, particularly in the BALB/c strain where NKT cells are responsible for the production of IL-4.

We previously demonstrated that a substantial percentage of CD8 SP thymocytes expressed Eomes in CIITA^{tg} mice and human, in which IL-4 is entirely responsible for its expression (12). In addition to this, recent identification of Eomes⁺ Th1 cells during thymic ontogeny has broadened the innate T cell repertoire, where

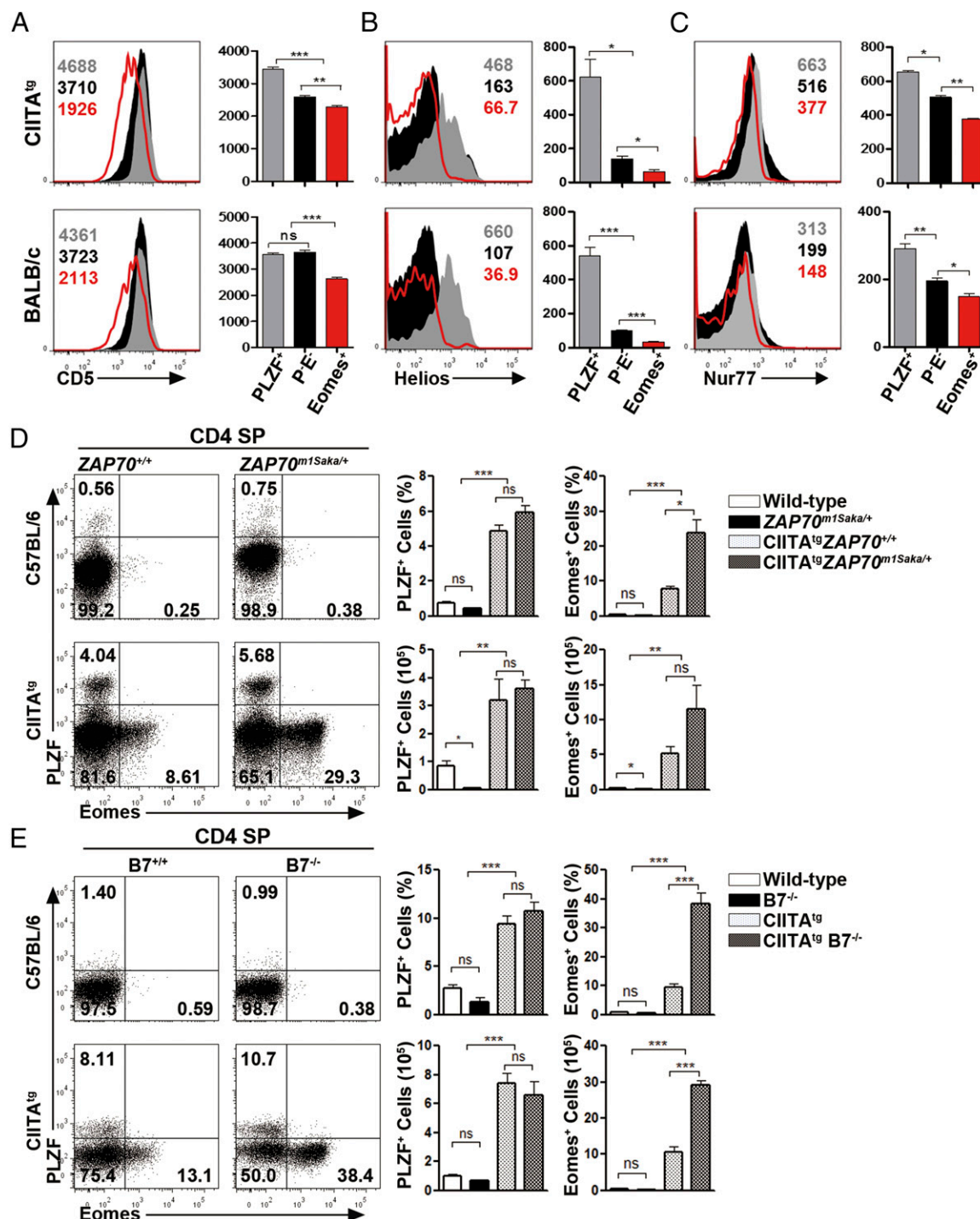


FIGURE 5. Low affinity/avidity TCR engagement prefers the generation of Eomes⁺ CD4 T cells. (A–C) Comparison of CD5 (A), Helios (B), and Nur77 (C) expression levels in PLZF⁺, Eomes⁺, or PLZF⁺Eomes⁻ (P⁺E⁻) subsets of CD4 SP thymocytes from CIITA^{tg} (*n* = 3) or BALB/c mice (*n* = 3). Representative histograms and summarized data are shown. Numbers in histograms indicate the MFI of the molecules; PLZF⁺ (filled gray), P⁺E⁻ (filled black), and Eomes⁺ (thick red line) CD4 SP thymocytes. Bars indicate the mean ± SEM. (D) Representative expression pattern of PLZF and Eomes in CD4 SP thymocytes from wild-type, *Zap70*^{m1Saka/+}, CIITA^{tg}, and CIITA^{tg}*Zap70*^{m1Saka/+} C57BL/6 mice (left panel), and