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

Linqiao Zhu,* Yu Qiao,* Esther S. Choi,* Joy Das,† Derek B. Sant’Angelo,‡ and Cheong-Hee Chang*

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.

The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6; BM, bone marrow; BMT, bone marrow transplantation; DN, double-negative; DP, double-positive; E-BMT, epithelial cell–selected CD4 bone marrow transplantation; E-CD4, epithelial cell–selected CD4; iNKT, invariant NKT; MSKCC, Memorial Sloan-Kettering Cancer Center; PLZF, promyelocytic leukemia zinc finger; SAP, signaling lymphocyte activation molecule–associated protein; SLAM, signaling lymphocyte activation molecule; SP, single-positive; T3, T-CD4 TCR transgenic; T-BMT, thymocyte-selected bone marrow transplantation; T-CD4, thymocyte-selected CD4; TEC, thymic epithelial cell; WT, wild-type.

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test the hypothesis, we generated a new line of TCR^T^ 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^T^) mice were previously described (22) and were bred to carry both the CD45.1 and CD45.2 congenic markers. Non-CIITA^T^ littermates from CIITA^T^ heterozygous breeding were used as wild-type (WT) controls. CD4^T^/V^a^ CD45.1^+^ C57BL/6 SJL mice and the MHC class II ^A^β–deficient mice (^A^β/−^) on the C57BL/6.J 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^V^β^8^2^+^ (23) BM and CD45.1^CD45.2^ CIITA^T^ BM coinjected into a CD45.1^A^β^+^ host. Two months after the transfer, CD45.2^V^β^2^V^β^8^2^ CD4 T cells were sorted, and cDNA was PCR amplified with a consensus V^β^2 forward primer (5′-AGT CGG ATG AGT TAC 3′) that binds all 10 V^β^2 family members and an additional unknown factor contributes to IL-4 and PLZF expression of the resulting T-CD4 T cells.

**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: CD3e (145-2C11), CD4 (L3T4), CD44 (IM7), CD45 (A20), CD45.2 (104), CD5 (53-7.3), CD2L2 (MEL-14), CD69 (H1.2F3), CD8a (53-6.7), NK1.1 (PK136), TCRβ (H57-597), Vε2 (B20.1), Vβ8 (F23.1), IFN-γ (XM81.2), 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 FACS Canto flow cytometer (BD Biosciences), and the data were analyzed with the FlowJo software (Tree Star). Sorting was performed using a FACS Aria (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 × 10^5/ml) were stimulated with 5 μg/ml plate-bound anti-CD3e (145-2C11), 1 μg/ml anti-CD28 (37.51), and 50 U/ml 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 monomycin (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.

**Statistical 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^T^ CD4 T cells were not selected by MHC class II–expressing thymocytes (6). Failure of these TCR^T^ 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^T^ mouse line, as described in Materials and Methods. The resulting line, designated T3, expresses a Vβ2 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^T^ 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^T^ BM to A^β^/−^ hosts [T3 + CIITA^T^→A^β^/−^] 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^T^ 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<sup>G</sup>→<sup>B</sup>]<sup>−/−</sup> T-BMT (Fig. 1A, right group). The cell number of T3 CD4 SP was greater in T-BMT than in E-BMT (Supplementary 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<sup>G</sup> partner donor cells even in T-BMT (Supplementary Table I, compare 0.46 ± 0.26 and 2.4 ± 0.75 million CD4 T cells originated T3 and CIITA<sup>G</sup>, 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<sup>G</sup> CD4 T cells were generated with comparable efficiency with the ratio of 1 in [WT + CIITA<sup>G</sup>→<sup>B</sup>]<sup>−/−</sup> chimeras, but WT CD4 T cells were not present in [WT + WT→<sup>B</sup>]<sup>−/−</sup> chimeras (6). Therefore, CIITA<sup>G</sup> 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<sup>G</sup> cells (Fig. 1D, top left panel), but because all three types of DP matured to CD4 SP, they expressed comparable levels of CD5β (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<sup>−/−</sup> + CIITA<sup>G</sup>→<sup>B</sup>]<sup>−/−</sup> chimeras (Supplementary Fig. 1A, 1B, Supplementary 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 V<sub>α</sub> paired with the transgenic V<sub>β</sub>8.2 and not by the transgene encoded V<sub>α</sub>. To test this, we crossed the T3 mice with TCRα<sup>−/−</sup> mice and constructed BM chimeras using T3/TCRα<sup>−/−</sup> BM cells. Consistent with the data from T3 mice, T3/TCRα<sup>−/−</sup> cells were also selected efficiently by thymocytes as evidenced in [T3/TCRα<sup>−/−</sup> + CIITA<sup>G</sup>→<sup>B</sup>]<sup>−/−</sup> T-BMT and almost all CD4 T cells expressed the transgenic TCR (Fig. 1E, right group). The CD4 T cell numbers of T3/TCRα<sup>−/−</sup> (0.51 ± 0.24 million) were also comparable to T3 (0.46 ± 0.26 million) (Supplementary Table I). In contrast, CD8 SP development was very poor (Supplementary Table I, compare 0.24 million for T3/TCRα<sup>−/−</sup> BM chimeras and 0.08 million for T3 BM chimeras). This difference is probably due to the competition of T3 T-CD8 T cells and the partner BM cells in T-BMT (Fig. 1F, first panels at the bottom). This result also indicates that T3 T-CD8 T cell development is TEC dependent as shown in previous studies (4, 11).
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 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<sup>Tg</sup> 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<sup>Tg</sup> models of E-CD4 T cells and also iNKT cells (28–31).

