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MHC Class II-Restricted Interaction between Thymocytes Plays an Essential Role in the Production of Innate CD8+ T Cells

Hye Sook Min,*1 You Jeong Lee,*†1 Yoon Kyung Jeon,* Eun Ji Kim,‡ Byung Hyun Kang,*‡ Kyeong Cheon Jung,*† Cheong-Hee Chang,* and Seong Hoe Park*†

We have recently shown that MHC class II-dependent thymocyte–thymocyte (T–T) interaction successfully generates CD4+ T cells (T–T CD4+ T cells), and that T–T CD4+ T cells expressing promyelocytic leukemia zinc finger protein (PLZF) show an innate property both in mice and humans. In this article, we report that the thymic T–T interaction is essential for the conversion of CD8+ T cells into innate phenotype in the physiological condition. CD8+ T cells developed in the presence of PLZF+ CD4+ T cells showed marked upregulation of eomesodermin (Eomes), activation/memory phenotype, and rapid production of IFN-gamma (T–T CD4+ T cells), and that T–T CD4+ T cells expressing promyelocytic leukemia zinc finger protein (PLZF) show an innate property. The same events may take place in humans, as a substantial number of Eomes expressing innate CD8+ CD8+ T cells into innate phenotype in the physiological condition. CD8+ T cells developed in the presence of PLZF+ CD4+ T cells have demonstrated the development of a distinct CD4+ T cell repertoire (T–T CD4+ T cells) via the MHC class II-dependent thymocyte–thymocyte (T–T) interaction (T–T CD4+, also referred to as T-CD4+) were added to the category of innate T cells (11–14).

Two transcriptional factors are known to regulate development of innate T cells: promyelocytic leukemia zinc finger protein (PLZF) and eomesodermin (Eomes). PLZF, which was first identified being expressed in iNKT cells and subsequently in T–T CD4+ T cells, modulates the cellular maturation and the cytokine production of both Th1 and Th2 types (15–17). In the absence of PLZF, iNKT cells remain immature and lose their innate function, whereas transgenic overexpression of PLZF converts naive T cells into the memory phenotype. In CD8 lineage cells, the T-box transcription factor Eomes, together with T-bet, is sufficient to invoke the attributes of the effector program including Th1 cytokine secretion, IL-2RB (CD122) upregulation, and IL-15 dependency (18–20). CD8αβ+ innate T cells in iNKT or iNKT−/− RIK−/− mice showed enhanced expression of Eomes during the thymic maturation process, indicating that Eomes is a marker of innate CD8+ T cells in the thymus (10).

By developing a mouse model in which MHC class II molecules are present only on T cells (CIITAβ+iPv−/− mice), we and others have demonstrated the development of a distinct CD4+ T cell repertoire (T–T CD4+ T cells) via the MHC class II-dependent homotypic interaction between thymocytes (12, 13). In recent subsequent studies, some proportion of T–T CD4+ T cells was found to express PLZF, which mainly exerts innate function (14, 21, 22). Most importantly, PLZF+ CD4+ T cells existed in humans, having a characteristic developmental kinetics in the early fetal stage. This strongly suggested that PLZF+ T–T CD4+ T cells might work as potent innate immune cells in the perinatal period, that is, before the establishment of sufficient memory pools elicited by conventional T cells.

In this study, we found that the novel CD8+ T cell population was generated in CIITAβ+iPv−/− mice, which acquired innate characteristics with the upregulation of Eomes via IL-4, which is mainly secreted by PLZF+ T–T CD4+ T cells. Interestingly, the CD8+ T cell population of the same phenotype was found in the
Materials and Methods

Mice

The plck-CIITA<sup>−/−</sup> mice were previously generated in our laboratory (12). CD45.1 congenic B6, PLZF<sup>−/−</sup>, TCR<sup>−/−</sup>, and IL-4<sup>−/−</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice carrying a deletion of promoter IV of the Mhc2ta gene (pIV<sup>−/−</sup>) were provided by Hua Gu (Columbia University, New York, NY). The plck-CIITA<sup>−/−</sup> mice were backcrossed to pIV<sup>−/−</sup>, CD1d<sup>−/−</sup>, PLZF<sup>−/−</sup>, TCR<sup>−/−</sup>, and IL-4<sup>−/−</sup> mice to generate CIITA<sup>pIV−/−</sup>, CIITA<sup>CD1d−/−</sup>, CIITA<sup>PLZF−/−</sup>, CIITA<sup>TCR−/−</sup>, and CIITA<sup>IL-4−/−</sup> mice, respectively. PLZF<sup>−/−</sup> mice driven by CD4 promoter were a kind gift from Dr. Albert Bendelac (Chicago, IL). All of the mice were maintained under specific pathogen-free conditions in the animal facility at the 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.

