

Generation of PLZF⁺ CD4⁺ T cells via MHC class II–dependent thymocyte–thymocyte interaction is a physiological process in humans

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Human thymocytes, unlike mouse thymocytes, express major histocompatibility complex (MHC) class II molecules on their surface, especially during the fetal and perinatal stages. Based on this observation, we previously identified a novel developmental pathway for the generation of CD4⁺ T cells via interactions between MHC class II–expressing thymocytes (thymocyte–thymocyte [T–T] interactions) with a transgenic mouse system. However, the developmental dissection of this T–T interaction in humans has not been possible because of the lack of known cellular molecules specific for T–T CD4⁺ T cells. We show that promyelocytic leukemia zinc finger protein (PLZF) is a useful marker for the identification of T–T CD4⁺ T cells. With this analysis, we determined that a substantial number of fetal thymocytes and splenocytes express PLZF and acquire innate characteristics during their development in humans. Although these characteristics are quite similar to invariant NKT (iNKT) cells, they clearly differ from iNKT cells in that they have a diverse T cell receptor repertoire and are restricted by MHC class II molecules. These findings define a novel human CD4⁺ T cell subset that develops via an MHC class II–dependent T–T interaction.

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Abbreviations used: α GalCer, α -galactosylceramide; DP, double positive; hu-mice, humanized mice; GA, gestational age; iNKT, invariant NKT; MAIT, mucosal-associated invariant T; NOG, NOD.SCID/ γ c^{-/-}; pIV, CIITA type IV promoter; PLZF, promyelocytic leukemia zinc finger protein; SLAM, signaling lymphocytic activation molecule; SP, single positive; T–E, thymocyte–epithelial cell; T–T, thymocyte–thymocyte.

Human thymocytes, unlike mouse thymocytes, express a substantial amount of MHC class II molecules on their surface, especially during the fetal and perinatal stages (Park et al., 1992). On the basis of this observation, we hypothesized that a subset of human CD4⁺ T cells develops via a homotypic thymocyte–thymocyte (T–T) interaction and performs unique functional roles in the periphery. In 1997, this hypothesis was first evidenced in an in vitro reaggregate thymic organ culture system in which mature human CD4⁺ T cells were efficiently generated only when MHC class II molecules were expressed on immature thymocytes (Choi et al., 1997). This was again confirmed in an in vivo system using mice that were transgenic for pIck-CIITA on a CIITA-deficient background; in these mice, MHC class II is only present on T cells (Choi et al., 2005; Li et al., 2005). Using

these mice, we have demonstrated that T–T interactions are as efficient as thymocyte–epithelial cell (T–E) interactions in the generation of functionally competent CD4⁺ T cells based on alloreactivity and diverse TCR V β usage. More recently, work from another laboratory has demonstrated that T–T interaction–derived CD4⁺ T cells share several functional properties with invariant NKT (iNKT) cells, including a rapid response upon antigen encounter and the secretion of several cytokines (Li et al., 2007b). The generation of CD4⁺ T cells via a T–T interaction is also dependent on the signaling lymphocytic activation molecule (SLAM)–associated protein signaling pathway (Li et al., 2007a; Veillette et al., 2007). However, T–T

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interaction-generated CD4⁺ T cells (T–T CD4⁺ T cells) are distinct from iNKT cells in that they express a diverse TCR repertoire and are restricted by polymorphic MHC class II molecules (Lee et al., 2009).

Recently, promyelocytic leukemia zinc finger protein (PLZF; encoded by *zbtb16*) was identified as a transcription factor necessary for the development of iNKT cells that is essential to direct the innate characteristics of iNKT cells (Kovalovsky et al., 2008; Savage et al., 2008). In addition, we have also demonstrated that in the thymus of plck-CIITA^{tg} mice with a CIITA type IV promoter (pIV)–null background (CIITA^{tg}pIV^{-/-} mice), a significant number of T–T CD4⁺ T cells express PLZF (Lee et al., 2009). Therefore, the expression of PLZF can be used as a marker to identify T–T CD4⁺ T cells as well as iNKT cells. However, the existence of CD4⁺ T cells that developed through an MHC class II–dependent T–T interaction in humans had been remained unknown.

In this study, we show that fetal human thymocytes and splenocytes isolated from second-trimester fetuses express PLZF and that PLZF⁺ CD4⁺ T cells acquire innate characteristics during their development. This finding defines a novel human CD4⁺ T cell subset that develops via an MHC class II–dependent T–T interaction.

RESULTS

PLZF⁺ T–T CD4⁺ T cells in mice

By backcrossing plck-CIITA^{tg} mice into a pIV–null background, we have generated CIITA^{tg}pIV^{-/-} mice in which thymocytes are positively selected solely by T–T interactions in the thymic cortex and undergo a physiological negative selection process in the medulla (Fig. S1; Waldburger et al., 2003; Lee et al., 2009). In these mice, PLZF was expressed in a significant proportion of CD4 single-positive (SP) thymocytes and splenic CD4⁺ T cells (Fig. 1 A and Fig. S2 A). Compared with WT mice, most PLZF⁺ cells in CIITA^{tg} and CIITA^{tg}pIV^{-/-} mice were negative for CD1d/α-galactosylceramide (αGalCer) tetramer staining, indicating that they are not iNKT cells. They are also unlikely to be type II NKT cells, as PLZF⁺ T–T CD4⁺ T cells normally develop under CD1d knockout conditions (Fig. 1 A). Collectively, these results demonstrate that the PLZF⁺ CD4⁺ T cells are generated via MHC class II–dependent T–T interactions.

We next asked whether PLZF directs T–T CD4⁺ T cells to have innate properties, similar to those of iNKT cells. The expression of memory markers and the ability of mature T cells to rapidly secrete IL-4 and/or IFN-γ after TCR stimulation are well-known hallmarks of innate T cells (Berg, 2007). In the thymus of CIITA^{tg} and CIITA^{tg}pIV^{-/-} mice, the majority of T–T CD4⁺ T cells expressed a high level of PLZF, but the expression level of this molecule was decreased in splenic CD4⁺ T cells (Fig. 1 A and Fig. S2 A). As was the case in iNKT cells, NK1.1 was expressed on a substantial number of CD4⁺ T cells, and they expressed a relatively low level of PLZF in the thymus and spleen. The PLZF^{lo} CD4⁺ T cells uniformly acquired the memory phenotype (CD-44^{hi}CD62L^{lo}CD122^{hi}; Fig. 1 B) and preferentially migrated

into the liver. However, the hepatic migration was not as dramatic as that of iNKT cells, only showing ~1.5 times that of spleen (Fig. S2, B and C). In addition, upon TCR stimulation, PLZF⁺ T–T CD4⁺ T cells produced both IFN-γ and IL-4, similar to iNKT cells (Fig. 1 C). Most of the cells secreting these two cytokines were not iNKT cells, as they did not bind to CD1d/αGalCer tetramers (Fig. S2 D). These findings suggest that PLZF⁺ T–T CD4⁺ T cells and iNKT cells follow a common maturation process and share innate phenotypes, which might be driven by PLZF expression.

