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The SLAM-Associated Protein (SAP)/Fyn/PKCθ Pathway is Required for Thymocyte-mediated CD4 T Cell Development

Wei Li[1], **M. Hanief Sofi**[2], **Svend Rietdijk**[3], **Ninghai Wang**[3], **Cox Terhorst**[3], and **Cheong-Hee Chang**[2]

[1]Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202

[2]Department of Microbiology and Immunology, The University of Michigan Medical School, Ann Arbor, MI 48109

[3]Division of Immunology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215

Summary

MHC class II-expressing double positive thymocytes induce progression of CD4 T cell development, as efficiently as cortical thymic epithelial cells (cTEC). Because double positive thymocytes expressing CD1d select NKT cells, we investigated whether thymocyte-selected CD4 (T-CD4) T cells require the same signaling components as NKT cells. We therefore examined the role of SAP, Fyn, PKCθ, one of the SAP binding receptors Ly108, T-bet, and IL-15Rα in T-CD4 T cell development. Using bone marrow chimeras, we found that SAP, Fyn and PKCθ are essential for T-CD4 T cell generation, whereas mutations in Ly108, IL-15Rα or T-bet had a marginal effect. Furthermore, SAP is critical for IL-4 production by T-CD4 T cells, but the PKCθ deficiency did not alter the ability of T-CD4 T cells to produce cytokines. T-bet is necessary to produce the maximum amount of IFN-γ for CD4 T cells regardless of the selection pathway. We conclude that, in contrast to mainstream epithelial cell-selected CD4 T cells, the two distinct lineages of T cells selected by thymocytes, *i.e.* T-CD4 and NKT cells both utilize the SAP/Fyn/PKCθ pathway for their development and function.

Introduction

It is well accepted that MHC class II expressed on cortical thymic epithelial cells (cTEC) plays a critical and unique role for positive selection of conventional CD4 T cells, and BM-derived hematopoietic cells cannot mediate positive selection of CD4 T cells. Recently, however, we and others identified an alternate pathway for CD4 T cell development that is mediated by MHC class II-expressing thymocytes (Choi et al., 2005; Li et al., 2005). To distinguish between the two CD4 T cell populations, we named them E- and T-CD4 T cells to reflect the cell type mediating selection: epithelial cell-selected (E-CD4) and thymocyte-selected CD4 (T-CD4) T cells, respectively. Unlike E-CD4 T cells that require an appropriate signal to differentiate to

Corresponding Authors: Cheong-Hee Chang, Department of Microbiology and Immunology, The University of Michigan Medical School, 5641 Medical Science Bldg II, Ann Arbor, MI 48109-0620, Phone: 734-647-7570, FAX: 734-764-3562, heechang@umich.edu, Wei Li, Department of Medicine, The Division of Infectious Diseases, Indiana University School of Medicine, 545 Barnhill Drive, 435 Emerson Hall, Indianapolis, IN 46202, Phone: 317-278-4691, FAX: 317-278-1587, wl1@iupui.edu.

effector Th1 or Th2 cells, T-CD4 T cells produce both Th1 and Th2 cytokines immediately after *in vivo* stimulation, which is very similar to NKT cells (Li et al., 2007). Surprisingly, IL-4 production by T-CD4 T cells does not require Stat6 that is also dispensable for NKT but not E-CD4 T cells in the generation of IL-4. Moreover, mice that had a T-CD4 population *in vivo* were protected from the development of allergen-induced allergic airway inflammation. This data, with another report showing that mice with T-CD4 T cells are protected from EAE (Park et al., 2004), suggest that T-CD4 cells have an important immunoregulatory role. A significant percentage of human fetal and neonatal thymocytes express MHC class II (Gilhus and Matre, 1983; Marinova et al., 2001; Park et al., 1992; Thulesen et al., 1999) and several observations support the presence of two selection pathways in humans (Godthelp et al., 2000; Klein et al., 1995; Markert et al., 2004; Markert et al., 2003; Rice et al., 2004). Moreover, human hematopoietic stem cells can generate CD4 single positive thymocytes on Delta-like 1-expressing OP9 bone marrow stromal cells *in vitro* (La Motte-Mohs et al., 2005), further supporting the co-existence of E- and T-CD4 T cells in humans.