Abs and flow cytometric analysis

The following fluorochrome- or biotin-labeled mAbs were purchased from BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA), or DiNonA (Seoul, Korea): anti–mouse CD4 (RM4.5), CD8α (53-6-7), CD8β (341), CD24 (M1/69), CD25 (3C7), I-<sup>A</sup> (AF6-120.1), CD62L (MEL-14), CD44 (IM7), Ki-67 (B56), TCRγδ (H57-597), TCRγδ (GL3), CD69 (H1.2F3), CD122 (TM-β1), CD127 (SB-199), IFN-γ (XMG1.2), IL-4 (1B11), and Eomes (WD1928) and anti-human CD4 (RPA-T4 or OKT-4), CD8α (RPA-T8 or OKT-8), CD8β (2ST.5H7), CD25 (M-A251), CD69 (FN50), CD45RA (HI100), CD45RO (UCHL1), CD122 (Mik-β3), CD161 (DX12), IFN-γ (45.15), and Eomes (Dan11-mag). The anti-mouse V<sub>B2</sub> (B2.60.6), V<sub>B3</sub> (KJ25), V<sub>B4</sub> (KT41), V<sub>B5.1</sub>&&5.2 (MR9-4), V<sub>B6</sub> (RR4-7), V<sub>B7</sub> (TR310), V<sub>B8</sub> (F23.1), V<sub>B10</sub> (B21.5), V<sub>B11</sub> (RR3-15), and V<sub>B13</sub> (MR12-3) Abs were purchased from BD Pharmaningen. FITC-conjugated anti-human V<sub>B1</sub> (BL37.3), V<sub>B2</sub> (MPB205), V<sub>B5.1</sub> (IMMU157), V<sub>B7</sub> (56c5.2), V<sub>B11</sub> (C21), V<sub>B13.1</sub> (IMMU222), V<sub>B17</sub> (E17.5F9.15.13), V<sub>B21.3</sub> (H1125), and V<sub>B22</sub> (IMMU546) Abs were purchased from Beckman Coulter. Allophycocyanin-conjugated anti-mouse α-galactosylceramide-loaded or unloaded CD1d tetramers were purchased from ProImmune (Bradenton, FL). Human samples were stained with mouse CD1d tetramers, as they are cross-reactive (23, 24). Fresh cell suspensions of thymocytes, splenocytes, or lymph node cells were resuspended in flow cytometry buffer, which consisted of PBS with 0.1% BSA and 0.01% sodium azide. After staining with fluorochrome-conjugated Abs for 30 min at 4°C, the cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuest Pro software (Becton Dickinson).

Adaptive T cell transfer

Splenic CD8<sup>+</sup> T cells from 8 wk-old wild-type (WT; 2.6 × 10<sup>7</sup>) and CIITA<sup>pIV−/−</sup> mice (2.17 × 10<sup>7</sup>) were purified using MACS according to manufacturer’s guide and injected into congenic mice (1 × 10<sup>6</sup>). One week later, recipient mice were sacrificed, and we analyzed the content of donor T cells in thymus, spleen, and lymph nodes.

Intracellular staining

For intracellular flow cytometry of PLZF and Eomes, cells were fixed with the fixation and permeabilization buffers from the Foxp3 staining buffer set (eBioscience). Intracellular PLZF was detected using mouse mAb D-9 (Santa Cruz, CA), and in some experiments, a biotin-conjugated D-9 Ab (DiNonA) was used. For cytokine staining, cells were stimulated with 50 ng/ml PMA and 1.5 μM ionomycin (Sigma, St. Louis, MO) for 5 h; 10 μg/ml brefeldin A (Sigma) was added during the last 3 h of stimulation. The stimulated cells were surface stained, fixed, and stained with anti-IL-4 (11B11), anti–IFN-γ (XMG1.2 or 45.15), anti–PLZF (D-9), or anti–Eomes (WD1928 or Dan11-mag), followed by fluorochrome-conjugated goat antimouse IgG1 (A85-1; BD Pharmingen) or streptavidin (BD Pharmingen) using the Foxp3 staining buffer set (eBioscience).