It is intriguing that PLZF is expressed only on a fraction of the population of mouse T–T CD4⁺ T cells (Fig. 1, A and B), whereas most of the iNKT cells in humans and mice universally express PLZF (Kovalovsky et al., 2008; Savage et al., 2008). To elucidate the environmental factors required for PLZF expression in T–T CD4⁺ T cells, we originally generated a mixed BM chimera in a 1:1 ratio that eventually resulted in a 1:3 ratio (CIITA^{tg}/OT-II), based on the comparative ratio of B cells derived from CIITA^{tg} and OT-II BM (Fig. S3 D). In this system, OT-II T cells develop only through the T–T interaction. In contrast to the polyclonal T cells in this chimera, the OT-II T cells expressed neither PLZF nor memory markers, suggesting that TCR interaction with endogenous cognate antigen is essential for PLZF expression (Fig. 1 D and Fig. S3).

T–T interaction in humanized mice (hu–mice)

To determine whether the MHC class II–dependent T–T interaction is a physiological event that normally occurs in rats and humans, where thymocytes express MHC class II molecules, we developed two kinds of chimeric mouse model systems. First, Rag^{-/-}γc^{-/-}MHCII^{-/-} mice were lethally irradiated and reconstituted with rat BM–derived stem cells. Despite the relatively low level of MHC class II expression of double-positive (DP) thymocytes in the WT rat (Fig. S4), the reconstituted rat thymocytes allowed the positive selection of rat T cells only via T–T interaction, because of the complete lack of T–E interaction in this chimera. 8 wk after the graft, we analyzed the developmental profile of rat CD4⁺ T cells from the spleen and thymus (Fig. 2 A, top; and Fig. S5 A, top). As expected, CD3⁺CD4⁺ T cells developed efficiently in Rag^{-/-}γc^{-/-}MHCII^{-/-} mice (4.7%; Fig. 2 A), indicating that CD4⁺ T cell development via T–T interaction normally occurs. When we analyzed the expression of PLZF, 24% of the developmental intermediates (CD4⁺CD8^{int} stage) from the Rag^{-/-}γc^{-/-}MHCII^{-/-} recipient mice showed up-regulated expression of PLZF, whereas expression was barely detected in the WT rat thymus (Fig. 2 A, bottom; and Fig. S5 A, bottom).

Similarly, we also developed hu–mice via transplantation of human cord blood CD34⁺ stem cells into irradiated NOD.SCID/γc^{-/-} (NOG) mice. In this system, it has been reported that developing human T cells have undergone selection only on human MHC (Traggiai et al., 2004). 14 wk after transplantation, when the human CD4⁺ T cells became fully mature in the periphery, expression of PLZF was evident in

the CD4⁺CD8^{int} population of human thymocytes (Fig. 2 B, top; and Fig. S5 B, top). These cells expressed a high level of CD3 and did not bind to the CD1d/αGalCer tetramer, which indicate that they are mature CD4⁺ T cells (Fig. S5, C and D). As in the spleen, the percentage of PLZF⁺ CD4⁺ cells also reduced in the liver (Fig. S5 E). To confirm that the expression of PLZF depends entirely on the T–T interaction, we transplanted a rat or fetal human thymus under the kidney

capsule of mice (Fig. 2 A, middle; and Fig. 2 B, bottom). With this procedure, it was possible to compare the PLZF⁺ population in the host thymus, in which only the T–T interaction can drive T cell development, with that in the grafted thymus, in which both the T–T and T–E interactions can drive T cell development. Although both host and grafted fetal thymi displayed almost identical developmental profiles in terms of the differentiation status of CD4⁺ and CD8⁺ T cells, the

