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A Transgenic TCR Directs the Development of IL-4$^+$ and PLZF$^+$ Innate CD4 T Cells

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MHC class II–expressing thymocytes can efficiently mediate positive selection of CD4 T cells in mice. Thymocyte-selected CD4 (T-CD4) T cells have an innate-like phenotype similar to invariant NKT cells. To investigate the development and function of T-CD4 T cells in-depth, we cloned TCR genes from T-CD4 T cells and generated transgenic mice. Remarkably, positive selection of T-CD4 TCR transgenic (T3) thymocytes occurred more efficiently when MHC class II was expressed by thymocytes than by thymic epithelial cells. Similar to polyclonal T-CD4 T cells and also invariant NKT cells, T3 CD4 T cell development is controlled by signaling lymphocyte activation molecule/signaling lymphocyte activation molecule–associated protein signaling, and the cells expressed both IL-4 and promyelocytic leukemia zinc finger (PLZF). Surprisingly, the selected T3 CD4 T cells were heterogeneous in that only half expressed IL-4 and only half expressed PLZF. IL-4– and PLZF-expressing cells were first found at the double-positive cell stage. Thus, the expression of IL-4 and PLZF seems to be determined by an unidentified event that occurs post-selection and is not solely dependent on TCR specificity or the selection process, per se. Taken together, our data show for the first time, to our knowledge, that the TCR specificity regulates but does not determine the development of innate CD4 T cells by thymocytes. *The Journal of Immunology*, 2013, 191: 737–744.

D uring an adaptive immune response, naive T cells go through activation-induced differentiation and then subsequent activation prior to producing effector molecules. In contrast, innate T cells such as invariant NKT (iNKT), mucosal-associated invariant T cells, and intestinal CD8αα intraepithelial lymphocytes release effector cytokines immediately upon stimulation (1–3). In addition to these innate T cells, we have identified CD4 T cells with similar characteristics (4). Unlike conventional CD4 T cells, which are selected by thymic epithelial cells, innate CD4 T cells are selected by MHC class II–expressing thymocytes (5, 6). To differentiate these two CD4 T cell populations, we named them epithelial cell-selected CD4 (E-CD4) and thymocyte-selected CD4 (T-CD4) T cells to reflect the selecting cell type of each. T-CD4 T cells show an effector/memory-like phenotype

and readily produce effector cytokines upon stimulation (4, 7). T-CD4 T cells were shown to inhibit airway inflammation (4) and also suppressed Ag-specific responses of CD8 or CD4 T cells during bacterial infections indicating an immune-suppressive function for T-CD4 T cells (8). Innate T-CD4 T cells are also reported to be present in humans (9).

Development of T-CD4 T cells requires signaling mediated by signaling lymphocyte activation molecule (SLAM)–associated protein (SAP) (7). SLAM is a family of receptors expressed on hematopoietic cells. Homotypic interactions between SLAM receptors expressed by thymocytes are necessary for iNKT cell development (10–12). Promyelocytic leukemia zinc finger (PLZF), a signature transcription factor expressed in iNKT and Vγ1$^+$Vδ6.3/Vδ6.4$^+$ cells (13–16), is also essential for the development of T-CD4 T cells (17). Overexpression of PLZF induces an innate-like phenotype in CD4 T cells (14, 18–20). Although the critical role of both SAP and PLZF for T-CD4 T cell development has been clearly demonstrated (7, 17), the underlying mechanisms as to how these two molecules regulate the developmental process are still unknown. In addition, T-CD4 T cells and iNKT cells share many similarities, but the TCR repertoire of the two T cell populations is different. A diverse TCR repertoire restricted to MHC class II is used by T-CD4 T cells (5), whereas iNKT cells express a limited set of TCRs that recognize the MHC-like molecule CD1d (21).

Previously, we have shown that thymocytes expressing the MHC class II–restricted DO11.10 TCR or the AND TCR were poorly selected by MHC class II expressed by other thymocytes (6). There are at least two possible explanations for poor selection of these TCR transgenic thymocytes. First, the specific MHC class II–peptide complexes required by these E-CD4 T cells might not be presented by thymocytes. Alternatively, the DO11.10 and AND E-CD4 T cells might require signaling delivered only by thymocyte–thymic epithelial cell (TEC) but not by thymocyte–thymocyte interactions. Clearly, these two explanations are not mutually exclusive. Regardless of the underlying mechanisms, we hypothesized that TCRs expressed by T-CD4 T cells would instruct the development of CD4 T cells by thymocytes instead of TEC. To

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test the hypothesis, we generated a new line of TCR^{Tg} mouse with a T-CD4–derived TCR. Remarkably, positive selection of T-CD4 TCR transgenic (T3) thymocytes occurred efficiently when MHC class II was expressed by thymocytes but not when expressed by thymic epithelial cells, which depends on SLAM/SAP signaling. However, only about half of the T3 T-CD4 single-positive (SP) thymocytes expressed IL-4 and PLZF, which are cardinal molecules expressed in innate T-CD4 T cells and iNKT cells. Therefore, TCR specificity plays a critical role for positive selection of T-CD4 T cells on thymocyte-expressed MHC class II, but an additional unknown factor contributes to IL-4 and PLZF expression of the resulting T-CD4 T cells.

Materials and Methods

Mice

CIITA transgenic (CIITA^{Tg}) mice were previously described (22) and were bred to carry both the CD45.1 and CD45.2 congenic markers. Non-CIITA^{Tg} littermates from CIITA^{Tg} heterozygous breeding were used as wild-type (WT) controls. CD45.1$^+$ C57BL/6.SJL mice and the MHC class II Aβ–deficient mice (Aβ$^{-/-}$) on the C57BL/6.SJL background carrying the CD45.1 congenic marker were purchased from Taconic. To generate T3 mice, thymocyte-selected bone marrow transplantation (T-BMT) chimera were constructed with CD45.2$^+$Vβ8.2^{Tg} (23) BM and CD45.1$^+$CD45.2$^+$ CIITA^{Tg} BM coinjected into a CD45.1$^+$Aβ$^{-/-}$ host. Two months after the transfer, CD45.2$^+$Vα2$^+$Vβ8.2$^+$ CD4 T cells were sorted, and cDNA was PCR amplified with a consensus Vα2 forward primer (5′-ATT CTG AAC TGC AGT TAT GAG-3′) that binds all 10 Vα2 family members and a TCR Cα reverse primer (5′-CCG AGG ATC TTT TAA CTG GTA-3′). The resulting PCR product was cloned in the TOPO TA vector (Invitrogen) and individually sequenced. The Vα2.7-Jα20 TCR, which was selected for construction of the transgene, was cloned into the pTris TCRα vector (provided by Dr. D. Mathis, Harvard Medical School, Boston, MA). The Vα2 and the Vβ8.2 transgenes were purified, mixed, and microinjected by Memorial Sloan–Kettering Cancer Center's (MSKCC) Mouse Genetics Core Facility into C57BL/6 fertilized eggs. Two T3 founder lines were characterized and one of them was crossed with TCRα$^{-/-}$ mice (Taconic), 4get (24), or SAP$^{-/-}$ (25) mice. PLZF^{GFP} mice carry a modified bacterial artificial chromosome transgene, expressing eGFP under the control of PLZF regulatory elements. A ~180-kb bacterial artificial chromosome clone (RP23 317 G17) carrying the complete PLZF locus was modified by replacing a 700-bp region at the ATG start site of the PLZF gene with the eGFP coding region, followed by a stop codon. The pLD53 SC-AB vector system (26) (provided by Dr. E. Pamer, MSKCC, New York, NY) was used for the modification. The purified bacterial artificial chromosome transgene was microinjected into fertilized C57BL/6 eggs by MSKCC's Mouse Genetics Core Facility. Three founder mice were identified and analyzed as shown in Supplemental Fig. 2. EGFP expression faithfully reproduced PLZF expression in all three founder lines. One was selected for further experiments. All animal work was done in compliance with the University of Michigan and MSKCC's Internal Animal Care and Use Committee and the guidelines of the Federal Office of Laboratory Animal Welfare. Animals were housed either in the Research Animal Resources Center of Memorial Sloan–Kettering or at the University of Michigan Medical School animal facility under specific pathogen-free conditions. Mice were used at 6–12 wk of age.