FIGURE 1. Innate CD8<sup>+</sup> T cell development in CIITA<sup>pIV−/−</sup> mice. A, Thymic T–T interaction facilitates the development of CD8 SP thymocytes. Single-cell suspensions of total thymocytes from the indicated mice were analyzed to assess CD4/CD8 ratios (n = 6, 8–12 wk old, respectively). Representative data from three independent experiments (left panel) and cumulative data (right panel) are shown; bars indicate the mean ± SEM. B, RT-PCR was performed to confirm the Eomes expression in CD8 SP thymocytes. Eomes expression was compared with that of β-actin. C, Eomes’ CD8 SP thymocytes from CIITA<sup>pIV−/−</sup> mice have the memory phenotype. Eomes<sup>+</sup> and Eomes<sup>−</sup> CD8 SP thymocytes from CIITA<sup>pIV−/−</sup> mice were analyzed for their expression of CD24, CD44, CD62L, CD122, CD5K, and CXCR3. The histograms show Eomes<sup>+</sup> (thick lines) or Eomes<sup>−</sup> (thin lines) CD8 SP populations. Data are representative of one of two experiments. D, Eomes’ CD8 SP thymocytes produce IFN-γ on ex vivo stimulation. MACS purified CD24<sup>+</sup> thymocytes from the WT and CIITA<sup>pIV−/−</sup> mice were stimulated with PMA and ionomycin for 5 h followed by intracellular IFN-γ staining. Data are representative of one of two experiments. Numbers in regions or quadrants indicate the percentage of cells in each. *p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant.
Quantitative real-time and RT-PCR

Total RNA was extracted from MACS-sorted thymocytes using the RNeasy mini kit (Qiagen, CA) according to the manufacturer’s instructions, and cDNA was synthesized using oligo(dT) primers. Sphingosine-1-phosphate 1 (S1P1) real-time RT-PCR was performed using premade primers from Applied Bioscience. The primers used for Eomes RT-PCR were: 5’-ACGGCCCGCCTGTAATCTGAA-3’ (forward) and 5’-AGAGCC-AGGCCTACAAACAGGCTG-3’ (reverse); and for β-actin, 5’-GGAAATCTGCGTGTGACATTAGG-3’ (forward) and 5’-GGCTTTTAGGATGGCAAGGGCAG-3’ (reverse).

Affymetrix gene array

The total RNA was prepared for the microarray and hybridized according to standard Affymetrix protocols (Affymetrix GeneChip Mouse Gene 1.0 ST Array: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26090, accession number GSE26090). 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).

BrdU incorporation

BrdU staining was performed with the “Allophycocyanin Mouse Anti-BrdU set” according to the manufacturer’s protocol (BD Pharmingen).

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 μm; 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 murine rIL-4 (1–10 ng/ml; PeproTech). After 7 d, the thymuses were harvested and single-cell suspensions were prepared and analyzed for the expression of CD4, CD8, PLZF, and Eomes by flow cytometry.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Accumulation of Eomes+ CD8 SP thymocytes in the thymus is not due to enhanced proliferation, retention, or recirculation of peripheral memory T cells. A and B, Proliferative capacity of CD8 SP thymocytes from indicated mice. After 40 min of BrdU injection (1 mg i.p.), 8-wk-old-mice (n = 3) of each strain were sacrificed and single-cell suspension of thymocytes was stained with anti-CD4, CD8, BrdU (A), and Ki-67 (B). Numbers in quadrants in DP thymocytes indicate the percentage of BrdU+ or Ki-67+ cells per TCRβlo cells, and numbers in quadrants in SP thymocytes indicate the percentage of BrdU+ or Ki-67+ cells/TCRβhigh cells. Representative data from two independent experiments (left panel) and cumulative data (right panel) are shown; bars indicate the mean ± SEM. C, Splenic CD8+ T cells (1 × 10^7) from WT and CIITA^−/− mice were adoptively transferred into 8-wk-old CD45.1 congenic hosts (n = 3, respectively). Seven days later, mice were sacrificed and CD45.1^+ donor CD8+ T cells were analyzed. Numbers in quadrants indicate the percentage of cells in each. Representative data from two independent experiments (left panel) and cumulative data (right panel) are shown; bars indicate the mean ± SEM. D, S1P expression in CD8 SP thymocytes from the WT and CIITA^−/− mice. Quantitative RT-PCR was performed with purified CD8 SP thymocytes from WT and CIITA^−/− mice. Results were expressed as ratios relative to the housekeeping gene GAPDH. Data shown are representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant; Sp, spleen; Thy, thymus.
Bone marrow chimeras

Recipient CIITA<sup>tg</sup>pIV<sup>−/−</sup> mice were exposed to 900 rad total body irradiation from a [137Cs] source administered in two doses given 4 h apart. The mice were rested for 4–24 h before receiving bone marrow (BM) cells. Total BM cells were prepared from the femurs and tibiae of donor mice, and mature T cells were depleted using a mixture of CD4 and CD8 microbeads and magnetic sorting (Miltenyi Biotech, Auburn, CA). Each recipient mouse received $3 \times 10^6$ T cell-depleted BM cells in a volume of 300 µl PBS via lateral tail vein injection. The mice were analyzed 5–12 wk later.