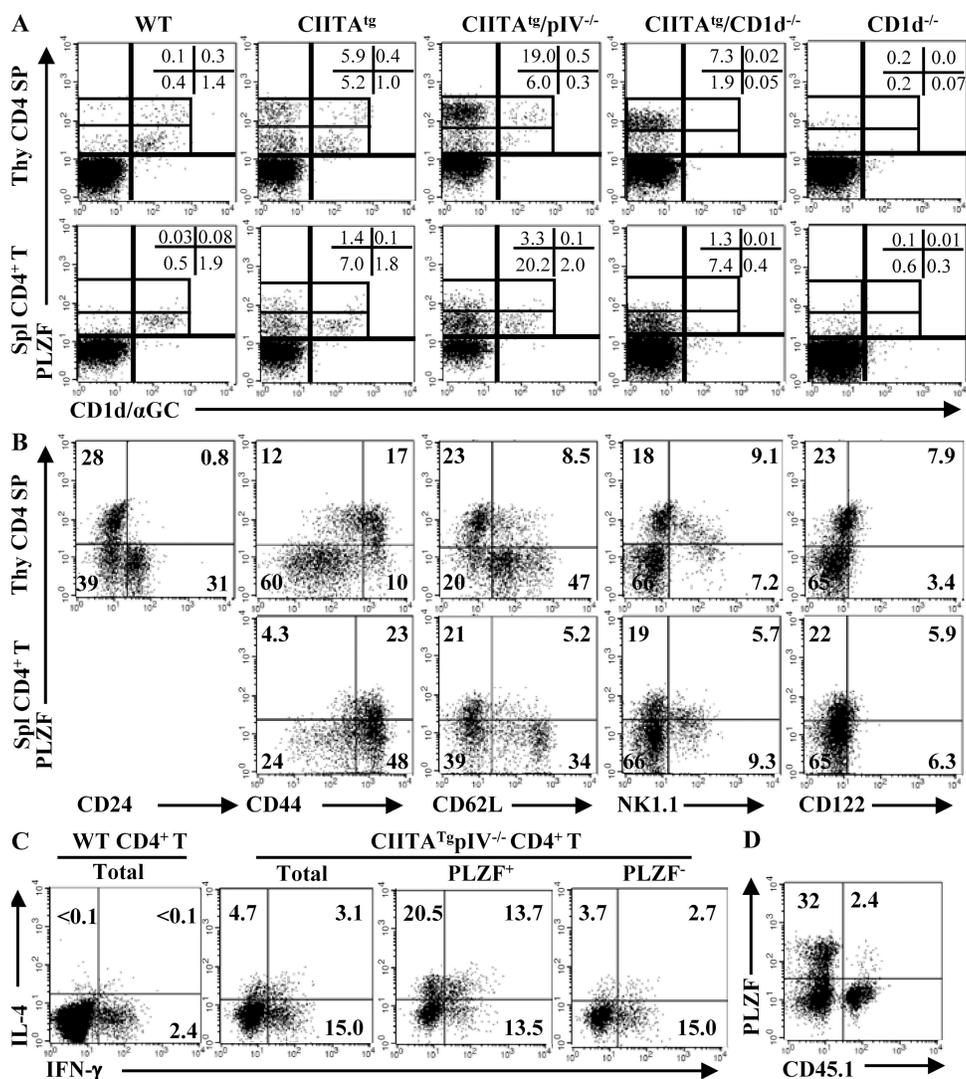


Figure 1. PLZF expression and acquisition of innate properties in mouse CD4⁺ T cells is dependent on MHC class II–dependent T–T interactions. (A) Flow cytometry of thymocytes and splenocytes from WT, CIITA^{tg}, CIITA^{tg}/pIV^{-/-}, CIITA^{tg}/CD1d^{-/-}, and CD1d^{-/-} mice assessing PLZF expression in permeabilized CD1d/αGalCer tetramer–positive (Tetr⁺) and –negative (Tetr⁻) populations. The numbers indicate the percentage of each PLZF⁺ subset (Tetr⁻PLZF^{hi}, Tetr⁻PLZF^{lo}, Tetr⁺PLZF^{hi}, and Tetr⁺PLZF^{lo}) among total cells. Representative data from two independent experiments are shown. (B) A representative profile of CD24, CD44, CD62L, NK1.1, and CD122 expression versus PLZF expression in gated CD4 SP thymocytes and splenic CD4⁺ T cells from CIITA^{tg}/pIV^{-/-} mice. PLZF^{hi} and PLZF^{lo} populations could be identified. The numbers indicate the percentage of cells in each quadrant. Representative data from six independent experiments are shown. (C) Intracellular flow cytometry for IL-4 and IFN-γ in WT and CIITA^{tg}/pIV^{-/-} mice. Splenic CD4⁺ T cells from each mouse were activated with PMA and ionomycin for 5 h and assessed for their cytokine secretion. The numbers in the dot plots indicate the percentage of cells in each quadrant. Representative data from three independent experiments are shown. (D) Flow cytometry of thymocytes to assess PLZF expression in OT-II TCR transgenic thymocytes after CD45.1/OT-II and CD45.2/CIITA^{tg} BM cells were mixed and transferred into lethally irradiated pIV^{-/-} hosts. The dot plot shows PLZF expression in CD4 SP thymocytes 7 wk after transfer. The numbers indicate the percentage of PLZF⁺ cells among the CD45.1⁺ (OT-II) and CD45.1⁻ (WT) CD4 SP thymocytes. Spl, spleen; Thy, thymus.

frequency of PLZF⁺ cells was drastically reduced in the grafted thymi (Fig. 2 A, middle; and Fig. 2 B, bottom), indicating that PLZF is expressed only in response to T–T interactions.

PLZF expression in CD4⁺ T cells of the human fetus

PLZF was known to be expressed exclusively in iNKT cells and mucosal-associated invariant T (MAIT) cells among adult human T cells (Savage et al., 2008). We extended the functional and anatomical analysis of PLZF expression to the fetal

and neonatal period. Overall, at 16 wk of gestation, 8.1% of CD4 SP thymocytes expressed PLZF (Fig. 3 A), and by 26 wk of gestation, the expression gradually decreased to 1.4% and was undetectable in neonatal thymocytes (Fig. 3, A and B). These cells were CD3 positive (Fig. S6 A), and in fetal spleens, PLZF expression was seen in ~8–15% of splenic CD4⁺ cells (Fig. 3, A and B). These T–T CD4⁺ T cells expressed CD161, the human equivalent of mouse NK1.1 (Fig. 3 C), similar to MAIT (Martin et al., 2009) and iNKT cells (Exley et al., 1998).