the accumulative data (*n* = 4–11; right panels). Numbers in quadrants indicate the cell percentages, and bars indicate the mean ± SEM. (E) Representative expression pattern of PLZF and Eomes in CD4 SP thymocytes from wild-type, B7-1- and B7-2-deficient (B7^{-/-}), CIITA^{tg}, and CIITA^{tg} B7^{-/-} C57BL/6 mice (left panel), and the accumulative data (*n* = 4; right panels). Numbers in quadrants indicate the cell percentages; bars indicate the mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. ns, not significant.

IL-4 produced by PLZF⁺ T-T CD4 T cells played a key role. This was confirmed by the absence of Eomes⁺ CD4 T cells in CIITA^{tg} PLZF^{Lu/Lu} and CIITA^{tg}IL-4^{-/-} mice (Figs. 2A, 3A). Eomes⁺ CD4 T cells are functionally and phenotypically equivalent to previously described innate T cells, particularly Eomes⁺ CD8 T cells

(12), in that they rapidly produce Th1 effector cytokines in response to PMA and ionomycin, and express surface molecules that are highly associated with CD44^{hi}CD62L^{hi}CD24^{lo} memory phenotype. These cells have a low level of GATA3 expression and virtually no T-bet expression (Fig. 1C). This type of innate CD4