Human tissues and samples

Postnatal thymuses were obtained during cardiac surgery at Seoul National University Hospital, and fetal spleen and thymus samples were obtained from aborted fetuses (16–26 wk of gestation) from Seoul National University Hospital or Jang’s Women’s Hospital (Seoul, Korea). Human umbilical cord blood 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. The gestational age of the fetus was calculated from the last menstrual period. Thymic tissues were teased into single-cell suspensions, and splenic mononuclear cells were separated by density gradient centrifugation over lympho M solution (Cedarlane, Burlington, ON, Canada). Cord blood 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 (GraphPad Software). Bar graphs denoting the percentage of each cell or concentration of each cytokine represent the mean value ± SEM, and the data were compared using an unpaired t test, one-way ANOVA, and Tukey’s multiple-comparison test.

FIGURE 3. PLZF is an essential factor for the induction of Eomes<sup>+</sup> CD8<sup>+</sup> SP thymocytes. A, Single-cell suspension of thymocytes from indicated mice was analyzed to compare Eomes and PLZF expression (n = 6; 8 to 12 wk old). CD4 SP (top left panels) and CD8 SP (bottom left panels) thymocytes were shown. Representative data from two independent experiments (left panel) and cumulative data (right panel) are shown; bars indicate the mean ± SEM. B, PLZF<sup>Lu/Lu</sup> (n = 4), CIITA<sup>tg</sup>PLZF<sup>Lu/Lu</sup> (n = 4), CIITA<sup>tg</sup>PLZF<sup>Lu/Lu</sup> (n = 3), and PLZF<sup>−/−</sup> mice (n = 4) were assessed for CD4/CD8 ratios (top panels) and the expression of Eomes and PLZF in CD4 (top middle panels) and CD8 (top bottom panels) SP thymocytes. Representative data from two independent experiments (top panel) and cumulative data (bottom panel) are shown; bars indicate the mean ± SEM. C, WT or PLZF<sup>Lu/Lu</sup> BM cells were mixed with CIITA<sup>tg</sup>TCR<sup>Δα/Δα</sup> BM cells in a 1:1 ratio and transferred into lethally irradiated CIITA<sup>tg</sup>pIV<sup>−/−</sup> hosts (n = 5). Thymocytes were analyzed to assess the expression of PLZF and Eomes in WT or PLZF<sup>Lu/Lu</sup> thymocytes after 12 wk of the transfer. Data are representative of five mice. Numbers in quadrants indicate the percentage of cells in each. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.
Results

The MHC class II-dependent T–T interaction facilitates the development of innate-like CD8+ T cells

In a mouse model system, where MHC class II molecules are present only on T cells to envisage MHC class II-dependent homotypic T–T interaction (CIITA tgpIV−/− mice) (12, 15), we unexpectedly found that the proportions and total numbers of CD8 single-positive (SP) thymocytes increased (CD4/CD8 ratio = 1:2–1:3) compared with the WT control (CD4/CD8 ratio = 2:1–3:1) (12, 13) (Fig. 1A, 1B). This phenomenon was not observed in CD1d−/− mice, in which iNKT cells are nearly absent (Fig. 1A).

The Affymetrix gene expression profile of these CD8+ SP thymocytes revealed a dramatic increase of Eomes transcripts (a 14.4-fold increase), which is normally expressed in NK, gd T, and memory CD8+ T cells (19, 20, 25). Most CD8 SP thymocytes in CIITA tgpIV−/− mice showed an activated/memory phenotype, showing increased levels of CD44, CD62L, CD122, and CXCR3 (Fig. 1C). These cells were distinct from intraepithelial CD8αα+ T cells, because this CD8+ T cell population expresses the CD8αβ heterodimer (data not shown). Splenic CD8+ T cells of CIITA+/+ and CIITA tgpIV−/− mice also showed a similar phenotype (Supplemental Fig. 1). Moreover, when these CD24loCD8 SP thymocytes were stimulated with PMA/ionomycin, Eomes+ CD8 SP thymocytes from both WT and CIITA tgpIV−/− mice immediately produced IFN-γ (Fig. 1D). It is notable that >90% of CD8+ cells were IFN-γ+ Eomes+ in thymi of CIITA+/+ mice, suggesting that these CD8 SP thymocytes belong to a category of innate CD8+ T cells.