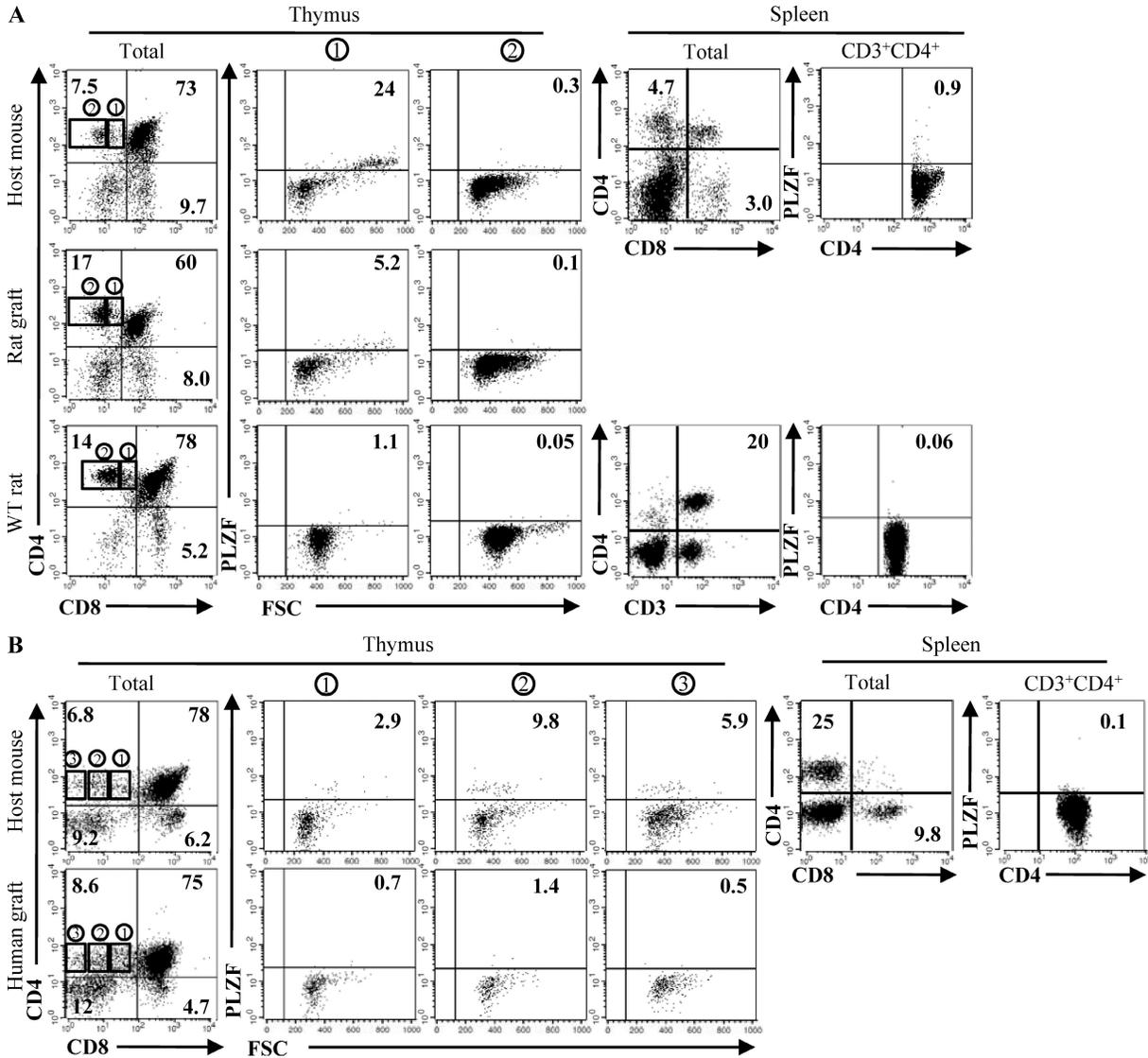


Figure 2. Rat and human thymocytes drive MHC class II-dependent T–T interaction and PLZF expression in a chimeric mouse system.

(A) Generation of PLZF⁺ CD4⁺ T cells in a rat→mouse BM chimera. Rat BM stem cells were transplanted into irradiated RAG-1^{-/-}γc^{-/-}MHCII^{-/-} mice with (middle) or without (top) fetal rat thymus graft. 8 wk after transplant, the recipient mouse thymus, the grafted rat thymus, and the recipient spleen were harvested, and PLZF expression in each subset of rat T cells was compared with that of WT rats. CD4 SP thymocytes were subdivided into two stages based on CD8 expression, as indicated. The numbers indicate the percentage of cells in each quadrant. Representative FACS data of two mice from two independent experiments are shown. The forward scatter (FSC) value is displayed as a linear scale. (B) T cell development and PLZF expression in CD3⁺ cord blood-reconstituted NOG mice with (bottom) or without a human fetal thymus graft (top). 14 wk after the transfer of cord blood cells, PLZF expression in human T cells was analyzed in the recipient mouse thymus and spleen as well as the grafted human thymus. CD4 SP thymocytes were subdivided into three populations based on CD8 expression, as indicated. The numbers indicate the percentage of cells in each quadrant. Representative FACS data from three independent experiments are shown.

Furthermore, upon stimulation with PMA and ionomycin, PLZF⁺ cells that produce IFN- γ were 14-fold greater in proportion than PLZF⁻ cells (Fig. 3 D). However, hardly any of the PLZF⁺ CD4⁺ T cells bound CD1d/ α GalCer tetramers (Fig. 3 E), and their frequency was from 42- to 290-fold higher than that of iNKT cells (Fig. S6 B).

TCR diversity of PLZF⁺ CD4⁺ T cells

Adaptive T cells are armed with a diverse repertoire of T cell receptors, whereas PLZF⁺ innate T cells, such as iNKT and MAIT cells, use a canonical V α chain and a limited number of V β chains (Treiner and Lantz, 2006). As PLZF⁺ T-T CD4⁺ T cells are restricted by self-peptide-MHC class II complexes