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<sup>+</sup>, IFN-γ<sup>+</sup>, and IL-4/IFN-γ<sup>+</sup> cells (Fig. 2B). Total IL-4<sup>+</sup> or IFN-γ<sup>+</sup> cells from T3 T-CD4 T cells were similar to that from CIITA<sup>Tg</sup> partner cells. In addition, the frequency of each subpopulation was comparable between T3 T-CD4 and CIITA<sup>Tg</sup> 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<sup>+</sup>. Surprisingly, however, we observed a CD4 SP population composed of both GFP<sup>+</sup> and GFP<sup>+</sup> 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<sup>+</sup> or only GFP<sup>+</sup> T cells. To test this, we generated T3/4get mice that cannot express endogenous TCRα-chains (T3/4get/TCRα<sup>−/−</sup>) and used them to construct [T3/4get/TCRα<sup>−/−</sup> + CIITA<sup>Tg</sup>/4get→A<sup>B<sub>2</sub></sup>/A<sup>α<sub>2</sub></sup>] T-BMT. As a control, [T3/4get + CIITA<sup>Tg</sup>/4get→A<sup>B<sub>2</sub></sup>/A<sup>α<sub>2</sub></sup>] 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<sup>Tg</sup> T-CD4 T cells. As shown in Fig. 3A, Vα2<sup>+</sup> thymic CD4 SP cells derived from T3/4get BM cells showed both GFP<sup>+</sup> and GFP<sup>+</sup> populations. T3/4get BM cells transferred with 4get to B6 hosts did not show a appreciable level of GFP<sup>+</sup> 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 monoaallelic expression of IL-4 is responsible for the segregation of the GFP allele. To test this, 4get mice were crossed with CIITA<sup>Tg</sup>/4get mice generating 4get<sup>+</sup> and CIITA<sup>Tg</sup>/4get<sup>+</sup> BM cells from these mice were used to construct BM chimeras and examined GFP expression. As shown in Fig. 3B, [CIITA<sup>Tg</sup>/4get→A<sup>B<sub>2</sub></sup>/A<sup>α<sub>2</sub></sup>] chimeras still produced GFP<sup>+</sup> and GFP<sup>+</sup> CD4 SP CD4 T cells. These data show that IL-4 expression is not solely controlled by the TCR specificity.
T cells. Introducing 10 times more CIITA<sup>Tg</sup> BM than 4get BM increased GFP<sup>+</sup> cells, suggesting that clonal competition for unidentified factors limits the development of GFP<sup>+</sup> cells.