BM chimeric mice

For BM transplantation experiments, the recipient mice were lethally irradiated with 960 rad 24 h before receiving BM transfers. BM cells were harvested from the femurs and tibias of donor mice and depleted of mature T cells, B cells, and MHC class II–positive lymphocytes by using a mixture of Abs containing anti-CD4 (RL172), anti-CD8 (TIB210), anti-CD19 (1D3), and anti-MHC class II (M5/114), followed by complement-mediated lysis. BM cells from two different types of donor mice were mixed at the indicated ratio. Each recipient mouse received 6 × 10^6 cells in 400 μl PBS through i.v. injection. In all experiments, CD45 congenic markers were used to distinguish cells derived from three different sources. All BM chimeras were reconstituted for 5–7 wk before analysis of T cell development and function.

Flow cytometry

Cells were preincubated with the anti-FcγR mAb (2.4G2) to block nonspecific Ab binding before they were stained with the following FITC-, PE-, PerCP-, PE-CY7–, allophycocyanin-, allophycocyanin-Cy7–, Pacific blue- or biotin-conjugated Abs: CD3ε (145-2C11), CD4 (L3T4), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD5 (53-7.3), CD62L (MEL-14), CD69 (H1.2F3), CD8α (53-6.7), NK1.1 (PK136), TCRβ (H57-597), Vα2 (B20.1), Vβ8 (F23.1), IFN-γ (XMG1.2), and IL-4 (11B11). Abs were purchased from BD Pharmingen or eBioscience. Allophycocyanin-conjugated α-galactosylceramide analog–loaded CD1d-tetramer was provided by the National Institutes of Health Tetramer Facility. Pacific blue–conjugated anti-mouse PLZF Ab (Mags.21F7) was generated as described previously (16). For PLZF staining, Foxp3 staining buffer set (eBioscience) was used for cell fixation and permeabilization following company's protocol. Events were acquired on a FACSCanto flow cytometer (BD Biosciences), and the data were analyzed with the FlowJo software (Tree Star). Sorting was performed using a FACSAria (BD Biosciences) to a final cell purity of ≥95%.

iNKT cell enrichment

Thymocytes were incubated with 2.5 μl allophycocyanin-conjugated PBS57–loaded CD1d-tetramer for 30 min at 4°C. Cells were then enriched with anti-allophycocyanin Microbeads (Miltenyi Biotec).

CD4 T cell preparation and differentiation

CD4 cells were enriched from single-cell suspensions of splenocytes with mouse CD4 MicroBeads (Miltenyi Biotec). CD4 T cells (1 × 10^6/ml) were stimulated with 5 μg/ml plate-bound anti-CD3ε (145-2C11), 1 μg/ml anti-CD28 (37.51), and 50 U IL-2 (Roche) for 5 d. For Th1 differentiation, 3.5 ng/ml IL-12 and 10 μg/ml anti–IL-4 (11B11) were added.

Cytokine intracellular staining

Fresh CD4$^+$ T cells or differentiated Th1 cells were stimulated with 50 ng/ml PMA and 1.5 μM ionomycin (Calbiochem) for 5 h. Monensin (Sigma-Aldrich) at 3 μM was added during the last 3 h of stimulation. When necessary, activated cells were stained with anti-CD45.1 and anti-CD45.2 Abs. Cells were then fixed in 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.2% saponin (Sigma-Aldrich), followed by staining with anti–IL-4 and anti–IFN-γ for flow cytometry.

Statistic analysis

The two-tailed Student t test was used to analyze the statistic significance of the difference between different groups as indicated. A p value < 0.05 was considered statistically significant.
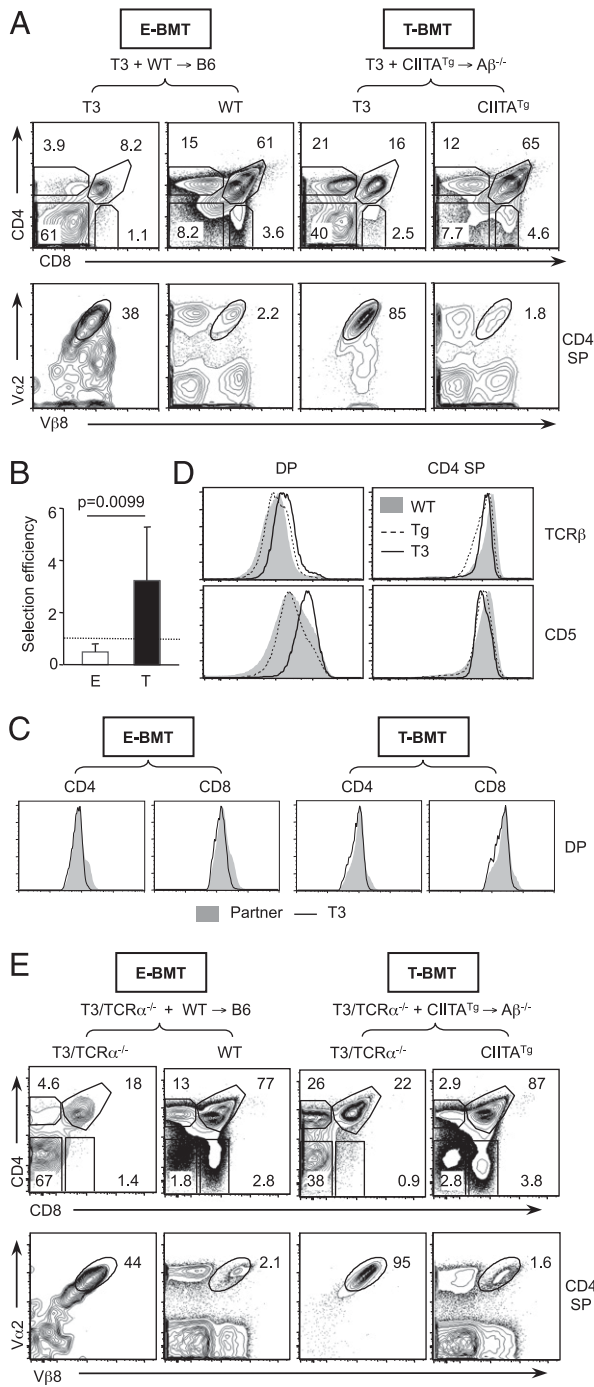
Results