NKT cells are a specialized subset of T lymphocytes that are positively selected by cortical thymocytes expressing non-classic MHC class I molecules CD1d. NKT cells express TCR/CD3 complexes, but their TCR repertoire is very restricted with most of them carrying the invariant Vα14-Jα18 α chain preferentially associated with Vβ8.2, Vβ7, or Vβ2. They co-express surface markers characteristic of both conventional T cells and several NK cell-associated receptors such as NK1.1, NK inhibitory and stimulatory receptors. Functionally, NKT cells resemble innate effector cells in that they can promptly produce large amounts of Th1- and Th2-type cytokines, such as IFN-γ and IL-4 upon TCR stimulation. The effect of these cytokines could bridge innate and adaptive immunity. Accumulating data demonstrated that NKT cells play a critical role in regulating anti-viral, anti-tumor immune responses, allergy, and autoimmunity (Bendelac et al., 2006; Kronenberg, 2005; Taniguchi et al., 2003).

Both mainstream E-CD4 T cells and NKT cells are derived from the same lymphocyte precursors and develop through the DP stage (Coles and Raulet, 2000; Dao *et al.*, 2004; Egawa *et al.*, 2005; Gapin *et al.*, 2001; Hammond *et al.*, 1998; Tilloy *et al.*, 1999). Yet, the requirements for E-CD4 T cell and NKT cell development are fundamentally different. NKT cells are positively selected by CD1d on cortical DP thymocytes in the presence of self glycolipid ligands, such as iGb3 (Zhou et al., 2004), whereas E-CD4 T cells develop on self-peptide/MHC class II complexes on cTEC (Starr et al., 2003). Although NKT cells share some development requirements with T cells selected on cTEC, recent studies have demonstrated that several signaling molecules, transcription factors, and cytokines are specifically required for NKT ontology at distinct differentiation stages (Bendelac et al., 2006; Matsuda and Gapin, 2005).

SAP (SLAM-Associated Protein) is selectively required for NKT but not E-CD4 T cell development in humans and mice (Chung et al., 2005; Nichols et al., 2005a; Pasquier et al., 2005). SAP binds to the specific tyrosine-based motifs (TxYxxV/I/L) within the cytoplasmic tail of six SLAM related receptors and relays the SLAM-elicited signaling through the recruitment and activation of the Src protein tyrosine kinase Fyn (Chan et al., 2003; Latour et al., 2003; Sayos et al., 1998). The SLAM family of hematopoietic-surface receptors comprises nine hematopoietic cell surface receptors. Whilst CD150 (SLAM), CD84, CD229 (Ly9), CD244 (2B4), Ly108 and CRACC bind the single SH2-domain adapters SAP and EAT-2, CD48, BLAME and SLAMF9 do not (Engel et al., 2003). These receptors are differentially expressed in various immune cells and all of them (except CD244 and CD48) serve as self-ligand or homophilic receptors (Bhat et al., 2005; Engel et al., 2003; Nichols et al., 2005b; Veillette and Latour, 2003). The SLAM/SAP/Fyn signaling pathway plays a critical role in the development, differentiation, and effector function of several leukocyte lineages in the innate and adaptive immune systems (Ma et al., 2007; Veillette, 2006). During T cell activation,

SLAM related receptors function as co-stimulatory molecules and the SLAM/SAP/Fyn pathway enhances TCR-mediated and PKCθ-dependent NF-κB activation (Cannons *et al.*, 2004). PKCθ/BCL10/NF-κB activation by Fyn has been implicated in NKT cell generation (Schmidt-Supprian *et al.*, 2004; Sivakumar et al., 2003; Stanic *et al.*, 2004a; Stanic *et al.*, 2004b). Because the SLAM/SAP/Fyn/PKCθ signaling cascade plays an important role in inducing TCR-mediated Th2 cytokine production (Cannons et al., 2004; Graham et al., 2006; Howie et al., 2005; Marsland et al., 2004; Wang et al., 2004; Wu et al., 2001), we have set out to test the role of this pathway in T-CD4 T cell selection and expansion in the thymus.

As T-bet, which due to its critical role in IFN-γ gene activation, is thought to regulate terminal maturation, survival, and effector function of NKT cells (Matsuda et al., 2006; Townsend et al., 2004) we examined its role in T-CD4 development. Similarly, as the IL-15/IL-15R signaling pathway is important for expansion/survival and functional maturation of NKT cells, but not required for NKT cell generation (Kennedy et al., 2000; Lodolce et al., 1998; Ohteki et al., 1997; Ranson et al., 2003; Schluns et al., 2004), the effect of a disruption in the IL-15Rα gene was evaluated.