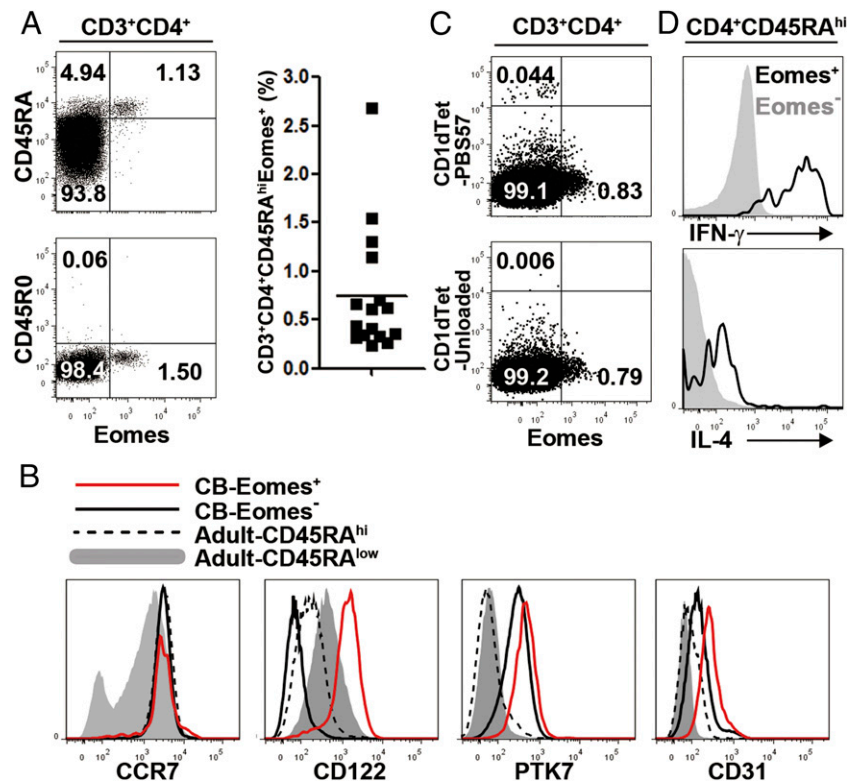


FIGURE 6. Presence of CD4 T cells with the innate phenotype in human CB cells. **(A)** Representative FACS profile showing Eomes expression in CD45RA⁺ or CD45R0⁻ populations of CD4⁺ T cells of umbilical CB and their cumulative data. **(B)** Expression patterns of surface molecules were compared between Eomes⁺ and Eomes⁻ CD4⁺ T cells from CB, and CD45RA^{hi} naive and CD45RA^{low} memory CD4⁺ T cells from human adult PBMCs. Data are representative of more than three independent experiments. **(C)** Flow-cytometric analysis of CB CD4⁺ T cells stained with anti-human Eomes Ab and PBS57 loaded or unloaded human CD1d tetramers (CD1dTet). **(D)** Cytokine production by Eomes⁺ and Eomes⁻ subsets of CD45RA^{hi} CD4⁺ T cells from human CB. Data are representative of two independent experiments.

T cell seems almost comparable with peripheral Th1 cells particularly with respect to functional properties (34), although the type of cytokines that are responsible for their development and subsequent transcriptional profile are somewhat different from conventional Th1 cells.

These Eomes⁺ CD4 T cells have extremely diverse TCR repertoire (Fig. 1D), similar to conventional CD4 T cells, whereas iNKT cells and recently described nTh17 cells have a restricted or skewed TCR repertoire (35).

Several recent reports have described that commitment to either the CD4 or the CD8 cell lineage results from interplay between the TCR signals and γ_c cytokines (36). An initial TCR signal in the DP cells causes downregulation of *Cd8a*, leading to loss of signaling through the MHC class I-restricted TCR (37, 38). Prolonged signaling through the MHC class II-restricted TCR leads to induction of Th-inducing POZ–Kruppel factor and GATA-3 transcription factors, which specify commitment to the CD4 lineage, whereas IL-7 signaling subsequent to cessation of TCR signal specifies commitment to the CD8 lineage (39). Immature CD4⁺ CD8⁺ DP thymocytes become responsive to cytokines with γ_c at the stage of intermediate CD4⁺CD8^{low} DP thymocytes immediately after positive selection (40). Because Eomes⁺ cells are predominantly skewed to the CD8 T cell phenotype in CIITA^{tg}pIV^{-/-} mice, detailed cell subset analysis for Eomes⁺ CD4 T cells would be much more difficult. Therefore, we used a CIITA^{tg}pIV^{-/-} $\beta 2m^{-/-}$ mouse system to turn off the CD8 developmental pathway. Another reason for using this system is to rule out the possibility that CD4 T cells come from an MHC class I/peptide and TCR interaction rather than from the MHC class II restriction pathway (36). In the thymus of CIITA^{tg}pIV^{-/-} $\beta 2m^{-/-}$ mice, PLZF⁺ and Eomes⁺ thymocytes first start to appear in the intermediate stage (CD4^{hi}CD8^{int}) of postselection thymocytes (Fig. 4B). Of the three TCR β ^{hi} cell subsets (CD4⁺CD8⁺ DP, CD4⁺CD8^{int} intermediate, CD4 SP), only DP thymocytes had the lowest Eomes expression, and substantial proportions of these cells were, in fact, Annexin V⁺ and down-