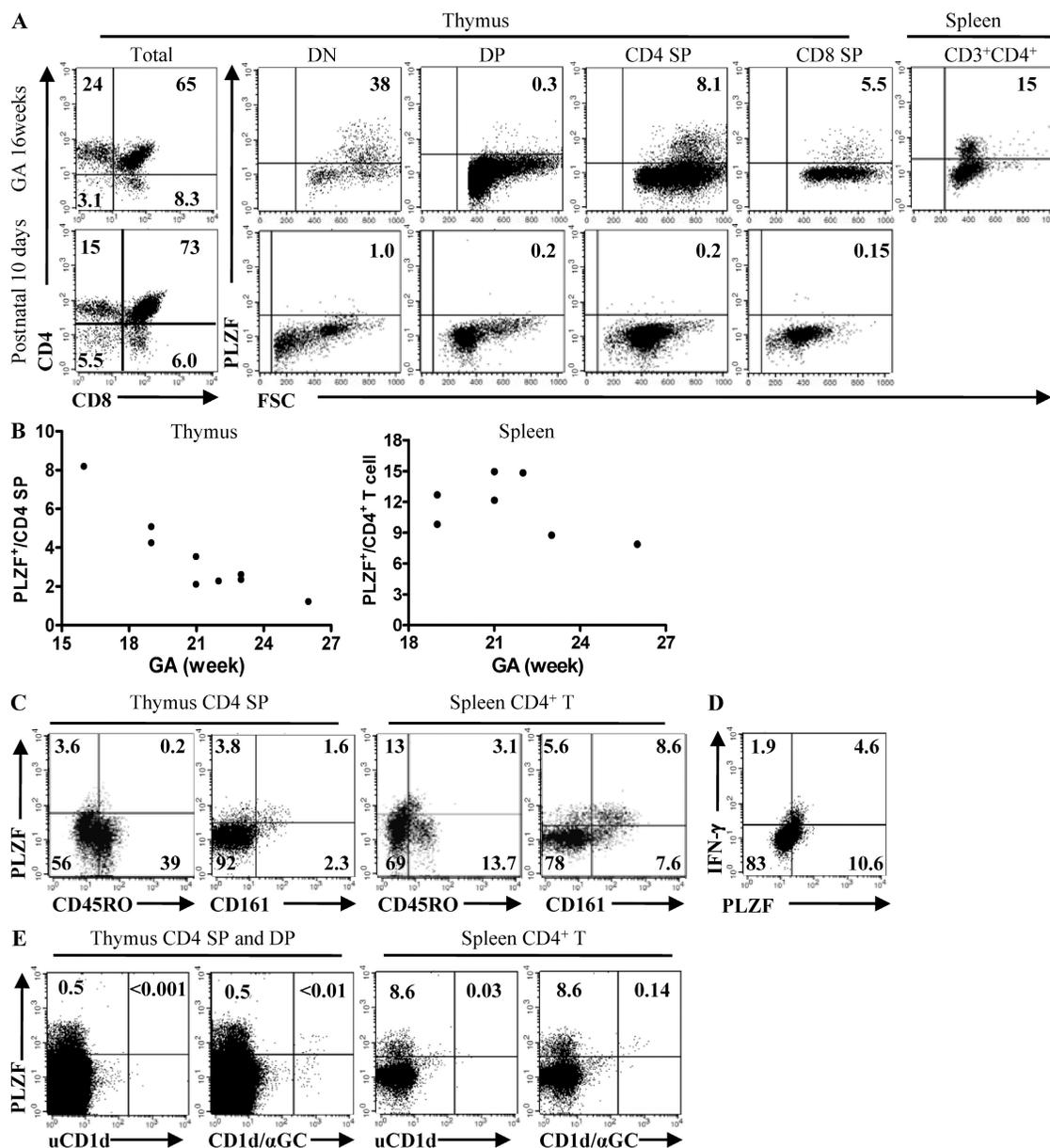


Figure 3. PLZF is expressed in a subset of fetal human CD4⁺ T cells that have properties similar to mouse T-T CD4⁺ T cells during the second trimester of gestation. (A) Representative expression profile of PLZF in the fetal thymus (GA = 16 wk) and spleen (GA = 23 wk), as well as the neonatal (day 10) thymus. The numbers in each quadrant indicate the percentage of cells present. The forward scatter (FSC) value is displayed as a linear scale. (B) Summary of PLZF⁺ cell frequency in the fetal human thymus and spleen. The PLZF⁺ populations in CD4 SP thymocytes and splenic CD4⁺ T cells were enumerated by flow cytometry at a GA of 16–26 wk. (C) CD45RO and CD161 expression on PLZF⁺ and PLZF⁻ CD4 SP thymocytes or splenic CD4⁺ T cells from human fetuses at a GA of 19 wk. (D) Intracellular flow cytometry of IFN- γ and PLZF in human fetal CD4⁺ T cells. MACS-enriched CD4⁺ T cells from human fetal splenocytes were activated with PMA and ionomycin for 5 h. The representative data of two independent experiments. (E) Flow cytometric assessment of the PLZF⁺ non-iNKT population in human fetal thymocytes (CD4 SP and DP gated) and CD4⁺ splenocytes. The iNKT cells were identified using CD1d/ α GalCer tetramers after comparison with cells stained with unloaded CD1d (uCD1d) tetramer. The numbers indicate the percentage of cells in each quadrant. DN, double negative.

in mice, we hypothesized that the TCR V β repertoire of PLZF⁺ T cells in humans would be wider than that of iNKT (V β 11) or MAIT (V β 2 and V β 13) cells (Treiner and Lantz, 2006). To confirm this, we analyzed their TCR V β chain usage. Based on their general diversity and frequency of individual V β chains, PLZF⁺ T–T CD4⁺ T cells and conventional CD4⁺ T cells isolated from the thymus or spleen of mice showed a similar TCR repertoire (Fig. 4 A). Furthermore, the diversity of TCR V β usage in human PLZF⁺ CD4⁺ T cells isolated from lymphoid tissue mimicked the V β usage of mouse T–T CD4⁺ T cells (Fig. 4 B). These data suggest that the development of PLZF⁺ CD4⁺ T cells in humans is similar to the development of T–T CD4⁺ T cells in mice.

DISCUSSION

We have previously identified a novel developmental pathway for the generation of CD4⁺ T cells via interactions between MHC class II–expressing thymocytes in a transgenic mouse system (Choi et al., 2005). Although T–T interactions have been suggested to be a physiological mechanism for the development of functional T cells in mice and humans (He and Kappes, 2006; Ladi et al., 2006; Martinic et al., 2006; Manz, 2007; Schwartzberg et al., 2009), the developmental dissection of this T–T interaction has not been possible because of the lack of known cellular molecules specific for T–T CD4⁺ T cells. Recently, PLZF had been shown to be a lineage–specific marker for iNKT cells, and work from our

laboratory has demonstrated that PLZF is also expressed in a subset of T–T CD4⁺ T cells (Lee et al., 2009). In this study, we demonstrated that PLZF can be used as a marker for cells originating from the T–T interaction in mice, and a substantial number of T–T CD4⁺ T cells were found in the fetal human thymus and spleen. Similar to their mouse counterparts, human PLZF⁺ CD4⁺ T cells had both innate immune properties and a diverse TCR V β usage. These data indicate that the MHC class II–dependent T–T interaction does exist and is involved in the generation of a unique subset of CD4⁺ T cells during fetal human lymphopoiesis.

For a detailed analysis, we developed a series of mouse model systems in which most of the CD4⁺ T cells were selected by engagement of their TCR with the MHC class II–peptide complex on thymocytes. In the first model, *plck–CIITA^{tg}* mice were backcrossed onto mice lacking *pIV*. When MHC class II expression on DP thymocytes was compared with that of B cells, immature thymocytes of both *CIITA^{tg}pIV^{-/-}* mice and a human fetus had a similar level of expression (Fig. S4 D; Choi et al., 2005). In these mice, APCs such as dendritic cells, macrophages, and B cells in the thymus also expressed MHC class II comparable to the level of WT mice (Fig. S1 B). We also developed a rat→mouse BM chimera via transplantation of rat hematopoietic stem cells into *RAG-1^{-/-} γ c^{-/-}MHCII^{-/-}* mice. In these mice, only rat thymocytes are able to provide the MHC class II–self-peptide complex for positive selection. Finally, to examine