Eomes+ CD8+ T cells of CIITA+/+ mice are preferentially committed and developed intrathymically

Next, we investigated whether the increased proportion of CD8 SP thymocytes is due to their enhanced proliferation after the completion of CD8 lineage commitment. The divisional capacity of the CD8 SP thymocytes from CIITA+/+ mice and CIITA tgpIV−/− mice, however, was even decreased compared with WT control mice (Fig. 2A, 2B), suggesting that the increase in CD8 SP thymocytes is not likely caused by the active expansion after the lineage commitment into CD8+ T cells. To further investigate the possibility that the peripheral memory CD8+ T cells in CIITA tgpIV−/− mice preferentially migrate into the thymus, we isolated splenic CD8+ T cells (CD45.2+) from WT and CIITA tgpIV−/− mice, and 1×10⁷ cells from each strain were adoptively transferred into CD45.1+ congenic host mice (Fig. 2C, Supplemental Fig. 2). However, few transferred cells were found within the host thymi, in contrast with the relative abundance in the periphery such as lymph nodes and spleens. Next, to investigate whether CD8 SP thymocytes in CIITA+/+ mice were accumulated in thymus, we evaluated S1P1 expression level, which is known to regulate T cell egress (26). The S1P1 level was not significantly different between CD8+ SP thymocytes from CIITA tgpIV−/− mice and
those from WT mice (Fig. 2D), which supported the assumption that the CD8+ SP thymocytes of CIITA<sup>pIV<sup>−/−</sup></sup> mice were not retained in thymus because of the block of the peripheral migration.

**The development of innate-like CD8+ T cells is dependent on the PLZF expression and IL-4 production of CD4+ T cells in CIITA<sup>pIV<sup>−/−</sup></sup> mice**

Recently, we showed that a substantial number of T-T CD4+ T cells in CIITA<sup>pIV<sup>−/−</sup></sup> mice expressed PLZF, which directs the innate characteristics of these CD4+ T cells as in the case of iNKT cells (15–17). We confirmed that most CD8 SP thymocytes in CIITA<sup>pIV<sup>−/−</sup></sup> mice, but not in B6 WT control and CD1<sup>d<sup>−/−</sup></sup> mice, expressed large amounts of Eomes (Fig. 3A, bottom), and we investigated the functional relation between PLZF<sup>+</sup> CD4+ T cells and Eomes<sup>+</sup> CD8+ T cells. To determine the role of PLZF expression in T-T CD4+ T cells for the development of Eomes<sup>+</sup> CD8+ T cells, we generated CIITA<sup>pIV<sup>−/−</sup></sup> and CIITA<sup>pIV<sup>Lu/Lu</sup></sup> mice in which the PLZF expression was fairly decreased in T-T CD4+ T cells (16). In these mice, the proportion of Eomes<sup>+</sup> CD8 SP thymocytes significantly decreased with the concomitant restoration of the CD4/CD8 ratio to the WT level, indicating that PLZF expression is essential for the generation of innate CD8<sup>+</sup> T cells (Fig. 3B). However, simple PLZF expression in the absence of T-T interaction was not sufficient to generate innate CD8<sup>+</sup> T cells, because Eomes<sup>+</sup> CD8<sup>+</sup> T cells were not enhanced in PLZF<sup>−/−</sup> mice (Fig. 3B). To further substantiate the role of PLZF in the Eomes<sup>+</sup> CD8<sup>+</sup> T cell development, PLZF<sup>Lu/Lu</sup> BM cells were mixed with CIITA<sup>pIV<sup>−/−</sup></sup> TCRC<sup>a<sup>−/−</sup></sup> BM cells and transferred into lethally irradiated CIITA<sup>pIV<sup>−/−</sup></sup> hosts. In these mice, because PLZF<sup>Lu/Lu</sup> thymocytes are to be selected by MHC class II on CIITA<sup>pIV<sup>−/−</sup></sup> TCRC<sup>a<sup>−/−</sup></sup> thymocytes, T-T CD4+ T cells would be generated in the absence of PLZF expression. Indeed, CD8 SP thymocytes drastically reduced Eomes expression despite being developed in the presence of T-T CD4+ T cells (Fig. 3C). Therefore, it is evident that PLZF expression in T-T CD4+ T cell is essential for the induction of Eomes in CD8 SP thymocytes.