expressed antiapoptotic molecule Bcl-2, suggesting that these cells have higher chances for cell death (Fig. 4B). CD4⁺CD8^{int} intermediate population expressing Eomes seems to be somewhat different from the typical CD4⁺CD8^{-/int} intermediate cells (41) in the expression level of TCR β /CD3 and CD8 (Supplemental Figs. 2, 3). This supports the hypothesis that this type of innate T cell underwent a distinct developmental pathway. To clearly visualize PLZF and Eomes-expressing cells, we have done immunohistochemical analysis, which revealed that Eomes⁺ cells were scattered in thymic cortex and a much higher number of these cells was in the medullary compartment of the thymus (Fig. 4C). We were also able to get the same picture with the expression of PLZF⁺ cells: mostly they reside in the medullary thymic region. Based on the fact that Eomes expression first appears right after positive selection, we suggest that Eomes⁺ cells in thymic cortex with relatively lower-level expression of Eomes migrate into thymic medulla where they express a much higher amount of Eomes molecules and become finally matured. Interestingly, this early time expression of Eomes was also found in the cells at intermediate stage of wild-type BALB/c thymus (Supplemental Fig. 2), whereas it totally disappeared in IL-4^{-/-} and CD1d-deficient mice, demonstrating that exactly the same intrathymic developmental process exists in the normal thymic compartment.

As mentioned previously, the strength of signal received by the T cells during thymic development seems to be the major determinant for the generation of Eomes⁺ CD4 T cells. This is really rather extraordinary, in that this type of cell is generated only when affinity/avidity generated during interaction between TCR and MHC/peptide is relatively low, whereas most of the innate T cells come from high affinity/avidity interactions during thymic development. The representative example of this is iNKT cells (31), nTh17 cells (24), and PLZF⁺ CD4 T cells (32, 33). Although regulatory T cells might well be more regulatory than immunogenic, they also result from high affinity/avidity interaction (31). It has been well-known for years that expression levels

of CD5, Helios, and Nur77 molecules are thought to reflect a prior binding force of TCR during thymic selection (29–31). As shown in Fig. 5A–C, these molecules are almost identical in their expression hierarchy, showing the highest expression in PLZF⁺ cells, followed by conventional T cells, and the lowest expression in Eomes⁺ cells. Plausible explanation comes from subsequent experiments, using two types of gene-manipulated mice: a SKG mouse and a B7^{-/-} mouse, which have defects in either TCR signaling or the costimulatory signaling pathway, respectively. There was a dramatic increase in the number of Eomes⁺ innate CD4 T cells in SKG mice with mutated *Zap70* gene where reduced TCR signaling are delivered inside the cells. These data dramatically illustrate the fact that reduced TCR signaling is really responsible for the generation of Eomes⁺ innate CD4 T cells. Additional evidence that reduced signal strength enhances the production of Eomes⁺ CD4 T cells comes from the experimental data using CIITA^{tg}B7^{-/-} mice. Because CD28-B7 interactions have been considered to be involved in deleting autoreactive thymocytes for self-tolerance (42), it raised the possibility that the increased number of Eomes⁺ CD4 T cells in CIITA^{tg} B7^{-/-} mice might be due to the defective negative selection. To address this issue, we further analyzed Eomes⁺ cell population in both intermediate (CD4^{hi}CD8^{int}) and CD4 SP stage to get a clue whether B7 deficiency affected positive or negative selection of Eomes⁺ CD4 T cells. As shown in Supplemental Fig. 3A, the frequency of Eomes⁺ cells at intermediate stage is maintained until these cells mature into CD4 SP cells both in CIITA^{tg} and in CIITA^{tg}B7^{-/-} thymuses. Moreover, syngeneic mixed lymphocytes reaction showed that the frequency of self-reactive clones between mature Eomes⁺ CD4 SP cells from CIITA^{tg} mice and CIITA^{tg}B7^{-/-} mice was not different (Supplemental Fig. 3B), providing substantial evidence that no autoreactive T cells escaped from thymic censorship. All these data suggest that enhanced positive selection, rather than a rescue from negative selection, is responsible for the increased number of Eomes⁺ CD4 T cells in the absence of CD28–B7 interaction.