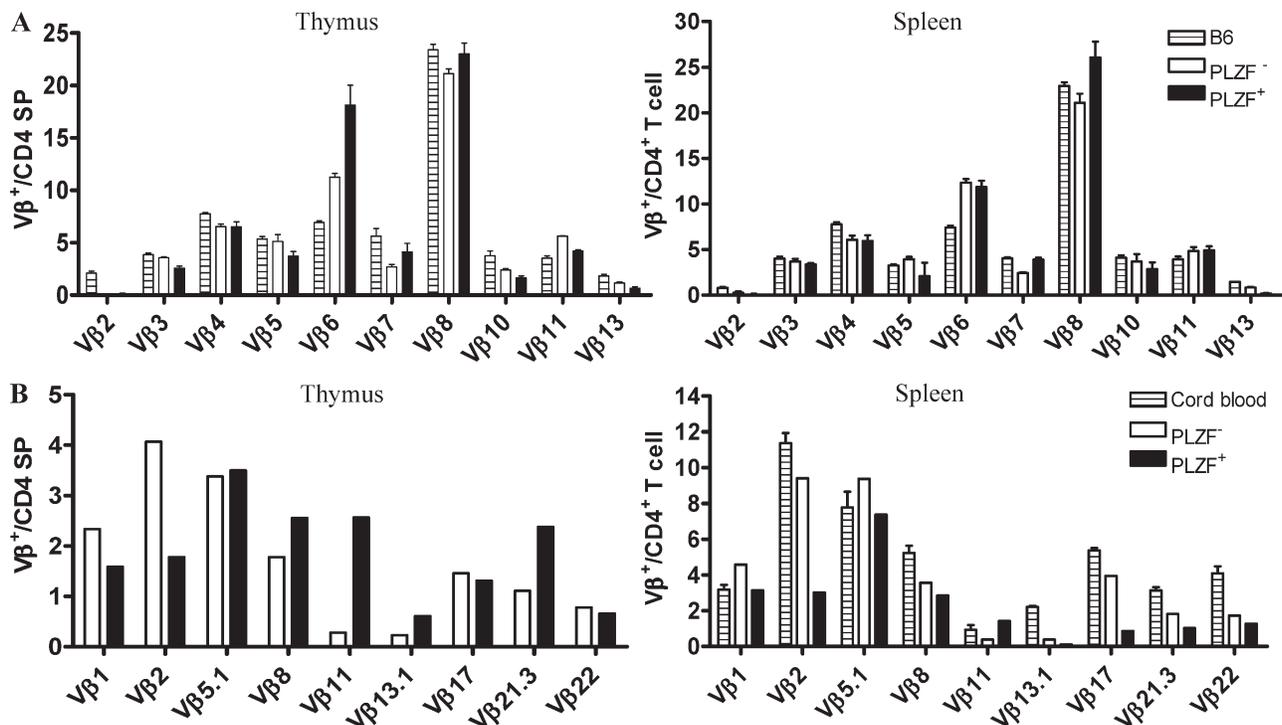


Figure 4. PLZF⁺ T–T CD4⁺ T cells in mice and humans have diverse TCR V β usage. The bar graph shows the V β chain distribution of PLZF⁺ and PLZF⁻ CD4 SP thymocytes and splenic CD4⁺ T cells from *CIITA^{tg}pIV^{-/-}* mice (A) and a human fetus (GA = 18 wk; B). This is compared with conventional CD4⁺ T cells from a WT mouse (A) and human cord blood (B). The data are mean values \pm SEM of four animals in each group of mice, with one human fetus and four cord blood samples.

the importance of the T–T interaction for the generation of PLZF⁺ CD4⁺ T cells in humans, similar to the rat→mouse chimera, we also developed a hu–mice system using cord-blood CD34⁺ stem cells. In this system, it has been reported that developing human T cells have undergone selection on human MHC, even if mouse MHC class II expression still occurs on host thymic epithelial cells (Traggiai et al., 2004). These three mouse models efficiently produced T–T CD4⁺ T cells in which PLZF was expressed in a significant proportion of CD4 SP thymocytes. Most of these PLZF⁺ cells were negative for CD1d/αGalCer tetramer staining, indicating that they were not iNKT cells. To establish a developmental environment that was close to the normal thymus, rat or fetal human thymic tissues were grafted into their respective chimeric mice to support the development of conventional T–E CD4⁺ T cells. The frequency of PLZF⁺ cells was drastically reduced in the grafted thymus, which contained abundant selecting thymic epithelial cells. In contrast, PLZF expression was observed in a significant population of CD4⁺ T cells from the host thymus, in which the selecting ligands were expressed exclusively on immature T cells. Therefore, the expression of PLZF can be used as a specific marker for T–T CD4⁺ T cells. One possibility is that non–T cells such as macrophages and dendritic cells involve the positive selection process. However, it is not likely that these cells efficiently select CD4 SP thymocytes because pIV^{-/-} mice, in which hematopoietic cells in the thymus still express MHC class II molecules, contained as few CD4 SP cells as CIITA^{-/-} mice (Fig. S1 A). Moreover, most of these CD4 SP cells (2.2%; Fig. S1) were iNKT cells (Waldburger et al., 2003), whereas PLZF⁺ CD4 SP thymocytes developed in hu–mice were not (Fig. S7 D). This has already been confirmed in WT→MH-CII^{-/-} BM chimera, where CD4⁺ T cells were not developed (Choi et al., 2005). Based on these results, it seems to be justifiable to extrapolate a notion that the T–T interaction efficiently develops human CD4⁺ T cells in hu–mice chimera. Another point of interest is that PLZF⁺ CD4⁺ T cells in the spleen and liver from xenochimeric mice were even smaller in number than those of CIITA^{tg}pIV^{-/-} mice. It can be explained by the assumption that the PLZF⁺ population is maintained by peripheral T–T interaction. CIITA^{tg}pIV^{-/-} mice would be a representative example for this, where there are more possibilities for T–T interactions in the periphery, because the MHC class II expression is almost always present in peripheral T cells.

Most importantly, to address whether this T–T event occurs normally and has physiological relevance in humans, we dissected the developmental profile of T cells in the fetal human thymus and spleen. The frequency of T–T CD4⁺ T cells in the fetal thymus was from 42- to 290-fold higher than that of CD1d/αGalCer tetramer–positive iNKT cells (Fig. S6 B). They are unlikely to be type II NKT cells, as T–T CD4⁺ T cells are normally developed under the knockout condition of CD1d in the mouse system (Fig. 1 A). Similarly, based on the frequency and TCR diversity of the T–T CD4⁺ T cells, they are unlikely to be MAIT cells, which have been reported

to be ~1/6,000 of the CD4⁺ T cell fraction in humans and have limited TCR Vβ usage (Vβ2 and Vβ13; Tilloy et al., 1999; Treiner and Lantz, 2006). When compared with TCR Vβ usage between the PLZF⁺ and PLZF⁻ populations, unlike other types of innate T cells, PLZF⁺ CD4⁺ T cells expressed a TCR Vβ repertoire as diverse as that of conventional CD4⁺ T cells in mice and humans (Fig. 4, A and B). Therefore, T–T CD4⁺ T cells are distinct from other types of innate T cells (such as iNKT and MAIT cells), particularly in terms of TCR Vβ usage and MHC class II restriction.