We previously reported that T-T CD4+ T cells produce abundant IL-4 in the thymus (14) and spleen, which was accompanied by PLZF expression (15). Also, it was recently found that IL-4 produced by KLF2-deficient CD4<sup>+</sup> T cells regulated Eomes in

![FIGURE 5. Presence of Eomes<sup>+</sup> innate CD8<sup>+</sup> T cells in the human fetus and spleen. A. Analysis of Eomes expression in the fetal thymocytes and the CD8<sup>+</sup> splenocytes of 18-wk human fetus. B. Eomes expression in CD8 SP thymocytes and splenic CD3<sup>+</sup>CD8<sup>+</sup> T cells at the indicated gestation ages and 3 mo after birth. C. Expression of memory markers in Eomes<sup>+</sup> CD8<sup>+</sup> T cells from human fetal spleen (top left panel, n = 2) and human cord blood (middle and bottom left panels, n = 4). Representative data (left panels) and cumulative data (right panel) are shown; bars indicate the mean ± SEM. D. IFN-γ production from Eomes<sup>+</sup> CD8<sup>+</sup> T cells. Total fetal splenocytes or Ficoll purified cord blood mononuclear cells were stimulated with PMA and ionomycin for 5 h and stained with IFN-γ. Representative data from two independent experiments (fetal splenocytes) and four independent experiments (cord blood) are shown. Numbers in quadrants indicate the percentages of cells in each.
bystander CD8 SP thymocytes (27). Based on these results, we speculated that IL-4 is a downstream signaling pathway of PLZF for the induction of Eomes. To investigate IL-4 effect on the development of Eomes+ CD8 SP thymocytes, we generated CIITA<sup>−/−</sup> IL-4<sup>−/−</sup> mice in which the IL-4 expression was abolished in T–T CD4<sup>+</sup> T cells. Interestingly, Eomes expression was barely detectable in these mice (Fig. 4A), and CIITA<sup>−/−</sup>IL-4<sup>−/−</sup> mice showed a 2-fold reduction of Eomes<sup>+</sup> CD8<sup>+</sup> T cells, suggesting a dose-dependent regulation by IL-4. Moreover, IL-4 alone was sufficient for the Eomes induction in CD8 SP thymocytes even in the absence of PLZF, as shown in WT B6 thymi (E15.5) cultured with variable concentrations of IL-4 for 7 d (Fig. 4B, Supplementary Fig. 3). Eomes was upregulated dramatically in CD8 SP thymocytes in the presence of IL-4 in a dose-dependent manner, although CD4 SP thymocytes did not express PLZF (Fig. 4C). The earlier results indicate that IL-4 is necessary and sufficient for the development of Eomes<sup>+</sup> CD8 SP thymocytes independent of PLZF expression.

The presence of CD8<sup>+</sup> T cells with the innate phenotype in the human fetus

Because PLZF-expressing T–T CD4<sup>+</sup> T cells were shown to be present in humans (15), we extended our study to humans to identify whether thymic CD8<sup>+</sup> T cells express Eomes and innate phenotype. At 18 wk of gestation, both double-negative and CD8 SP populations clearly expressed Eomes (Fig. 5A), which were not stained with α-galactosylceramide–loaded CD1d tetramers (data not shown). However, the Eomes<sup>+</sup> CD8<sup>+</sup> T cells were gradually decreased in the course of human fetal thymopoiesis and had almost disappeared 3 mo after birth (Fig. 5B). This pattern correlates well with the expression kinetics of PLZF in CD4 SP thymocytes (15). Fetal splenocytes showed a greater percentages of Eomes<sup>+</sup> CD8<sup>+</sup> T cells (31, 31, and 21% of CD8<sup>+</sup> T cells at 18, 21, and 22 wk of gestational age, respectively) with partial upregulation of CD122 and CD161 (Fig. 5C, top panel), and a substantial fraction of cord blood CD3<sup>+</sup>CD8<sup>+</sup> T cells (1.7–5.4%, n = 4) still expressed Eomes (Fig. 5C, middle panel). Furthermore, most Eomes-expressing CD3<sup>+</sup>CD8<sup>+</sup> T cells were CD45RA<sup>−</sup>CD45RO<sup>+</sup>, and the significant proportion of them expressed CD122 and CD161 (Fig. 5C, bottom panel). These Eomes<sup>+</sup> T cells immediately produced IFN-γ on ex vivo stimulation (Fig. 5D), showing their functional competence.