Several recent reports have suggested that there is an inverse correlation between signal strength received by T cells and the susceptibility of γ_c cytokine (36, 43–45). Our data are, in fact, in favor of this idea, in that T cells that are positively selected at the low level of affinity/avidity interaction become extraordinarily susceptible to IL-4, which subsequently controls downstream phosphorylation of Stat6. The phosphorylation status of Stat6 in Eomes⁺ cells was much higher than that of Eomes⁻ cells and there was concomitant upregulation of IL-4R α when an individual subset of cells was treated with various amounts of IL-4.

These cells were hardly detected in the peripheral lymphoid organs and tissues. As shown in Supplemental Fig. 4, the Eomes⁺ CD62L⁺CD4⁺ T cells were barely detected in the spleen of C57BL/6 and B7^{-/-}, and even in CIITA^{tg} mice. This might be due to the absence of proper surface marker to distinguish these cells from other types of innate T cells. Another possibility is that the expression of Eomes might vanish when they migrate into the periphery. This notion was partially supported by the results from the spleen of CIITA^{tg}B7^{-/-} mice: relatively higher frequency of Eomes⁺ CD4 T cells was seen in the splenic CD4 T compartments of these mice than those of CIITA^{tg} mice. These data suggest that CD28–B7 interaction seemed to negatively regulate Eomes expression during peripheral homeostasis, as well as thymic development. To expand our knowledge of the generation of this type of T cell, we investigated the presence of human counterparts of murine Eomes⁺ nTh1 cells. Because of the difficulty in obtaining more human fetal tissues, we were able to investigate the presence of these T cells only in human umbilical CB cells. Interestingly,

we found that the 0.2–2.6 percentage of CB-CD3⁺CD4 T cells expressed Eomes and they showed Th1-like properties, rapid production of IFN- γ , but none of IL-4 (Fig. 6D), suggesting their functional competence. These cells also showed an expression pattern of molecules associated with naive T cells (CD45RA^{hi}, CD45RO^{low}, and CCR7^{hi}) and recent thymic emigrants (PTK7⁺ and CD31⁺), as well as memory phenotype (CD122^{hi}). These phenotypic characteristics are reminiscences of recently reported stem-like memory T cells to be found in the peripheral blood of human (46) and nonhuman primates (47). Further investigation of the possible relationship between PLZF⁺ innate T cells and the generation of stem-like memory T cells is required in the human immune system. Considering the previous report of developmental kinetics of CD8 fetal thymic T cells (12), we speculate the generation of Eomes⁺ CD4 human fetal thymic T cells might be in gear with the developmental kinetics of fetal thymic PLZF⁺CD4 T cells (11), that is, the peak frequency in early gestation (second trimester), the declination after that, and almost absence after birth.

We conclude that a key aspect for the generation of nTh1 cells would be a high susceptibility of these cells to IL-4, which comes from the low affinity/avidity interaction during thymic selection. As a consideration of what this may mean, from a developmental point of view, we think that this is really a unique developmental type of pathway for the generation of a novel T cell subset. These nTh1 cells are functionally equivalent to Th1 cells in the periphery.

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Disclosures

The authors have no financial conflicts of interest.