We compared GFP<sup>+</sup> and GFP<sup>−</sup> T3 T-CD4 T cells to assess their potential to express cytokines with the expectation that GFP<sup>+</sup> cells will produce more IL-4 than GFP<sup>−</sup> cells because of having preformed IL-4 mRNA. To do this, we sorted GFP<sup>+</sup> and GFP<sup>−</sup> T3 T-CD4 T cells from [T3/4get + CIITA<sup>Tg</sup>/4get→Ab<sup>−/−</sup>] T-BMT, differentiated under Th1 conditions, and assessed IL-4 and IFN-γ expression. In agreement with the presence of IL-4 mRNA, GFP<sup>+</sup> cells produced more IL-4 than GFP<sup>−</sup> cells under Th1 skewing conditions (Fig. 3C). The increase in IL-4<sup>+</sup> cells was from both IL-4<sup>+</sup> and IL-4<sup>+</sup>IFN-γ<sup>+</sup> populations (Supplemental Fig. 1G). However, GFP<sup>+</sup> and GFP<sup>−</sup> cells showed similar levels of IFN-γ-expressing cells, suggesting that GFP<sup>+</sup> T3 T-CD4 cells are more potent Th2 cytokine producers than GFP<sup>−</sup> 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 similar to 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<sup>Tg</sup>/4get→Ab<sup>−/−</sup>] chimeras were sorted into GFP<sup>+</sup> and GFP<sup>−</sup>, cultured under Th1-skewing conditions, and assayed for IFN-γ and IL-4 expression. Results are from three pairs of mice. **p < 0.01.

![FIGURE 3. Heterogeneous expression of IL-4 mRNA in T3 T-CD4 T cells.](http://www.jimmunol.org/)
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<sup>Tg</sup>/4get→<sup>−</sup>A<sup>−</sup>−/−] 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<sup>Tg</sup>/4get. When we examined the phenotype of DP thymocytes, the GFP+ DP thymocytes from CIITA<sup>Tg</sup>/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<sup>Tg</sup>/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<sup>Tg</sup>/4get→<sup>−</sup>A<sup>−</sup>−/−] T-BMT were stained for PLZF, and then, CD4 and CD8 expressions of PLZF<sup>+</sup> cells were examined. Similar to IL-4 expression, PLZF<sup>+</sup> cells were present in CD4 SP primarily and also DP

**FIGURE 4.** Coexpression of PLZF and IL-4 in T3 T-CD4 T cells. (A) Thymic CD4 SP thymocytes from [T3/4get + CIITA<sup>Tg</sup>/4get→<sup>−</sup>A<sup>−</sup>−/−] 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<sup>+</sup>4get GFP<sup>+</sup> and tetramer<sup>−</sup>4get GFP<sup>−</sup> cells. Tetramer 4get GFP<sup>−</sup> cells from the same mice (labeled as E-CD4) were used as control. All were stained for PLZF. Shown are representative of three groups of mice. **(D) PLZF expression of 4get GFP<sup>+</sup> and 4get GFP<sup>−</sup> T3 cells from [T3/4get + CIITA<sup>Tg</sup>/4get→<sup>−</sup>A<sup>−</sup>−/−] T-BMT were compared with WT E-CD4 T cells from [T3 + WT→B6].

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

**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<sup>Tg</sup>/4get BM from [T3/4get + CIITA<sup>Tg</sup>/4get→<sup>−</sup>A<sup>−</sup>−/−] 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<sup>Tg</sup>→<sup>−</sup>A<sup>−</sup>−/−] 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 TCRαβ 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), Vβ8/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<sup>tg</sup> mice (T3/CIITA<sup>tg</sup>) in which MHC class II is expressed in thymocytes showed the similar frequency of CD4 T cells to CIITA<sup>tg</sup>-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<sup>tg</sup> 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<sup>tg</sup> 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<sup>GFP</sup> 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<sup>tg</sup> thymocytes at the DP stage (Fig. 1D). Therefore, we envision that T3 cells are selected by thymocytes in T3/CIITA<sup>tg</sup> 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<sup>tg</sup> 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 T cells constitutively transcribed the IL-4 gene when BM cells were transferred at 1:1 ratio. We ruled out the possibility that monopolar 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<sup>+</sup> 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<sup>+</sup> or PLZF<sup>+</sup>, but not IL-4<sup>+</sup> or PLZF<sup>+</sup>, 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<sup>+</sup> (IL-4 mRNA expressing) cells from T3/4get T-BMT generated a mixture of three different types of effector cells (IL-4<sup>+</sup>, IFN-γ<sup>+</sup>, and IL-4<sup>+</sup>/IFN-γ<sup>+</sup>) under Th1 differentiation conditions (Fig. 3C). In contrast, PLZF expression distinguished effector cells into IL-4<sup>+</sup> or IFN-γ<sup>+</sup>-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
**TCR-SPECIFIC DEVELOPMENT OF INNATE CD4 T CELLS**

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). Unquestionably, 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.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


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14. Min, H. S., J. Y. Lee, Y. J. 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 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). Unquestionably, 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.