The experiments in the current study are designed to test the hypothesis that selection and progression of T-CD4 T cells and NKT cells depend on similar signaling pathways. To investigate the role of SAP, Fyn, PKCθ, Ly108, T-bet, and IL-15Rα in regulating T-CD4 cell development, survival, expansion, terminal differentiation, or effector function, we utilized bone marrow chimeras and mice in which these genes had been disrupted.

Results

Essential role of SAP and Fyn in T-CD4 T cell development

SAP is expressed in T cells, NK cells, and several other hematopoietic cells (Ma et al., 2007; Veillette, 2006) and its expression in BM-driven cells has been shown to be necessary and sufficient to mediate NKT cell development in both humans and mice (Chung et al., 2005; Nichols et al., 2005a; Pasquier et al., 2005). Since both NKT and T-CD4 T cells are developed through interactions between thymocytes, we hypothesized that SAP might also be essential for T-CD4 T cell generation. To test this, we utilized transgenic mice expressing the MHC class II transactivator (CIITA) as a transgene (denoted as Tg from hereon) (Patel et al., 2005). Because CIITA can activate the expression of MHC class II and other molecules that participate in MHC class II-restricted antigen presentation (Reith et al., 2005), thymocytes in Tg mice express MHC class II (Patel et al., 2005). Using Tg mice, we have demonstrated that T-CD4 T cells can be developed efficiently by MHC class II-expressing thymocytes (Li et al., 2005).

To examine the potential role of signaling molecules in T-CD4 T cell development and function, we utilized the notion that MHC class II expressing (Tg) thymocytes can select non-MHC expressing thmocytes *in trans* (Choi et al., 2005). To demonstrate the ability of Tg thymocytes to mediate development of the other thymocytes and to assess the co-selection efficiency in the mixed BM chimeric mice, BM from Tg and non-transgenic (WT) mice were co-transferred into the MHC class II Aβ deficient hosts (Tg+WT→$A\beta^{-/-}$). As shown in Figure 1A and 1B, the percentages of CD4 SP thymocytes originated from Tg and WT BM (5.5 ± 1.7 vs. 3.9 ± 1.3) were not significantly different. In the periphery, splenic T-CD4 cells accumulated to similar levels, although the percentage of LN T-CD4 T cells from Tg BM was slightly higher than that of the WT cells (Figure 1A and 1B). WT BM transferred to $A\beta^{-/-}$ host in the absence of Tg BM generated very few CD4 T cells, similar to $A\beta^{-/-}$ mice (Li et al., 2005). As we have reported previously (Patel et al., 1995), CD8 T cells originated from Tg BM were over- and under-represented in the thymus and the periphery, respectively (Figure 1A and 1B). This change seems to be due to elevated IL-4 in the $CIITA^{Tg}$ thymus since

CIITA^{Tg} mice deficient in IL-4 gene expression did not show the alteration in CD8 T cell development (Patel et al., 2005). The results shown here set the premise that T-CD4 T cells are generated in Aβ$^{-/-}$ recipients when Tg thymocytes are present. In all experiments, CD45 congenic markers were used to distinguish cells derived from different BM sources and the recipients.

We next tested the role of SAP in T-CD4 T cell development. Unlike the WT BM, generation of SAP$^{-/-}$ T-CD4 cells in Tg+SAP$^{-/-}$→Aβ$^{-/-}$ mice was very poor in the thymus as well as in the peripheral lymphoid organs (Figure 1C, left group; 1D, top left graph). In the control chimeras (WT+SAP$^{-/-}$→B6), both WT and SAP$^{-/-}$ E-CD4 T cells were selected equally well on MHC class II expressed in host cTEC (Figure 1C, right group; 1D, top right graph). The numbers of CD4 T cells were consistent with these results (Supplemental Table 1). In contrast, SAP did not influence the generation of CD8 SP cells in the thymus, and SAP$^{-/-}$ CD8 T cells tended to accumulate at a higher percentage than the Tg counterparts in the Aβ$^{-/-}$ hosts (Figure 1C, left group; 1D, bottom left graph). The percentages and the numbers of CD8 T cells in the thymus, spleen and the LN were summarized in Supplemental Table 2 and 3, respectively. In addition, we analyzed NKT cell development in Tg+SAP$^{-/-}$→Aβ$^{-/-}$ and WT+SAP$^{-/-}$→WT chimeric mice. The proportion of NKT cells was greatly diminished, if BM cells were from SAP$^{-/-}$ but not Tg or WT BM cells (Figure 1E). These results were consistent with the previous studies showing a critical role of SAP in NKT cell ontology but not in the generation of E-CD4 T cells. More importantly, SAP is essential for not only NKT but also T-CD4 T cell development.