References

- Berg, L. J. 2007. Signalling through TEC kinases regulates conventional versus innate CD8⁺ T-cell development. *Nat. Rev. Immunol.* 7: 479–485.
- Urdahl, K. B., J. C. Sun, and M. J. Bevan. 2002. Positive selection of MHC class Ib-restricted CD8⁺ T cells on hematopoietic cells. *Nat. Immunol.* 3: 772–779.
- Lamboltz, F., M. Kronenberg, and H. Cheroutre. 2007. Thymic differentiation of TCR $\alpha\beta$ ⁺ CD8 $\alpha\alpha$ ⁺ IELs. *Immunol. Rev.* 215: 178–188.
- Veillette, A., Z. Dong, and S. Latour. 2007. Consequence of the SLAMFAP signaling pathway in innate-like and conventional lymphocytes. *Immunity* 27: 698–710.
- Xiong, N., and D. H. Raulet. 2007. Development and selection of gammadelta T cells. *Immunol. Rev.* 215: 15–31.
- Lee, Y. J., K. C. Jung, and S. H. Park. 2009. MHC class II-dependent T-T interactions create a diverse, functional and immunoregulatory reaction circle. *Immunol. Cell Biol.* 87: 65–71.
- Martin, E., E. Treiner, L. Duban, L. Guerri, H. Laude, C. Toly, V. Premel, A. Devys, I. C. Moura, F. Tilloy, et al. 2009. Stepwise development of MAIT cells in mouse and human. *PLoS Biol.* 7: e54.
- Godfrey, D. I., D. G. Pellicci, O. Patel, L. Kjer-Nielsen, J. McCluskey, and J. Rossjohn. 2010. Antigen recognition by CD1d-restricted NKT T cell receptors. *Semin. Immunol.* 22: 61–67.
- Alonzo, E. S., and D. B. Sant'Angelo. 2011. Development of PLZF-expressing innate T cells. *Curr. Opin. Immunol.* 23: 220–227.
- Treiner, E., and O. Lantz. 2006. CD1d- and MR1-restricted invariant T cells: of mice and men. *Curr. Opin. Immunol.* 18: 519–526.
- Lee, Y. J., Y. K. Jeon, B. H. Kang, D. H. Chung, C. G. Park, H. Y. Shin, K. C. Jung, and S. H. Park. 2010. Generation of PLZF⁺ CD4⁺ T cells via MHC class II-dependent thymocyte-thymocyte interaction is a physiological process in humans. *J. Exp. Med.* 207: 237–246.
- Min, H. S., Y. J. Lee, Y. K. Jeon, E. J. Kim, B. H. Kang, K. C. Jung, C. H. Chang, and S. H. Park. 2011. MHC class II-restricted interaction between thymocytes plays an essential role in the production of innate CD8⁺ T cells. *J. Immunol.* 186: 5749–5757.
- Park, S. H., Y. M. Bae, T. J. Kim, I. S. Ha, S. Kim, J. G. Chi, and S. K. Lee. 1992. HLA-DR expression in human fetal thymocytes. *Hum. Immunol.* 33: 294–298.
- Choi, E. Y., W. S. Park, K. C. Jung, D. H. Chung, Y. M. Bae, T. J. Kim, H. G. Song, S. H. Kim, D. I. Ham, J. H. Hahn, et al. 1997. Thymocytes positively select thymocytes in human system. *Hum. Immunol.* 54: 15–20.
- Choi, E. Y., K. C. Jung, H. J. Park, D. H. Chung, J. S. Song, S. D. Yang, E. Simpson, and S. H. Park. 2005. Thymocyte-thymocyte interaction for efficient positive selection and maturation of CD4 T cells. *Immunity* 23: 387–396.