Development of monoclonal T-CD4 T cells by thymocytes

We previously showed that AND and DO11.10 TCR^{Tg} CD4 T cells were not selected by MHC class II–expressing thymocytes (6). Failure of these TCR^{Tg} CD4 T cells to develop via thymocyte–thymocyte interactions suggested that the TCR repertoire expressed by T-CD4 T cells was distinct from E-CD4 T cells. To begin to address this possibility, we cloned TCRs from T-CD4 T cells and used one to generate a new TCR^{Tg} mouse line, as described in *Materials and Methods*. The resulting line, designated T3, expresses a Vα2 and Vβ8.2 TCR.

To direct the development of T3 CD4 T cells by MHC class II–expressing thymocytes only, we constructed mixed BM chimeras. We showed previously that expression of CIITA as a transgene confers MHC class II expression in thymocytes and that thymocytes developed from CIITA^{Tg} BM support CD4 T cell development from cotransferred WT BM in MHC class II–deficient hosts (6). BM cells from T3 mice were cotransferred with WT BM to C57BL/6 (B6) [T3 + WT→B6] or with CIITA^{Tg} BM to Aβ$^{-/-}$ hosts [T3 + CIITA^{Tg}→Aβ$^{-/-}$] to direct selection of T3 cells by TEC or thymocytes, respectively. Two T3 founder lines behaved similarly, and therefore, we show the results from one of them. WT mice were CIITA transgene–negative littermates from CIITA^{Tg} breeding. In all experiments, CD45 congenic markers were used to distinguish cells derived from three different sources.

In [T3 + WT→B6] chimera, called E-BMT (E-CD4 BMT), few T3-derived E-CD4 T cells were present in the thymus, and <50%

of these cells expressed the transgenic TCR (Fig. 1A, *left group*). In contrast, T3 T-CD4 T cells were efficiently generated in [T3 + CIITA^{Tg}→Aβ$^{-/-}$] T-BMT (Fig. 1A, *right group*). The cell number of T3 CD4 SP was greater in T-BMT than in E-BMT (Supplemental Table I, compare 0.46 ± 0.26 and 0.08 ± 0.06 million T3 CD4 T cells in T- and E-BMT mice, respectively). However, the T3-derived cells were not as competitive as CIITA^{Tg} partner donor cells even in T-BMT (Supplemental Table I, compare 0.46 ± 0.26 and 2.4 ± 0.75 million CD4 T cells originated T3 and CIITA^{Tg}, respectively). To quantify the selection efficiency from several BM chimeras, the ratio of [%CD4 SP of T3/%CD4 SP of the partner thymocytes] was calculated. Previously, we have shown that WT and CIITA^{Tg} CD4 T cells were generated with comparable efficiency with the ratio of 1 in [WT + CIITA^{Tg}→Aβ$^{-/-}$] chimeras, but WT CD4 T cells were not present in [WT + WT→Aβ$^{-/-}$] chimeras (6). Therefore, CIITA^{Tg} or WT partner cells served as internal controls for T3 cell selection in the two groups of chimeras. Selection efficiency of T3 CD4 T cells was higher in T-BMT than E-BMT (Fig. 1B), supporting more efficient selection of T3 CD4 T cells by thymocytes than TEC.

Although double-positive (DP) thymocytes originated from T3 BM appeared to express lower CD4 and CD8 than the partner cells, the levels of CD4 and CD8 on T3 BM–driven DP were comparable to partner cells (Fig. 1C). We further examined the phenotype of DP in detail to test the maturity of T3 DP by comparing the expression of TCRβ and CD5 because these two molecules are known to increase as thymocytes are selected and mature (27). T3 DP expressed higher TCRβ than WT or CIITA^{Tg} cells (Fig. 1D, *top left panel*), but because all three types of DP matured to CD4 SP, they expressed comparable levels of TCRβ (Fig. 1D, *top right panel*). CD5 expression was also similar to TCRβ such that CD5 expressed at low levels in T3 DP and then increased in T3 CD4 SP (Fig. 1D, *bottom panels*). On the basis of these observations, T3 DP are likely at the preselection stage. We also observed that double-negative (DN) populations developed from T3 BM were increased in both E- and T-BMT (Fig. 1A, *first* and *third panels* at the top).

T-CD4 T cell development is controlled by SLAM/SAP signaling (7, 11), and therefore, we asked whether same signaling is necessary for T3 T-CD4 T cell development. Indeed, development of T3 T-CD4 T cells relies on SLAM/SAP signaling because the frequency of T3 T-CD4 cells was severely decreased in both the percentages and the cell numbers in the absence of SAP as shown in [T3/SAP$^{-/-}$ + CIITA^{Tg}→Aβ$^{-/-}$] chimeras (Supplemental Fig. 1A, 1B, Supplemental Table I). These data indicate that T3 T-CD4 T cell development is similar to that of polyclonal T-CD4 T cells. However, it is possible that the T3 T-CD4 T cells might have been selected by TCR species composed of an endogenous Vα paired with the transgenic Vβ8.2 and not by the transgene encoded Vα2. To test this, we crossed the T3 mice with TCRα$^{-/-}$ mice and constructed BM chimeras using T3/TCRα$^{-/-}$ BM cells. Consistent with the data from T3 mice, T3/TCRα$^{-/-}$ cells were also selected efficiently by thymocytes as evidenced in [T3/TCRα$^{-/-}$ + CIITA^{Tg}→Aβ$^{-/-}$] T-BMT and almost all CD4 T cells expressed the transgenic TCR (Fig. 1E, *right group*). The CD4 T cell numbers of T3/TCRα$^{-/-}$ (0.51 ± 0.24 million) were also comparable to T3 (0.46 ± 0.26 million) (Supplemental Table I). In contrast, CD8 SP development was very poor (Supplemental Table