Fyn and PKCθ are essential for T-CD4 T cell development

Fyn, the adaptor protein of SAP, is shown to be important for NKT but not E-CD4 T cell development (Eberl et al., 1999; Gadue et al., 1999). Since the deficiency of SAP dramatically reduced the T-CD4 T cell compartment, it is possible that Fyn is also necessary for T-CD4 T cell generation. In addition, TCR engagement activates several PKC isoforms that transduce signals to downstream events. Among them, PKCθ is selectively recruited to the immunological synapse upon TCR stimulation. Furthermore, this recruitment is enhanced by co-activation of TCR and SLAM (Cannons et al., 2004). Although PKCθ plays an essential role in T cell activation and survival (Cannons et al., 2004; Coudronniere et al., 2000; Manicassamy et al., 2006), it is dispensable for E-CD4 T cell development. However, a recent study revealed a critical role of PKCθ in the generation of functional NKT cells (Stanic et al., 2004b). Therefore, we investigated the role of Fyn and PKCθ in T-CD4 T cell development.

We constructed Tg+Fyn$^{-/-}$→Aβ$^{-/-}$ and WT+Fyn$^{-/-}$→WT chimeric mice and compared the development of E-, T-CD4 T cells, and NKT cells. Similar to SAP, the deficiency in Fyn also diminished T- but not E-CD4 T cell development (Figure 2A and 2B). In agreement with the published studies, Fyn deficient BM cells did not reconstitute NKT cells as efficient as wild type BM (Figure 2C). Likewise, in Tg+PKCθ$^{-/-}$→Aβ$^{-/-}$ mice, Tg T-CD4 T cells were efficiently generated, but the reconstitution of PKCθ$^{-/-}$ T-CD4 T cells was significantly reduced (Figure 2D and 2E, left group). In contrast, the thymic generation of E-CD4 T cells was not impaired in the absence of PKCθ in comparison to the WT E-CD4 T cells shown in WT+PKCθ$^{-/-}$→WT chimeric mice (Figure 2D and 2E, right group). However, there were fewer PKCθ$^{-/-}$ E-CD4 T cells than WT ($p<0.05$) in LN and spleens of WT+PKCθ$^{-/-}$→WT chimeric mice, suggesting that PKCθ plays a role in the peripheral homeostasis of E-CD4 T cells (Figure 2D and 2E, right group). Thymic PKCθ$^{-/-}$ NKT cells were greatly reduced in both kinds of chimeric mice (Figure 2F). Taken together, both Fyn and PKCθ that functions downstream of SAP govern T-CD4 T cell as well as NKT cell development.

Minimal involvement of Ly108, T-bet and IL-15Rα in regulating T-CD4 T cell ontology

The SLAM related surface receptors recruit SAP and several family members, including CD84, CD150, CD229, and Ly108 ((Ma et al., 2007; Veillette, 2006) and the co-submitted manuscript)), are expressed on DP thymoyctes. Therefore, it is possible that they participate in the positive selection of T-CD4 T cells as well as NKT cells. The deficiency of CD229 did not cause the impairment of NKT cell development (Graham et al., 2006). However, the role of Ly108 has not been demonstrated in either NKT or CD4 T cell generation. To address this, we co-transferred Tg and Ly108$^{-/-}$ BM into Aβ$^{-/-}$ hosts and the chimeras were examined. We found a lower representation of Ly108$^{-/-}$ T-CD4 T cells than Tg T-CD4, but the differences were not significant (Figure 3A and Supplemental Figure 1A, left group). In addition, Ly108 was not required for E-CD4 development (Figure 3A and Supplemental Figure 1A, right group). Similarly, NKT cells were present in the absence of Ly108 although the NKT cell population was slightly reduced (Figure 3B). Thus, lack of Ly108 did not greatly affect the development of NKT or T-CD4 T cells, which was in contrast to the phenotype caused by the SAP deficiency. It is, however, likely that SAP controls cooperating signals initiated by several of the SLAM-family receptors (Engel et al., 2003; Graham et al., 2006; Howie et al., 2005), which are requisite for T-CD4 cell development.