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Supplemental Figure Legends

Supplemental Figure 1. Characterization of T3 cells.

(A, B) Development of SAP⁻/⁻ T3 T-CD4 T cells. Thymocytes from T3/SAP⁻/⁻ T-BMT chimera were analyzed for CD4 and CD8 profile (A) and T3 TCR expression on CD4 SP. Shown are representatives of 3 mice. (C, D) IFN-γ⁺, IFN-γ⁺IL-4⁺ and IL-4⁺ subpopulations were compared between T3 (solid) and CIITA⁺ Tg (open) derived T-CD4 T cells after short-stimulation (C) and Th1 differentiation (D). (E) Poor GFP⁺ cell generation in E-BMT. CD4 SP thymocytes from the indicated donor from [T3/4get +4get →B6] E-BMT were analyzed for GFP expression. It should be noted that T3/4get cell development was poor in E-BMT. (F) Generation of GFP⁺ cells in mixed BM chimeras. Different ratios of BM cells from 4get and CIITA Tg mice as indicated at the top were co-transferred and GFP⁺ cells were compared. (G) IFN-γ⁺, IFN-γ⁺IL-4⁺ and IL-4⁺ subpopulations were compared between 4get⁺ (solid) and 4get⁻ (open) T3 T-CD4 T cells under Th1 differentiation.

Supplemental Figure 2. Development and analysis of a PLZF GFP reporter mouse line.

(A) Schematic of the BAC transgene showing where eGFP was inserted. (B) iNKT cells were present at normal numbers in PLZF GFP mice (right panel) as compared to transgene negative littermates (left panel). (C) iNKT cells from PLZF GFP mice expressed eGFP (green line) as compared to iNKT cells from a wild type littermate (black line). (D) Splenic GFP⁺ (green) and GFP⁻ T cells (blue)
were sorted from the PLZF\textsuperscript{GFP} reporter mice and then stained for PLZF. T Cells from the PLZF\textsuperscript{-/-} mice (red) were served as a negative control. (E) PLZF\textsuperscript{GFP} mimics PLZF expression in iNKT cells. Different stages of thymic iNKT cells were identified using CD44 and NK1.1 (left panel) and then GFP expression of the cells was compared (right panel). Consistent with published data, immature stage 1 iNKT cells (blue) have the highest levels, stage 2 cells (green) down-regulate GFP and stage 3 (orange) have the lowest levels.

**Supplemental Figure 3.** Characterizing of T3 BM derived DN cells, T3/TCR\textalpha{^-/-} T-CD4 SP cells and thymocytes from T3 or T3/CIIA\textsuperscript{Tg} mice. (A) DN cells from both donors of the [T3/4get + CIIA\textsuperscript{Tg}/4get\rightarrow A\beta\textsuperscript{-/-}] T-BMT mice were compared for 4getGFP expression together with V\textalpha{2}. (B) Total thymocytes of T3 and T3/CIIA\textsuperscript{Tg} were analyzed for CD4 and CD8. Each subset of thymocytes was analyzed for V\textalpha{2} and V\textbeta{8} expression. (C) Summary of T3 TCR expressing CD4 SP thymocytes in (B). (D) CD4 SP thymocytes from [T3/TCR\textalpha{^-/-} + CIIA\textsuperscript{Tg}\rightarrow A\beta\textsuperscript{-/-}] chimeras were analyzed for PLZF expression. All data are from 3 mice separately.
## Supplemental Table I. Generation of T3 CD4 T cells in BM chimeric mice

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The numbers (x10<sup>6</sup>) shown are mean±SD.
Supplemental Figure 3, Zhu et al

A

T3/4get + CIITA\textsuperscript{Tg}/4get \rightarrow A\beta^{-/-}

T3/4get  

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D

T3/TCR\alpha^{-/-} + CIITA\textsuperscript{Tg} \rightarrow A\beta^{-/-}

T3/TCR\alpha^{-/-} CIITA\textsuperscript{Tg}

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