FIGURE 1. The selection of T3 cells by thymocytes. (**A**) Representative CD4 and CD8 profiles of total thymocytes derived from different BM donors (*upper panels*). CD4 SP thymocytes were further analyzed for Vα2 and Vβ8 expression (*lower panels*). BM cells from T3 mice (CD45.2$^+$) were cotransferred with WT BM (CD45.1$^+$CD45.2$^+$) into B6.SJL hosts (CD45.1$^+$) mice to generate E-CD4 T cells and cotransferred with CIITA^{Tg} BM (CD45.1$^+$CD45.2$^+$) to Aβ$^{-/-}$ (CD45.1$^+$) mice to generate T-CD4 T cells. Data shown are representatives of six pairs of chimeras. (**B**) More efficient selection of T3 T-CD4 T cells than E-CD4 T cells. Selection efficiency was calculated using a formula [%CD4 SP of T3/%CD4 SP of the partner] as described in the text. The dotted line indicates the ratio of 1. Shown are mean ± SD; *n* = 6. (**C**) DP thymocytes of the T3 or partner donor from the BMT chimera shown in (A) were analyzed for CD4 and CD8 levels. (**D**) DP and CD4 SP thymocytes of the WT, T3, or CIITA^{Tg} (Tg) donor from the BMT chimera shown in (A) were analyzed for TCRβ and CD5 expression. (**E**) CD4 and CD8 populations of total thymocytes (*upper panels*) and Vα2 and Vβ8 profiles of CD4 SP thymocytes

(*lower panels*) from T3/TCRα$^{-/-}$ BM chimeras. Data shown are representatives of three pairs of chimeras. In all of the experiments, T3 or T3/TCRα$^{-/-}$ and the partner BM cells were transferred at 4:1.

I), and only a fraction of the residual CD8 SP expressed T3 TCR. Therefore, the data showed that the transgenic TCR supported efficient selection of T3 T-CD4 T cells on thymocyte-expressed MHC class II.

T3 T-CD4 T cells possess innate effector functions

To examine T3 CD4 T cells in the periphery, CD3$^+$ T cells from total splenocytes were gated, and the presence of T3 CD4 T cells was analyzed. T-BMT reconstituted T3 T-CD4 T cells more efficiently than E-BMT, and most of CD4 T cells in T-BMT expressed the transgenic TCR (Fig. 2A). However, although the main population of the partner CIITA^{Tg} T cells was CD4 T cells, the majority of T3 T-CD4 T cells expressed neither the CD4 nor the CD8 coreceptor (Fig. 2A, *top panels*), as observed in other TCR^{Tg} models of E-CD4 T cells and also iNKT cells (28–31).
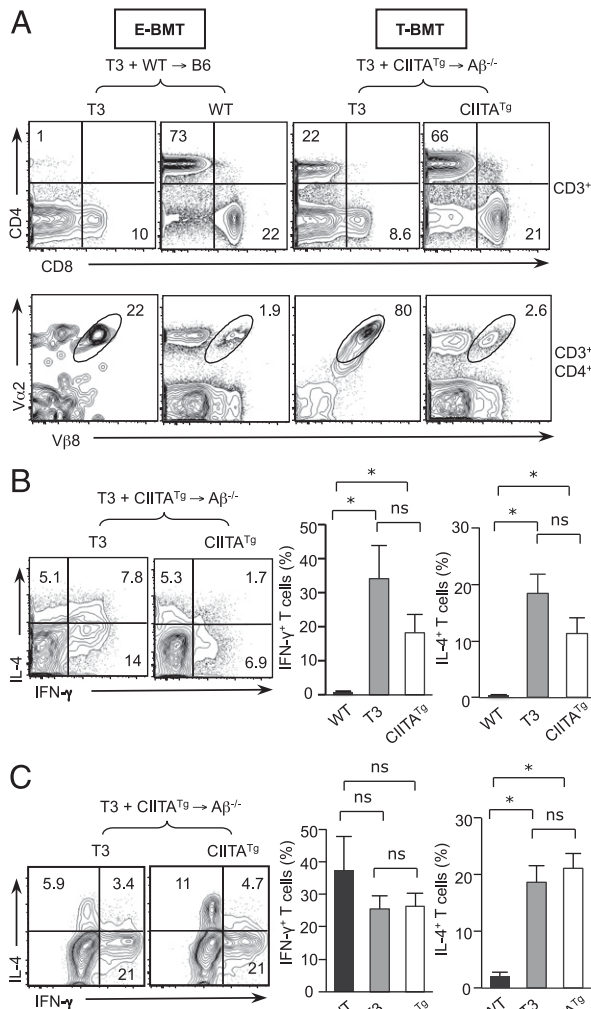
FIGURE 2. The cytokine production pattern of splenic T3 CD4 T cells. (**A**) CD3$^+$ cells from total splenocytes from indicated chimeras were gated and analyzed for CD4 and CD8 profiles (*upper panels*) and Vα2 and Vβ8 expression of CD4$^+$ cells (*lower panels*). (**B** and **C**) Cytokine expression of splenic CD4 T cells after short stimulation (B) or Th1 differentiation (C). Splenic CD4 T cells were enriched as described in *Materials and Methods* and stimulated with PMA and ionomycin for 5 h (B) or cultured under Th1 differentiation conditions for 5 d and restimulated for 5 h with PMA and ionomycin (C). All samples were then subjected to intracellular staining for IFN-γ and IL-4. Bar graphs show the summary of cytokine production upon short stimulation (B) and Th1 differentiation (C). WT indicate WT-derived CD4 T cells from E-BMT. Results are from six pairs of chimeras. *$p < 0.05$.

Next, we evaluated cytokine production by T-CD4 T cells following short stimulation (4). For this purpose, we studied T3 cells from T-BMT but not E-BMT because the numbers of T3 CD4 T cells in E-BMT were too few to examine. Instead, we used WT-derived E-CD4 T cells as a control. When splenic CD4 T cells from T-BMT were enriched and stimulated with PMA and ionomycin for 5 h, we observed three types of cytokine-producing cells that were IL-4$^+$, IFN-γ$^+$, and IL-4$^+$IFN-γ$^+$ cells (Fig. 2B). Total IL-4$^+$ or IFN-γ$^+$ cells from T3 T-CD4 T cells were similar to that from CIITA^{Tg} partner cells. In addition, the frequency of each subpopulation was comparable between T3 T-CD4 and CIITA^{Tg} CD4 T cells (Supplemental Fig. 1C). WT E-CD4 T cells produced little IL-4 or IFN-γ under this condition (see bar graphs in Fig. 2B). Another key feature of T-CD4 T cells is the expression of IL-4 under Th1 differentiation conditions (4). T3 T-CD4 T cells cultured under Th1 differentiation conditions expressed IL-4 as well as IFN-γ, which were similar to the partner cells in total as well as in each subpopulation of cytokine-producing cells (Fig. 2C, Supplemental Fig. 1D). Again, WT CD4 T cells produced predominantly IFN-γ not IL-4 (bar graphs in Fig. 2C). Therefore, T3 T-CD4 T cells are functionally innate effector CD4 T cells.