Using a similar scheme, we investigated the role of T-bet in T-CD4 T cell development. T-bet regulates terminal differentiation of NKT cells and its deficiency results in only a small number of immature NKT cells. If the maturation programs between NKT cells and T-CD4 T cells are similar, T-bet may play a similar role in T-CD4 cell development. The results shown in Figure 3C indicated otherwise. The absence of T-bet did not significantly affect thymic development or peripheral accumulation of T-CD4 as well as E-CD4 T cells, although in lymph nodes, T-bet$^{-/-}$ T-CD4 T cells were slightly decreased compared to Tg T-CD4 T cells (Figure 3C and Supplementary Figure 1B). As expected from the published results, T-bet$^{-/-}$ NKT cells were greatly under-represented, compared to the Tg or WT counterparts (Figure 3D).

IL-15 through its receptor IL-15R plays a critical role in the proliferation and expansion of mature NKT cells and CD8 T cells that constitutively express the IL-2/IL-15Rβ (CD122) chain. When we tested the significance of this signaling pathway, the absence of IL-15Rα had a marginal effect on development of T- or E-CD4 T cells (Figure 3E and Supplementary Figure 1C). Consistent with these data, T-CD4 T cells did not express a measurable level of CD122, whereas a significant proportion of NKT cells expressed CD122 (Supplemental Figure 2). However, maximum NKT cell generation seemed to require IL-15Rα since IL-15α$^{-/-}$ NKT cells were decreased in the chimeric mice in comparison to their Tg or WT controls (Figure 3F). Our results indicate that IL-15Rα is dispensable for both T- and E-CD4 T cell generation.

Differential role of signaling molecules on the cytokine production potential of T-CD4 T cells

Our data revealed that T-CD4 T cells did not completely overlap with NKT cells or E-CD4 T cells in their developmental requirements. Next, we investigated whether the effector function of T-CD4 T cells is altered by the deficiency of those signaling molecules. Although the absence of signaling molecules affected T-CD4 T cell development, the residual population of CD4 T cells in the periphery was detectable and hence was used to assess their cytokine production potential. The key feature of T-CD4 T cell effector function is the immediate secretion of cytokines (Li et al., 2007). Similar to NKT cells, T-CD4 cells produce both IFN-γ and IL-4 shortly after TCR stimulation without being skewed to either Th1 or Th2 lineage. In contrast, E-CD4 T cells express little cytokines under the same condition.

Previously, we have shown that cytokine producing T-CD4 T cells exhibit the effector/memory phenotype by expressing a high level of CD44 (Li et al., 2007) and that the majority of peripheral T-CD4 T cells are CD44 ^{hi} (Li et al., 2005). When we examined the expression of

CD44, the deficiency of the above studied signaling molecules had no apparent effect on the CD44 phenotype of either E- or T-CD4 T cells in the mixed BM chimeric mice (Supplemental Figure 3A and 3B). To characterize the cytokine production, splenic CD4 T cells from the mixed BM chimeras shown in Figures 1-3 were purified and stimulated for 5 hours in the absence of exogenous cytokines. The cytokine production pattern of this type of cultures would show the propensity of CD4 T cells. Cells were then stained with the CD45.1, CD45.2, CD4, and NK1.1 antibodies followed by intracellular cytokine staining (ICS) to measure IFN-γ and IL-4 production in NK1.1$^-$ CD4 T cells.

We first examined the role of SAP using CD4 T cells from Tg+SAP$^{-/-}$→Aβ$^{-/-}$ and WT+SAP$^{-/-}$→WT mice. Although the number of SAP$^{-/-}$ T-CD4 T cells in the periphery was dramatically reduced in these chimeras, the residual SAP$^{-/-}$ T-CD4 T cells were able to produce both IFN-γ and IL-4 cytokines shortly after activation (Figure 4A, compare Tg vs. SAP$^{-/-}$ T-CD4 cells in the left chimera). The proportions of IFN-γ$^+$ cells were comparable between SAP$^{+/+}$ (Tg) vs. SAP$^{-/-}$ (KO) T-CD4 T cells, whereas percentages of IL-4$^+$ cells were decreased in the absence of SAP. Nevertheless, E-CD4 T cells showed little IL-4 expression and small numbers of IFN-γ$^+$ cells regardless of the SAP status (Figure 4A, right group). Similarly, Ly108 deficient T-CD4 T cells produced both IFN-γ and IL-4 at a comparable level to that of Ly108 sufficient cells (Figure 2B). Therefore, SAP and Ly108 are not essential for T-CD4 T cells to produce cytokines, but SAP appears to be required for optimal production of IL-4 by T-CD4 T cells.