16. Li, W., M. G. Kim, T. S. Gourley, B. P. McCarthy, D. B. Sant'Angelo, and C. H. Chang. 2005. An alternate pathway for CD4 T cell development: thymocyte-expressed MHC class II selects a distinct T cell population. *Immunity* 23: 375–386.
17. Savage, A. K., M. G. Constantinides, J. Han, D. Picard, E. Martin, B. Li, O. Lantz, and A. Bendelac. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29: 391–403.
18. Kang, B. H., H. S. Min, Y. J. Lee, B. Choi, E. J. Kim, J. Lee, J.-R. Kim, K.-H. Cho, T. J. Kim, K. C. Jung, and S. H. Park. 2015. Analyses of the TCR repertoire of MHC class II-restricted innate CD4⁺ T cells. *Exp. Mol. Med.* 47: e154.
19. Broussard, C., C. Fleischacker, R. Horai, M. Chetana, A. M. Venegas, L. L. Sharp, S. M. Hedrick, B. J. Fowlkes, and P. L. Schwartzberg. 2006. Altered development of CD8⁺ T cell lineages in mice deficient for the Tec kinases Itk and Rlk. *Immunity* 25: 93–104.
20. Jordan, M. S., J. E. Smith, J. C. Burns, J. E. Austin, K. E. Nichols, A. C. Aschenbrenner, and G. A. Koretzky. 2008. Complementation in trans of altered thymocyte development in mice expressing mutant forms of the adaptor molecule SLP76. *Immunity* 28: 359–369.
21. Fukuyama, T., L. H. Kasper, F. Boussouar, T. Jeevan, J. van Deursen, and P. K. Brindle. 2009. Histone acetyltransferase CBP is vital to demarcate conventional and innate CD8⁺ T-cell development. *Mol. Cell. Biol.* 29: 3894–3904.
22. Vervakakis, M., M. D. Boos, A. Bendelac, and B. L. Kee. 2010. SAP protein-dependent natural killer T-like cells regulate the development of CD8⁺ T cells with innate lymphocyte characteristics. *Immunity* 33: 203–215.
23. Weinreich, M. A., O. A. Odumade, S. C. Jameson, and K. A. Hogquist. 2010. T cells expressing the transcription factor PLZF regulate the development of memory-like CD8⁺ T cells. *Nat. Immunol.* 11: 709–716.
24. Marks, B. R., H. N. Nowyhed, J. Y. Choi, A. C. Poholek, J. M. Odegard, R. A. Flavell, and J. Craft. 2009. Thymic self-reactivity selects natural interleukin 17-producing T cells that can regulate peripheral inflammation. *Nat. Immunol.* 10: 1125–1132.
25. Caraux, G., and S. Pinloche. 2005. PermutMatrix: a graphical environment to arrange gene expression profiles in optimal linear order. *Bioinformatics* 21: 1280–1281.
26. Lee, Y. J., K. L. Holzapfel, J. Zhu, S. C. Jameson, and K. A. Hogquist. 2013. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat. Immunol.* 14: 1146–1154.
27. McDonald, B. D., M. G. Constantinides, and A. Bendelac. 2013. Polarized effector programs for innate-like thymocytes. *Nat. Immunol.* 14: 1110–1111.
28. Lai, D., J. Zhu, T. Wang, J. Hu-Li, M. Terabe, J. A. Berzofsky, C. Clayberger, and A. M. Krensky. 2011. 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.* 208: 1093–1103.
29. Azzam, H. S., A. Grinberg, K. Lui, H. Shen, E. W. Shores, and P. E. Love. 1998. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J. Exp. Med.* 188: 2301–2311.
30. Daley, S. R., D. Y. Hu, and C. C. Goodnow. 2013. Helios marks strongly autoreactive CD4⁺ T cells in two major waves of thymic deletion distinguished by induction of PD-1 or NF- κ B. *J. Exp. Med.* 210: 269–285.
31. Moran, A. E., K. L. Holzapfel, Y. Xing, N. R. Cunningham, J. S. Maltzman, J. Punt, and K. A. Hogquist. 2011. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* 208: 1279–1289.