Heterogeneous expression of IL-4 in T3 T-CD4 T cells

The ability of T-CD4 T cells to express IL-4 upon short stimulation and also under Th1 differentiation conditions is due to the presence of preformed IL-4 mRNA (4, 32). Preformed IL-4 mRNA can be detected using an IL-4 reporter system known as 4get (24). Previously, we have shown that polyclonal T-CD4 T cells express the 4get GFP reporter (32) and anticipated that all CD4 T cells that have undergone thymocyte-mediated selection would be GFP$^+$. Surprisingly, however, we observed a CD4 SP population composed of both GFP$^+$ and GFP$^-$ cells (32). We attributed this observation to potentially different TCR repertoires between the two populations. If so, monoclonal T-CD4 T cells should consist of only GFP$^+$ or only GFP$^-$ cells. To test this, we generated T3/4get mice that cannot express endogenous TCRα-chains (T3/4get/TCRα$^{-/-}$) and used them to construct [T3/4get/TCRα$^{-/-}$ + CIITA^{Tg}/4get→Aβ$^{-/-}$] T-BMT. As a control, [T3/4get + CIITA^{Tg}/4get→Aβ$^{-/-}$] T-BMT chimeras were made. In these chimeras, both donor cells carry the 4get allele, which allowed us to compare the expression of IL-4 in monoclonal T3 versus polyclonal CIITA^{Tg} T-CD4 T cells. As shown in Fig. 3A, Vα2$^+$ thymic CD4 SP cells derived from T3/4get BM cells showed both GFP$^+$ and GFP$^-$ populations. T3/4get BM cells transferred with 4get to B6 hosts did not show an appreciable level of GFP$^+$ CD4 T cells (Supplemental Fig. 1E). T3/4get lacking TCRα-chain also showed the similar GFP expression pattern in T-BMT (Fig. 3A, *right group*). These data show that IL-4 expression is not solely controlled by the TCR specificity.

We next asked whether monoallelic expression of IL-4 is responsible for the segregation of the GFP allele. To test this, 4get mice were crossed with CIITA^{Tg}/4get mice generating 4get$^{+/+}$ and CIITA^{Tg}/4get$^{+/+}$. BM cells from these mice were used to construct BM chimeras and examined GFP expression. As shown in Fig. 3B, [CIITA^{Tg}/4get$^{+/+}$→Aβ$^{-/-}$] chimeras still produced GFP$^+$ and GFP$^-$ CD4 SP thymocytes at an equal ratio. To explore whether the percent chimerism determines the frequency of GFP$^+$ cells in T-CD4 T cells, we transferred 4get and CIITA^{Tg} BM cells at 10:1, 1:1, and 1:10 ratio to Aβ$^{-/-}$ hosts. When 4get BM cells were transferred 10 times more than CIITA^{Tg} BM cells, the percentages of GFP$^+$ cells were much lower than 1:1 ratio (Supplemental Fig. 1F), which is likely due to 10-fold less MHC class II$^+$ thymocytes to select 4get cells resulting in inefficient selection of 4get CD4
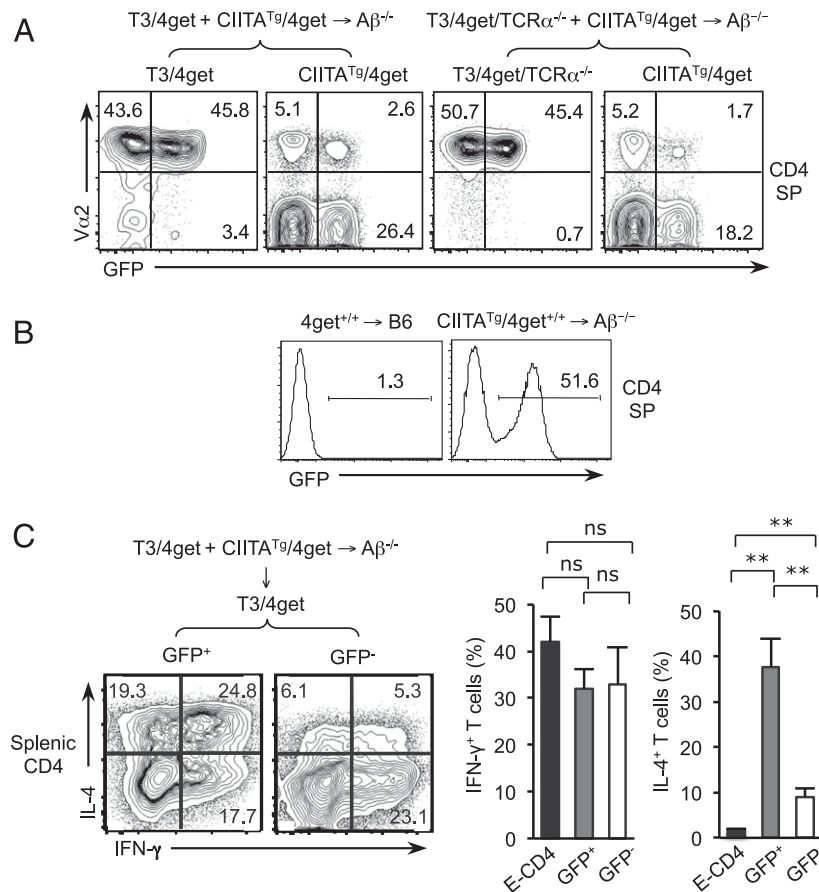
FIGURE 3. Heterogeneous expression of IL-4 mRNA in T3 T-CD4 T cells. (**A**) CD4 SP thymocytes from indicated chimeras were gated and then analyzed for Vα2 and 4get^{GFP} expression. (**B**) 4get^{GFP} expression in T-CD4 T cells from 4get homozygous mice. BM chimeras were constructed as shown, and GFP expression in CD4 SP thymocytes was examined. (**C**) Splenic T3 T-CD4 T cells from [T3/4get + CIITA^{Tg}/4get→Aβ$^{-/-}$] chimeras were sorted into GFP$^+$ and GFP$^-$, cultured under Th1-skewing conditions, and assayed for IFN-γ and IL-4 expression. Results are from three pairs of mice. **$p < 0.01$.

T cells. Introducing 10 times more CIITA^{Tg} BM than 4get BM increased GFP$^+$ cells, suggesting that clonal competition for unidentified factors limits the development of GFP$^+$ cells.

We compared GFP$^+$ and GFP$^-$ T3 T-CD4 T cells to assess their potential to express cytokines with the expectation that GFP$^+$ cells will produce more IL-4 than GFP$^-$ cells because of having preformed IL-4 mRNA. To do this, we sorted GFP$^+$ and GFP$^-$ T3 T-CD4 T cells from [T3/4get + CIITA^{Tg}/4get→Aβ$^{-/-}$] T-BMT, differentiated under Th1 conditions, and assessed IL-4 and IFN-γ expression. In agreement with the presence of IL-4 mRNA, GFP$^+$ cells produced more IL-4 than GFP$^-$ cells under Th1 skewing conditions (Fig. 3C). The increase in IL-4$^+$ cells were from both IL-4$^+$ and IL-4$^+$IFN-γ$^+$ populations (Supplemental Fig. 1G). However, GFP$^+$ and GFP$^-$ cells showed similar levels of IFN-γ–expressing cells, suggesting that GFP$^+$ T3 T-CD4 cells are more potent Th2 cytokine producers than GFP$^-$ T3 T-CD4 cells.