In contrast with other innate T cells including iNKT cells and MAIT cells (8), the TCR repertoire of the Eomes<sup>+</sup> CD8<sup>+</sup> T cells from both CIITA<sup>−/−</sup>plIV<sup>−/−</sup> mice and humans was as diverse as that of conventional CD8<sup>+</sup> T cells (Fig. 6A). Single-cell suspension of total thymocytes from WT B6 and CIITA<sup>−/−</sup>plIV<sup>−/−</sup> mice were stained with Abs recognizing CD4, CD8, Eomes, and different TCR V<sub>b</sub>-chains (Supplemental Fig. 3), which were not detectable in these mice (Fig. 4A), and CIITA<sup>−/−</sup>IL-4<sup>−/−</sup> mice showed a 2-fold reduction of Eomes<sup>+</sup> CD8<sup>+</sup> T cells, suggesting a dose-dependent regulation by IL-4. Moreover, IL-4 alone was sufficient for the Eomes induction in CD8 SP thymocytes in the presence of IL-4 in a dose-dependent manner, although CD4 SP thymocytes did not express PLZF (Fig. 4C). The earlier results indicate that IL-4 is necessary and sufficient for the development of Eomes<sup>+</sup> CD8 SP thymocytes independent of PLZF expression.

Discussion

This study showed that Eomes<sup>+</sup> innate CD8<sup>+</sup> T cells are intrathymically developed and preferentially committed into innate CD8<sup>+</sup> T cells in the presence of MHC class II-dependent T–T interaction. In this process, IL-4 produced by PLZF<sup>+</sup> T–T CD4<sup>+</sup> T cells and the PLZF expression in T–T CD4<sup>+</sup> T cells are essential for controlling the development of Eomes<sup>+</sup> CD8<sup>+</sup> T cells. Overall, these findings indicate that the MHC class II-dependent T–T interaction has a key role in generating PLZF<sup>+</sup> CD4<sup>+</sup> T cells and subsequently Eomes<sup>+</sup> CD8<sup>+</sup> T cells, both of which take on an innate phenotype.

In CIITA<sup>−/−</sup> and CIITA<sup>−/−</sup>plIV<sup>−/−</sup> mice, a strikingly large population of CD8 SP thymocytes expresses Eomes (85 and 93%, respectively). This proportion is not reduced in CIITA<sup>−/−</sup>CD1d<sup>−/−</sup> mice, indicating that the iNKT cells are not involved in the development of this CD8<sup>+</sup> T cell population. It strongly suggests that the PLZF<sup>+</sup> T–T CD4<sup>+</sup> T cell population generated in mice with CIITA<sup>−/−</sup> background (CIITA<sup>−/−</sup>, CIITA<sup>−/−</sup>plIV<sup>−/−</sup>, and CIITA<sup>−/−</sup>plIV<sup>−/−</sup>) is a key resource of IL-4 in thymus, which directly controls the generation of Eomes<sup>+</sup> CD8<sup>+</sup> T cells as shown in the fetal thymic organ culture (FTOC) with IL-4 (Fig. 4B). The adoptive transfer experiment supports the finding that Eomes<sup>+</sup> T cells are bona fide intrathymic T cells, based on the results that mature CD8<sup>+</sup> T cells have little chance of returning to the thymus and the subsequent expansion. The S1P<sub>1</sub> expression level was not altered in CD8 SP thymocytes from CIITA<sup>−/−</sup>plIV<sup>−/−</sup> mice compared with those from WT mice, suggesting that the increased number of CD8<sup>+</sup> SP thymocytes from CIITA<sup>−/−</sup>plIV<sup>−/−</sup> mice was not due to the defective migration to the periphery. However, the higher expression of CXCR3 on CD8<sup>+</sup> SP thymocytes from CIITA<sup>−/−</sup>plIV<sup>−/−</sup> mice (Fig. 1C) seemed to be against this, because CXCR3 in mice may be associated with accumulation of thymocytes, as in the case of mature iNKT thymocytes (28). Nevertheless, it is conceivable that the Eomes<sup>+</sup> innate CD8<sup>+</sup> T cells are preferentially committed during the late stage of CD8<sup>+</sup> T cell development.