32. Qiao, Y., L. Zhu, H. Sofi, P. E. Lapinski, R. Horai, K. Mueller, G. L. Stritesky, X. He, H. S. Teh, D. L. Wiest, et al. 2012. Development of promyelocytic leukemia zinc finger-expressing innate CD4 T cells requires stronger T-cell receptor signals than conventional CD4 T cells. *Proc. Natl. Acad. Sci. USA* 109: 16264–16269.
33. Zhu, L., Y. Qiao, E. S. Choi, J. Das, D. B. Sant'angelo, and C. H. Chang. 2013. A transgenic TCR directs the development of IL-4⁺ and PLZF⁺ innate CD4 T cells. *J. Immunol.* 191: 737–744.
34. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7: 145–173.
35. Park, S. H., A. Weiss, K. Benlagha, T. Kyin, L. Teyton, and A. Bendelac. 2001. The mouse CD1d-restricted repertoire is dominated by a few autoreactive T cell receptor families. *J. Exp. Med.* 193: 893–904.
36. Singer, A., S. Adoro, and J. H. Park. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat. Rev. Immunol.* 8: 788–801.
37. Brugnera, E., A. Bhandoora, R. Cibotti, Q. Yu, T. I. Guintier, Y. Yamashita, S. O. Sharrow, and A. Singer. 2000. Coreceptor reversal in the thymus: signaled CD4⁺8⁺ thymocytes initially terminate CD8 transcription even when differentiating into CD8⁺ T cells. *Immunity* 13: 59–71.
38. Singer, A. 2002. New perspectives on a developmental dilemma: the kinetic signaling model and the importance of signal duration for the CD4/CD8 lineage decision. *Curr. Opin. Immunol.* 14: 207–215.
39. Park, J. H., S. Adoro, T. Guintier, B. Erman, A. S. Alag, M. Catalfamo, M. Y. Kimura, Y. Cui, P. J. Lucas, R. E. Gress, et al. 2010. Signaling by intra-thymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. *Nat. Immunol.* 11: 257–264.
40. Yu, Q., J. H. Park, L. L. Doan, B. Erman, L. Feigenbaum, and A. Singer. 2006. Cytokine signal transduction is suppressed in preselection double-positive thymocytes and restored by positive selection. *J. Exp. Med.* 203: 165–175.
41. Lundberg, K., W. Heath, F. Köntgen, F. R. Carbone, and K. Shortman. 1995. Intermediate steps in positive selection: differentiation of CD4⁺8^{int} TCR^{int} thymocytes into CD4⁺8⁺TCR^{hi} thymocytes. *J. Exp. Med.* 181: 1643–1651.
42. Pobezinsky, L. A., G. S. Angelov, X. Tai, S. Jeurling, F. Van Laethem, L. Feigenbaum, J. H. Park, and A. Singer. 2012. Clonal deletion and the fate of autoreactive thymocytes that survive negative selection. *Nat. Immunol.* 13: 569–578.
43. Zhu, J., H. Huang, L. Guo, T. Stonehouse, C. J. Watson, J. Hu-Li, and W. E. Paul. 2000. Transient inhibition of interleukin 4 signaling by T cell receptor ligation. *J. Exp. Med.* 192: 1125–1134.
44. Park, J. H., S. Adoro, P. J. Lucas, S. D. Sarafova, A. S. Alag, L. L. Doan, B. Erman, X. Liu, W. Ellmeier, R. Bosselut, et al. 2007. 'Coreceptor tuning': cytokine signals transcriptionally tailor CD8 coreceptor expression to the self-specificity of the TCR. *Nat. Immunol.* 8: 1049–1059.
45. Koenen, P., S. Heinzel, E. M. Carrington, L. Happo, W. S. Alexander, J.-G. Zhang, M. J. Herold, C. L. Scott, A. M. Lew, A. Strasser, and P. D. Hodgkin. 2013. Mutually exclusive regulation of T cell survival by IL-7R and antigen receptor-induced signals. *Nat. Commun.* 4: 1735.
46. Gattinoni, L., E. Lugli, Y. Ji, Z. Pos, C. M. Paulos, M. F. Quigley, J. R. Almeida, E. Gostick, Z. Yu, C. Carpenito, et al. 2011. A human memory T cell subset with stem cell-like properties. *Nat. Med.* 17: 1290–1297.
47. Lugli, E., M. H. Dominguez, L. Gattinoni, P. K. Chattopadhyay, D. L. Bolton, K. Song, N. R. Klatt, J. M. Brenchley, M. Vaccari, E. Gostick, et al. 2013. Superior T memory stem cell persistence supports long-lived T cell memory. *J. Clin. Invest.* 123: 594–599.