Coordinated expression of IL-4 and PLZF in T3 T-CD4 T cells

Another characteristic of T-CD4 T cells is the expression of PLZF, the key transcription factor that regulates the development and function of iNKT and T-CD4 T cells (13, 14, 33). Because ectopic expression of PLZF can change the phenotype of E-CD4 T cells similar to T-CD4 T cells including the activation of IL-4 (14, 18–20) we asked whether PLZF expression would be different in GFP$^+$ and GFP$^-$ CD4 SP thymocytes. We constructed [T3/4get + CIITA^{Tg}/4get→Aβ$^{-/-}$] chimeras, sorted GFP$^+$ and GFP$^-$ CD4 SP thymocytes from each donor cell type, and compared PLZF expression among the four groups of cells. For CD4 SP thymocytes, two observations were made. First, PLZF$^+$ cells resided mostly in GFP$^+$ and not in GFP$^-$ cells from both T3 and CIITA^{Tg} BM–driven cells (Fig. 4A). Second, when GFP$^+$ cells were compared, a

majority of GFP$^+$ T3 T-CD4 T cells expressed PLZF, whereas GFP$^+$ CIITA^{Tg} cells were composed of PLZF$^+$ and PLZF$^-$ cells at an equivalent ratio (compare the first and the third panel in Fig. 4A and Fig. 4B). Because NKT cells express different levels of IL-4 and PLZF during their development (13, 14), we examined 4get GFP$^+$ and 4get GFP$^-$ iNKT cells for their PLZF expression. We found that, unlike T3 T-CD4 T cells, both GFP$^+$ and GFP$^-$ iNKT cells expressed PLZF albeit at different levels (Fig. 4C). To eliminate the possibility that 4get GFP$^-$ cells express PLZF at a low level, we compared the PLZF expression among WT E-CD4, 4get GFP$^+$, and 4Get GFP$^-$ T3 T-CD4 T cells. The PLZF level of GFP$^-$ T3 cells was very similar to that of WT E-CD4 T cells, indicating that, at most, only a small population of GFP$^-$ T3 T-CD4 T cells expresses PLZF (Fig. 4D).

Coexpression of IL-4 and PLZF in T3 T-CD4 T cells raised the question of whether PLZF confers IL-4 expression in T-CD4 T cells. To investigate this possibility, we used a PLZF^{GFP} reporter mouse strain, described in *Materials and Methods*. GFP expression in PLZF^{GFP} mice faithfully followed the expression of endogenous PLZF protein (Supplemental Fig. 2). T3 mice were crossed with PLZF^{GFP} mice (T3/PLZF^{GFP}) and used to construct BM chimeras. In [T3/PLZF^{GFP} + CIITA^{Tg}→Aβ$^{-/-}$] T-BMT, T3 T-CD4 T cells expressed PLZF in the thymus and also in the spleen evidenced by the GFP signal (Fig. 5A). Interestingly, similar to 4get^{GFP} expression, we also observed PLZF^{GFP}-positive and PLZF^{GFP}-negative T3 T-CD4 T cells at an equivalent ratio in the thymus. We then sorted PLZF^{GFP}-positive and PLZF^{GFP}-negative cells from the spleen of [T3/PLZF^{GFP} + CIITA^{Tg}→Aβ$^{-/-}$] T-BMT followed by differentiation under Th1 conditions. PLZF^{GFP}-positive and -negative cell populations showed two opposing profiles of IL-4 and IFN-γ expression after Th1 differentiation. PLZF^{GFP}-positive CD4 T cells expressed predominantly
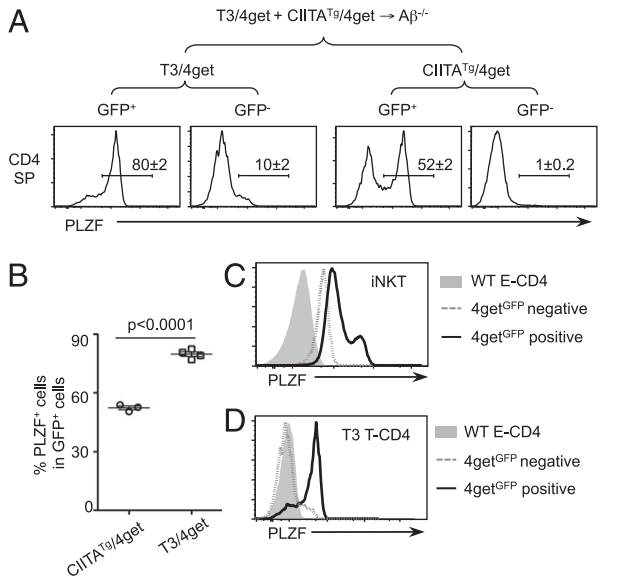
FIGURE 4. Coexpression of PLZF and IL-4 in T3 T-CD4 T cells. (**A**) Thymic CD4 SP thymocytes from [T3/4get + CIITA^{Tg}/4get→Aβ$^{-/-}$] chimeras were sorted into GFP$^+$ and GFP$^-$ populations and analyzed for PLZF expression. (**B**) Summary of PLZF-expressing cells shown in (A). (**C**) Total thymocytes from 4get mice were used to enrich iNKT cells with PBS57-loaded CD1d tetramers and then sorted into tetramer$^+$4get GFP$^+$ and tetramer$^+$4get GFP$^-$ cells. Tetramer$^-$ 4get GFP$^-$ cells from the same mice (labeled as E-CD4) were used as control. All were stained for PLZF. Shown are representatives of three groups of mice. (**D**) PLZF expression of 4get GFP$^+$ and 4get GFP$^-$ T3 cells from [T3/4get + CIITA^{Tg}/4get→Aβ$^{-/-}$] T-BMT were compared with WT E-CD4 T cells from [T3 + WT→B6].