Similar to CD8<sup>+</sup> T cells generated in the I<sub>hk</sub>−/− or I<sub>hk</sub>−/−Rlk<sup>−/−</sup> mice, Eomes<sup>+</sup> CD8<sup>+</sup> T cells in CIITA<sup>−/−</sup> and CIITA<sup>−/−</sup>plIV<sup>−/−</sup> mice showed innate-like characteristics, including the surface expression of CD4<sub>49b</sub>CD122<sup>+</sup> and the rapid production of cytokine. These features are also shared by thymic CD8<sup>+</sup> T cells from the CREB binding protein (CBP)-deficient mice (29), and it was proposed that I<sub>hk</sub>, Rlk, and CBP are likely to regulate TCR signaling pathway to develop innate CD8<sup>+</sup> T cells. However, one article that was published recently demonstrated that the development of innate CD8<sup>+</sup> T cells in I<sub>hk</sub>−/− or I<sub>hk</sub>−/−Rlk<sup>−/−</sup> mice and CBP-deficient mice was attributable to the IL-4–dependent mechanism in common (30). In that report, KL2-deficient mice...
developed an innate-like CD8⁺ T cell population via IL-4 pathway, which was regulated by PLZF⁺ T cells in mice. However, it is not clear whether and how KLF deficiency causes the expansion of the PLZF⁺ population in this model (27, 30).

These recent evidences bear a striking similarity to our current findings on the role of IL-4 for the innate CD8⁺ T cell development; however, the important difference between the earlier models and the CIITA⁰ mice is that the CIITA⁰ condition can mimic human thymic ontogeny. Unlike mouse thymocytes, human thymocytes normally express MHC class II molecules on their surface, and it facilitates the cell interactions between thymocytes via MHC class II to produce PLZF⁺ CD4⁺ T cells. It strongly suggests that PLZF⁺ CD4⁺ T cells selected by MHC class II-dependent T–T interaction would be one of the main PLZF⁺ populations for IL-4 production in human thymus, which drives the development of innate CD8⁺ T cells as shown by our current findings. This possibility is supported by the developmental kinetics of both Eomes⁺ CD8⁺ T cells and PLZF⁺ CD4⁺ T cells, and by the presence of a considerable proportion of PLZF⁺ but CD1d tetramer⁺ CD4⁺ T cells (15) and Eomes⁺ CD8⁺ T cells in human fetuses. Based on these results, we believe that the innate CD8⁺ T cells develop in an early human developmental stage, which is regulated by IL-4 secreted from PLZF⁺ CD4⁺ T cells. This whole process seems to be intrinsically controlled by MHC class II-dependent T–T interaction, which occurs only in the human system.

In addition, the diverse TCRβ usage of these Eomes CD8⁺ T cells, as well as PLZF⁺ CD4⁺ T cells (15), in both the human and the mouse models repeatedly suggests the difference between these populations from the previously established innate T cells, including iNKT and MAIT cells. This result proposes that Eomes⁺ CD8⁺ T cells are likely to be selected by MHC class II molecules, and that this population actively participates in the innate immune response against various pathogens, such as viruses.

Eomes⁺ CD8⁺ T cells, though small in number, are also present in the thymus of WT B6 mice. It has been shown that MHC class Ib-restricted CD8 SP thymocytes are present in K bDb knockout mice (5), and these cells were known to have memory markers in thymus, seemingly innate in their phenotype. Based on this finding, it is assumed that small number of Eomes⁺ CD8⁺ T cells in thymus of WT mice might be identical cells in nature with CD8⁺ T cells produced by iNKT cells regulates the generation of innate-like CD8⁺ T cells. This possibility was verified by the recent report that IL-4 produced by iNKT cells selected by homotypic thymocyte interaction. The effector function of CD8+ T cell developing thymocyte-expressed MHC class II selects a distinct T cell population. Immunity 23: 375–386.

Taken together, although the physiological role of Eomes⁺ CD8⁺ T cells is less clear, one can easily envision that these cells may function as a first line of defense on an infection during the perinatal period of human. Regarding PLZF⁺ T–T CD4⁺ T cells, they might function in at least two distinct aspects. First, they serve as effector CD4⁺ T cells in the periphery by producing Th1 and Th2 cytokines immediately upon an immune response. The other function that was discovered in this study is to facilitate the generation of Eomes⁺ CD8⁺ SP thymocytes in the thymus by producing IL-4. In this sense, these two populations developed via MHC class II-dependent T–T interaction might offer a comprehensive coverage of the innate immune response before the full-scale activation of adaptive immunity, which is more critical in the early stage of human development.

Disclosures

The authors have no financial conflicts of interest.
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