It is intriguing that PLZF is expressed only in a fraction of the population of mouse T–T CD4⁺ T cells, whereas most of the iNKT cells in humans and mice universally express PLZF. This discrepancy can be partially explained by the differences in the diversity of selecting ligands. The limited TCR repertoire of iNKT cells is mainly shaped by a cortical event in which only cells with a high-affinity interaction between the TCR and a very limited number of endogenous lipid ligands (e.g., iGb3) are positively selected and survive (Bendelac et al., 2007). In this situation, the resulting repertoire is extremely skewed and narrowed down to a particular TCR usage. On the contrary, T–T CD4⁺ T cells interact with a broad range of self-peptide ligands and, even though T cells do not encounter agonistic peptides, they still have possibilities for positive selection and survival by multiple low-affinity interactions. Among these interactions, cells that are engaged by self-peptides of relatively high affinity may undergo PLZF up-regulation, thereby providing T–T CD4⁺ T cells with an innate property. In support of this hypothesis, OT-II CD4⁺ T cells selected via a T–T interaction failed to express PLZF. In this system, the possibility that an OT-II TCR encounters an agonistic selecting ligand is extremely low; therefore, they are not able to receive a sufficient signal to induce PLZF expression (Fig. 1 D and Fig. S3).

As for a biological relevance of T–T CD4⁺ T cells, we speculate that T–T CD4⁺ T cells might serve as immediate effector cells against diverse pathogens, owing to their rapid effector function and extreme TCR diversity as in conventional T cells. Although other innate-like T cells such as iNKT or MAIT cells participate in this process, they have canonical TCRs restricted by nonpolymorphic MHC class Ib molecules, which limit specificity against foreign antigens. Thus, T–T CD4⁺ T cells would provide a strong defense mechanism, especially for antiviral resistance before the establishment of sufficient memory pools elicited by conventional T cells (Lee et al., 2009). T–T CD4⁺ T cells are able to explain the pathogenesis of X-linked lymphoproliferative disorder in SLAM-associated protein–deficient patients, in whom T–T interactions during thymic ontogeny do not take place (Li et al., 2007a). In the absence of rapid effector cells secreting the antiviral cytokine IFN-γ, we speculate that the uncontrolled initial viral load leads to a massive lymphoproliferation.

Both T–T CD4⁺ T cells and iNKT cells are most frequent in the thymus early in gestation. However, their relative frequency declines with age and they became rarer in the neonatal thymus (Sandberg et al., 2004). To address the issue

of why the generation of PLZF⁺ CD4⁺ T cells is highest during the second trimester, we analyzed the molecules that are involved in the T–T interaction, such as SLAM (CD150), CD1d, and MHC class II. However, they remained constant during the fetal and postnatal periods (Fig. S7), indicating that factors other than these surface molecules are required for T–T interaction. One of the probable candidate is thymus-specific serine protease (*prss16*), which is involved in the processing of endogenous proteins in cortical thymic epithelial cells, thereby regulating the positive selection of CD4⁺ T cells. The alternatively spliced transcripts of thymus-specific serine protease have been reported to be differentially expressed, depending on the fetal and postnatal age of the human thymus (Luther et al., 2005; Gommeaux et al., 2009). Therefore, purely on a theoretical basis, it is assumed that during a particular developmental stage of the thymus, T–E interaction would be much less efficient and, subsequently, T–T interaction would dominate over T–E interaction. This issue is currently being investigated in this laboratory.

A report from one independent laboratory showed drastic developmental suppression of iNKT cells in CIITA^{tg} mice using CD4 promoter (Li et al., 2009), whereas this was not the feature in our mouse model (Fig. 1 A; Fig. S2 A; and Fig. S8, #16 CIITA^{tg}) used in experiments. This discrepancy seems to be caused by a different MHC class II expression level, because the similar suppression of iNKT cell development was also seen in another founder line from this laboratory (Fig. S8, #1 CIITA^{tg}) that expresses a much higher level of MHC class II on DP thymocytes (Fig. S8).

Another point we would like to emphasize is that the number of PLZF⁺ T–T CD4⁺ T cells was four- to fivefold higher than that of PLZF⁺ T–T CD4⁺ T cells in CIITA^{tg}pIV^{-/-} mice, and the same might be true in humans. This data suggests that the actual number of T–T CD4⁺ T cells that developed from the T–T interaction might be greater than the number of T–T CD4⁺ T cells expressing PLZF.

In summary, we have shown that the MHC class II-dependent T–T interaction normally takes place during human thymopoiesis, and these events could be tracked by PLZF expression. We speculate that the biological relevance of T–T CD4⁺ T cells is the generation of early effector T cells against diverse foreign pathogens, particularly viruses. It has been generally accepted that the T–E interaction is the only pathway for the generation of functional CD4⁺ T cells. However, this developmental pathway might be revised by the addition of a novel CD4⁺ T cell subset, T–T CD4⁺ T cells.

MATERIALS AND METHODS

Mice. The plck-CIITA^{tg} mice were previously generated in our laboratory (Choi et al., 2005). RAG-1^{-/-}, γ c^{-/-}, MHC class II^{-/-}, CIITA^{-/-}, CD45.1 congenic B6, OT-II TCR transgenic, and NOG mice were purchased from the Jackson Laboratory. Mice carrying a deletion of promoter IV of the *Mhc2ta* gene (pIV^{-/-}) were provided by H. Acha-Orbea (University of Lausanne, Lausanne, Switzerland). B6.CD1d^{-/-} mice were provided by H. Gu (Columbia University, New York, NY). The plck-CIITA^{tg} mice were backcrossed to pIV^{-/-} or CD1d^{-/-} mice to generate CIITA^{tg}pIV^{-/-} or CIITA^{tg}CD1d^{-/-} mice, and RAG-1^{-/-} mice were bred with MHCII^{-/-}

and γ c^{-/-} mice to generate RAG^{-/-} γ c^{-/-}MHCII^{-/-} mice. OT-II mice were bred with CD45.1 mice to generate CD45.1 OT-II mice. All mice were maintained under specific pathogen-free conditions at the animal facility at the Center for Animal Resource Development, Seoul National University College of Medicine. Experiments were performed after receiving the approval of the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Resources, Seoul National University.