IL-4, whereas IFN-γ$^+$ CD4 T cells were the main population in PLZF^{GFP}-negative cell culture under Th1 differentiation conditions (Fig. 5B). Therefore, PLZF correlates with IL-4 gene expression in T-CD4 T cells.
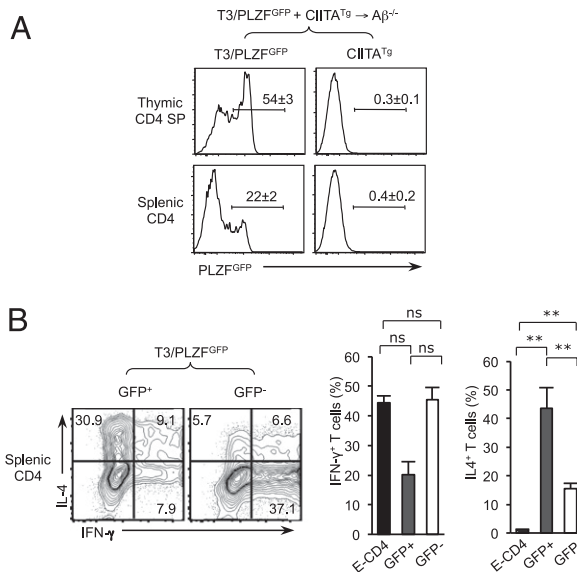

FIGURE 5. PLZF correlates with IL-4 gene expression in T-CD4 T cells. (**A**) Expression of the PLZF^{GFP} reporter in CD4 SP thymocytes (*upper panels*) and splenic CD4 T cells (*lower panels*) from [T3/PLZF^{GFP} + CIITA^{Tg}→Aβ$^{-/-}$] BM chimera. (**B**) Splenic T3 T-CD4 T cells from [T3/PLZF^{GFP} + CIITA^{Tg}→Aβ$^{-/-}$] chimeras as in (A) were sorted into PLZF^{GFP}-positive and PLZF^{GFP}-negative cells. Sorted cells were then differentiated under Th1 conditions followed by IFN-γ and IL-4 intracellular staining. Data shown are representatives of results obtained from three pairs of mice. ****p < 0.01.

IL-4 and PLZF expression starts in double positive thymocytes after selection

To gain further insight into the acquisition of effector functions during the development of T3 T-CD4 T cells, we examined 4get GFP$^+$ cells (active transcription of the IL-4 locus) for coreceptor expression using [T3/4get + CIITA^{Tg}/4get→Aβ$^{-/-}$] chimeras. GFP$^+$ cells from each donor-driven thymocytes were gated, and then, CD4 and CD8 expression was analyzed. As shown in Fig. 6A, the majority of GFP$^+$ cells from both BM donors were CD4 SP thymocytes. However, DP as well as DN cells were also found to express GFP (Fig. 6A). Consistent with the data that thymocytes developed from T3 BM cells have a large population of DN, T3/4get cells had more GFP$^+$ DN than the partner CIITA^{Tg}/4get. When we examined the phenotype of DP thymocytes, the GFP$^+$ DP thymocytes from CIITA^{Tg}/4get BM cells expressed higher levels of TCRβ, CD5, and CD69 as compared with GFP$^-$ DP cells, strongly suggesting that GFP$^+$ DP thymocytes had undergone positive selection (Fig. 6B, *right panels*). In contrast, T3/4get BM–derived DP thymocytes expressed high levels of TCRβ and CD5 regardless of GFP expression (Fig. 6B, *left panels*), which can be attributed to their early expression of TCRα. The expression level of CD69 was higher in GFP$^+$ cells than GFP$^-$ T3/4get DP cells (Fig. 6B, *left panels*). We also examined DN thymocytes and found that relative levels of TCRβ expressed on GFP$^+$ versus GFP$^-$ DN thymocytes from T3/4get or CIITA^{Tg}/4get donor were similar to that of DP thymocytes (Fig. 6B, *bottom panels*).

We then investigated whether PLZF expression also starts at the same stage. Total thymocytes from [T3 + CIITA^{Tg}→Aβ$^{-/-}$] T-BMT were stained for PLZF, and then, CD4 and CD8 expressions of PLZF$^+$ cells were examined. Similar to IL-4 expression, PLZF$^+$ cells were present in CD4 SP primarily and also DP
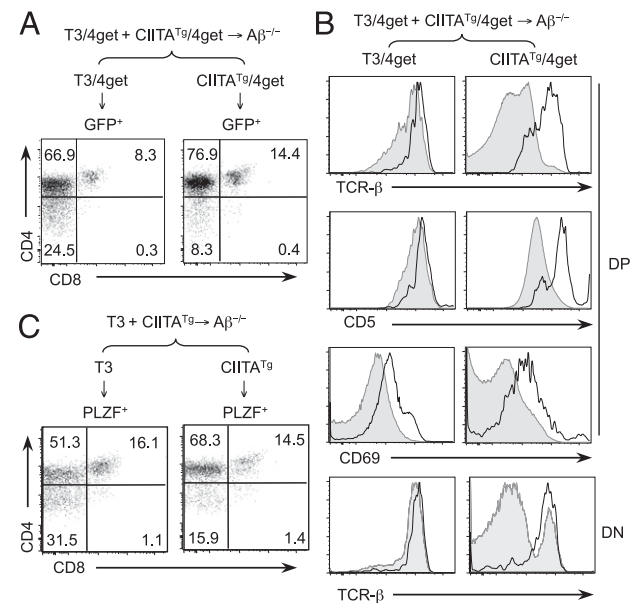
FIGURE 6. Expression of IL-4 and PLZF during thymic development. (**A**) IL-4 gene expression in different subsets of thymocytes. GFP$^+$ thymocytes originated from T3/4get or CIITA^{Tg}/4get BM from [T3/4get + CIITA^{Tg}/4get→Aβ$^{-/-}$] mice were gated and then analyzed for CD4 and CD8 expression. (**B**) Expression of TCRβ, CD5, and CD69 on GFP$^+$ (open line) and GFP$^-$ (shaded) thymocytes from cells indicated at the top. (**C**) CD4 and CD8 expression of PLZF$^+$ thymocytes. [T3 + CIITA^{Tg}→Aβ$^{-/-}$] mice were constructed, and total thymocytes were stained for PLZF. PLZF$^+$ cells from each donor-driven cell were gated and analyzed for CD4 and CD8 expression. Data shown are representatives of results from three pairs of mice.

thymocytes, indicating that both monoclonal and polyclonal T-CD4 T cells gain the effector function immediately after selection (Fig. 6C).

Discussion

The T3 model clearly shows that, similar to iNKT cells, T-CD4 T cell selection is supported by thymocytes more efficiently than by TEC and that the T3 TCR plays a key role for the fate of selection. Unlike invariant usage of TCR by iNKT cells (34), however, the TCR repertoire of T-CD4 T cells is diverse, suggesting that the TCR specificity is not directly involved in choosing E- versus T-CD4 T cell selection pathway. It is possible that the T3 TCR recognizes a MHC class II–peptide complex expressed on thymocytes that is not available on TEC. Although CIITA is known to induce gene expression of molecules necessary for Ag processing and presentation by MHC class II (35), it is possible that CIITA expression in thymocytes may not completely mimic Ag presentation by TECs resulting in a different pool of peptides for MHC class II in thymocytes. This possibility is supported by a report that MHC class II–expressing human CD4 T cells present not only peptides derived from the same pool of self-protein as B cells but also their own unique peptides (36). It is equally possible that T3 T-CD4 T cell development requires signaling that can only be delivered by thymocyte–thymocyte interaction during selection. For example, iNKT cells rely upon SLAM receptor engagement for full development (10–12). Indeed, this current study along with our previous report demonstrated that signals provided by the SLAM/SAP pathway are critical for T-CD4 T cell development (7). It is also tempting to speculate that other receptor-mediated signaling via thymocyte–thymocyte interactions also contributes to the generation of innate T-CD4 T cells.

T3 DP cells expressed higher TCRβ and CD5 than the partner cells but lower than CD4 SP, suggesting that they have not undergone selection. We also observed that T3 TCR$^+$ DN populations were increased in the thymus and also in the periphery of T3 T-BMT. Similar increases in DN populations were reported in NKT TCR transgenic models expressing Vα14-Jα18 TCR (29), Vβ9/Vα3.2 TCR (30), and human Vα24-JαQ TCR (31). In addition, "conventional" TCR transgenic models also showed more DN cells than nontransgenic mice, although the increase was less dramatic (28, 37). These observations suggest that forced expression of the transgenic TCR at the early stage of development might have contributed to increased DN populations. In addition, NKT TCR transgenic models showed the presence of functionally mature NKT cells in the DN population (29). Similarly, ~10% of total T3 DN cells in T-BMT looked to be functionally mature T cells as evidenced by IL-4 mRNA expression (Fig. 6A, Supplemental Fig. 3A). Mature iNKT cells in WT mice are composed of CD4$^+$ and CD4$^-$ cells. Although the developmental relationship between CD4$^+$ and CD4$^-$ NKT cells is poorly understood, CD4$^-$ iNKT cells seem to be derived from CD4$^+$ NKT cells because CD4$^-$ NKT cells appear to develop later than CD4$^+$ NKT cells (38). Therefore, IL-4$^+$ or PLZF$^+$ DN T3 cells might have originated from CD4$^+$ T3 cells.

Although T3 CD4 T cells developed efficiently by thymocytes in T-BMT mice, T3 mice crossed with CIITA^{Tg} mice (T3/CIITA^{Tg}) in which MHC class II is expressed in thymocytes showed the similar frequency of CD4 T cells to CIITA^{Tg}-negative mice (Supplemental Fig. 3B). It is peculiar that T3 cells were present in T3 mice in which MHC class II–expressing thymocytes should be absent. We suspect that these T3 cell might have been selected by other MHC class II–expressing hematopoietic cells. In T3/CIITA^{Tg} mice, more MHC class II–expressing hematopoietic cells are available, and therefore, more transgenic Vα2- and Vβ8.2-positive CD4 SP

thymocytes were generated than T3 mice (Supplemental Fig. 3C). The data also suggest that when both TEC- and thymocyte-mediated pathways are available such as in T3/CIITA^{Tg} mice, selection by TEC is more efficient than by thymocytes. It is not clear what controls the choice of the pathway for a given thymocyte and the selection efficiency of each pathway. Nevertheless, we suspect that thymocytes selected by thymocytes are more susceptible to negative selection than by TEC because of strong TCR signaling received during selection processes. Our recent study using a Nur77^{GFP} reporter showed that, similar to iNKT cells, T-CD4 T cells receive stronger TCR signaling than E-CD4 T cells during selection and decreased TCR signaling strength enhanced T-CD4 T cell development (39). Moreover, the T3 TCR may represent the high end of the spectrum of the TCR signaling strength among T-CD4 TCRs. This is supported by the data showing that T3 thymocytes expressed higher levels of TCRβ and CD5 than polyclonal CIITA^{Tg} thymocytes at the DP stage (Fig. 1D). Therefore, we envision that T3 cells are selected by thymocytes in T3/CIITA^{Tg} mice, but a majority of those selected cells die, resulting in poor development of T3 T-CD4 T cells. This constraint is relieved in the T-BMT environment allowing T3 cells to develop efficiently. Even in T-BMT, however, because T3 DP thymocytes receive stronger TCR signaling than the partner CIITA^{Tg} DP thymocytes, T3 thymocytes are less competitive to reconstitute the CD4 T cell compartment than the partner polyclonal cells.

Interestingly, we found that only half of T-CD4 cells constitutively transcribed the IL-4 gene when BM cells were transferred at 1:1 ratio. We ruled out the possibility that monoallelic expression of IL-4 is responsible for the generation of heterogeneous population. Furthermore, unlike iNKT cells that express PLZF at different levels depending on their developmental stages (13, 14), only half of the T3 T-CD4 T cells expressed PLZF regardless of the endogenous TCRα status (Fig. 5A, Supplemental Fig. 3D). Because a similar pattern was observed with polyclonal T-CD4 T cells and with the monoclonal T3 expressing T cells, it is highly unlikely that TCR specificity dictates this substantial difference. Therefore, the different pattern of IL-4 or PLZF expression of T-CD4 T cells does not seem to be associated with the selection process itself. Instead, clonal competition for unidentified factors seems to limit the development of IL-4$^+$ T-CD4 T cells. Similar developmental constraint has been reported for the generation of Foxp3$^+$ regulatory T cells (40, 41), which suggests a potential role of TCR for survival of regulatory T and T-CD4 T cells, both of which have strong TCR signaling for the development (39, 42).

Although IL-4 and PLZF expression coincided in T3 CD4 T cells, expression of the two was not fully coordinated in polyclonal T-CD4 T cells. T3 CD4 T cells can produce both IL-4 and IFN-γ upon short stimulation. However, mostly IL-4$^+$ or PLZF$^+$, but not IL-4$^-$ or PLZF$^-$, cells produced IL-4 under Th1 differentiation, showing that at least two subpopulations of T-CD4 T cells bearing distinct functions may develop. Moreover, T3 cells exhibit different patterns of cytokine production potential depending on the criteria. 4get GFP$^+$ (IL-4 mRNA expressing) cells from T3/4get T-BMT generated a mixture of three different types of effector cells (IL-4$^+$, IFN-γ$^+$, and IL-4$^+$/IFN-γ$^+$) under Th1 differentiation conditions (Fig. 3C). In contrast, PLZF expression distinguished effector cells into IL-4 or IFN-γ–expressing cells under the same conditions (Fig. 5B). Therefore, PLZF shows a strong correlation with IL-4 expression.

IL-4 transcript can be expressed in the presence or absence of PLZF when thymocytes are selected by other thymocytes. However, neither IL-4 nor PLZF expression is induced in CD4 T cells selected by TEC. Taken together, thymocyte–thymocyte interaction delivers signaling necessary for the induction of IL-4 and

PLZF, but the underlying regulatory mechanisms of the gene expression are distinct. Recently, we showed that T-CD4 T cells play an immunosuppressive role during bacterial infections (8). Undoubtedly, further studies and characterizations of the subpopulations of T-CD4 T cells and signaling requirements for the activation of IL-4 and PLZF are warranted to gain a full understanding of their development and function during immune responses.

Acknowledgments

Disclosures

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