Antibodies and flow cytometric analysis. The following fluorochrome- or biotin-labeled monoclonal antibodies were purchased from BD, eBioscience, or Dinona: anti-mouse CD4 (RM4.5), CD8 (53-6.7), CD11b (M1/70), CD11c (HL3), CD24 (M1/69), B220 (RA3-6B2), I-A^b (AF6-120.1), CD62L (MEL-14), CD44 (IM7), Ki-67 (B56), NK1.1 (PK136), TCR β (H57-597), CD69 (H1.2F3), CD122 (TM- β 1), IFN- γ (XMG1.2), and IL-4 (11B11); anti-human CD1d (CD1d42), CD4 (RPA-T4 or OKT-4), CD8 (RPA-T8 or OKT-8), CD45RO (UCHL1), CD161 (DX12), HLA-DR (YG18), CD150 (A12), and IFN- γ (45.15); and anti-rat CD4 (Ox-35), CD8 (Ox-8), CD3 (G4.18), CD45R (HIS24), and RT1B (OX-6). The anti-mouse V β 2 (B20.6), V β 3 (KJ25), V β 4 (KT4), V β 5.1&5.2 (MR9-4), V β 6 (RR4-7), V β 7 (TR310), V β 8 (F23.1), V β 10 (B21.5), V β 11 (RR3-15), and V β 13 (MR12-3) antibodies were purchased from BD. FITC-conjugated anti-human V β 1 (BL37.3), V β 2 (MPB2D5), V β 5.1 (IMMU157), V β 8 (56C5.2), V β 11 (C21), V β 13.1 (IMMU222), V β 17 (E17.5F3.15.13), V β 21.3 (IG125), and V β 22 (IMMU546) antibodies were purchased from Beckman Coulter. Allophycocyanin-conjugated anti-mouse α GalCer-loaded or unloaded CD1d tetramers were gifts from A. Bendelac (University of Chicago, Chicago, IL). Human samples were also stained with mouse CD1d tetramers, as they are cross-reactive (Benlagha et al., 2000; Karadimitris et al., 2001). Fresh cell suspensions of thymocytes or splenocytes were resuspended in flow cytometry buffer, i.e., PBS with 0.1% BSA and 0.1% sodium azide. Hepatic lymphocytes were prepared as previous described (Curry et al., 2000). In brief, liver samples were homogenized and hepatocyte-rich matrix was removed by centrifugation for 1 min at 30 g. The supernatants were harvested and lymphocytes were separated by density gradient centrifugation over lympho M solution (Cedarlane). After staining with fluorophore-conjugated antibodies for 30 min at 4°C, the live cells (gated as the propidium iodide-negative population; Sigma-Aldrich) were analyzed using a flow cytometer (FACSCalibur; BD) and CellQuest Pro software (BD).

Intracellular staining. For intracellular flow cytometry of PLZF, thymocytes and splenocytes were stained as described previously (Savage et al., 2008). In brief, cells were fixed with the fixation and permeabilization buffers from the Foxp3 Staining Buffer Set (eBioscience). Intracellular PLZF was detected using the mouse monoclonal antibody D-9 (Santa Cruz Biotechnology, Inc.), and in some experiments, a biotin-conjugated D-9 antibody (Dinona) was used. For cytokine staining, cells were stimulated with 50 ng/ml PMA and 1.5 μ M ionomycin (Sigma-Aldrich) for 5 h; 10 μ g/ml brefeldin A (Sigma-Aldrich) was added during the last 3 h of stimulation. The stimulated cells were surface stained with anti-CD4 and α GalCer-loaded or unloaded CD1d tetramers. Cells were then fixed and stained with anti-IL-4 (11B11), anti-IFN- γ (XMG1.2 or 45.15), and anti-PLZF (D-9), followed by fluorophore-conjugated goat anti-mouse IgG1 (A85-1; BD) or streptavidin (BD) using the Foxp3 Staining Buffer Set.

BM chimeras. Recipient RAG^{-/-} γ c^{-/-}MHCII^{-/-} and pIV^{-/-} mice were exposed to 800 rad of total body irradiation from a ¹³⁷Cs source. The irradiation was split into two doses separated by 4 h, and the mice were rested for 4–24 h before receiving BM cells. Total BM cells were prepared from the femurs and tibias of donor mice, and mature T cells were depleted using a cocktail of CD4 and CD8 microbeads and magnetic sorting (MACS; Miltenyi Biotec). Each recipient mouse received 3 \times 10⁶ T cell-depleted BM cells in a volume of 300 μ l PBS via lateral tail vein injection. The mice were analyzed 4–12 wk later. For the generation of the rat \rightarrow mouse chimera, rat BM cells were prepared from the femurs and tibias of Sprague Dawley

rats, and the stem-cell population was obtained after centrifugation onto a 28% BSA cushion gradient, as described previously (Prakapas et al., 1993).

hu-mice system. hu-mice were generated as described previously (Ito et al., 2002). In brief, NOG mice were irradiated with 220 rad, and at least 2×10^5 CD34⁺ cells separated from human cord blood using MACS were intravenously inoculated into mice via the lateral tail vein. In some experiments, fetal human thymic tissue, which was frozen and thawed once, was engrafted under the kidney capsule of mice.

Human tissues and samples. Postnatal thymi 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. 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 (GA) 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. Cord blood mononuclear cells were isolated from whole blood using Ficoll-Hypaque density gradient centrifugation (GE Healthcare), and CD34⁺ stem cells were separated with CD34 magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. CD34⁺ stem cells with >95% purity were used for transplantation.

Statistical analysis. All data were analyzed using Prism software (GraphPad Software, Inc.). Bar graphs denoting the percentage of each cell or concentration of each cytokine represent mean values \pm SEM, and data were compared using an unpaired *t* test.

Online supplemental material. Fig. S1 shows thymocyte development and MHC class II expression on APCs in the thymi and spleens of WT, pIV^{-/-}, and CIITA^{-/-} mice. Fig. S2 shows a summary of the development and function of PLZF⁺ T–T CD4⁺ T cells in mice. Fig. S3 shows that OT-II T cells generated by T–T interaction do not express PLZF. Fig. S4 shows the level of MHC class II expression on cortical thymocytes of the human, mouse, and rat. Fig. S5 shows anatomical localization of PLZF⁺ T cells that exclusively belong to the category of the CD4⁺ CD1d/αGalCer tetramer-negative T cell population. Fig. S6 shows CD3 expression on human PLZF⁺ CD4⁺ SP thymocytes and the ratio of PLZF⁺ CD4⁺ SP thymocytes and splenic CD4⁺ T cells to iNKT cells. Fig. S7 shows SLAM, CD1d, and MHC class II expression on fetal and postnatal human thymocytes. Fig. S8 shows the effect of MHC class II expression of DP thymocytes on the development of iNKT cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091519/DC1>.

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