The downstream T cell signaling events that are important for IL-4 production and also known to be affected by SAP deficiency include PKCθ recruitment to lipid rafts, downstream IκBα degradation, and NF-κB1 nuclear translocation (Cannons et al., 2004). However, PKCθ deficiency had little effect on IL-4 or IFN-γ production by T-CD4 T cells (Figure 4C, left group), which indicates other signaling pathways might compensate PKCθ deficiency in mediating SAP signal transduction to the IL-4 gene.

T-bet is a critical transcription factor for IFN-γ gene expression in E-CD4 T cells (Szabo et al., 2000). Similar to E-CD4 T cells (Figure 4D, right group), there was a reduction in IFN-γ$^+$ cells that were from the T-bet$^{-/-}$ origin in Tg+T-bet$^{-/-}$→Aβ$^{-/-}$ chimeras (Figure 4D, left group). However, when we compared the IFN-γ production between T-bet deficient E- vs. T-CD4 T cells, the two showed a difference. As reported, E-CD4 T cells generated no detectable IFN-γ-producing cells without T-bet, whereas T-bet$^{-/-}$ T-CD4 T cells expressed IFN-γ, albeit at a low level. Therefore, T-bet is important for IFN-γ production by both E- and T-CD4 T cells but less critical for T-CD4 T cells. Finally, we analyzed the cytokine production potential by IL-15Rα$^{-/-}$ T-CD4 T cells. IFN-γ production was reduced slightly in both IL-5Rα$^{-/-}$ T- and E-CD4 T cells, whereas the ability to produce IL-4 by T-CD4 T cells was not affected by IL-15Rα deficiency (Figure 4E).

The Role of the signaling molecules in Th1 and Th2 differentiation

The results shown in Figure 4 demonstrated the differential potential of CD4 T cells to produce cytokines in the absence of exogenous cytokines. We next examined Th1 or Th2 effector cells to ascertain the function of signaling molecules during Th cell differentiation of T-CD4 T cells. Among several signaling molecules, we chose to examine SAP, PKCθ and T-bet that are known to be critical for Th cell differentiation. Splenic CD4 T cells from the mixed BM chimeric mice that generate either E- or T-CD4 T cells were differentiated under Th1- and Th2-inducing conditions for 6 days as described in the Experimental Procedures and their cytokine production profiles were quantified by ICS.

Based on the published reports (Cannons et al., 2004; Wu et al., 2001), we expected that SAP$^{-/-}$ E-CD4 T cells would respond normally to the Th2-skewing conditions and express IL-4.

Indeed, differentiation of $SAP^{-/-}$ E-CD4 T cells to either Th1 or Th2 cells was not affected (Figure 5A, right group). One of the characteristics of T-CD4 T cells is their ability to produce Th2 cytokines including IL-4 even under the Th1 skewing condition (Li et al., 2007; Patel et al., 2005). In fact, Th1 cells generated from SAP sufficient T-CD4 T cells produced IL-4 (Figure 5A, left group). In contrast, T-CD4 T cells lacking SAP produced a reduced amount of IL-4 under Th1 as well as Th2 conditions (Figure 5A). Nevertheless, $SAP^{-/-}$ Th1 cells produced IL-4 under the Th1 skewing conditions, if they were selected on thymocytes but not on epithelial cells (compare KO cells between the two chimeras).

PKCθ has shown to be important for IL-4 production by E-CD4 T cells (Marsland et al., 2004). Indeed, when we assessed IL-4 production, E-CD4 T cells were not able to make the maximum amount of IL-4 without PKCθ (Figure 5B, right group). However, PKCθ seems to be dispensable for T-CD4 T cells to make IL-4 since T-CD4 T cells produced an equivalent level of IL-4 under either the Th1 or Th2 skewing condition (Figure 5B, left group).

T-bet is critical for Th1 differentiation of E-CD4 T cells (Szabo et al., 2000) and Th1 cells lacking T-bet barely expressed IFN-γ when they were selected on TEC (Figure 5C, right group). When T-CD4 T cells were examined, $T\text{-}bet^{-/-}$ Th1 cells could make IFN-γ but much less than T-bet sufficient T-CD4 T cells (Figure 5C, left group). Therefore, IFN-γ production is compromised in both E- and T-CD4 T cells, if T-bet is not expressed.

Discussion

T-CD4 T cells are positively selected by MHC class II-expressing thymocytes, similar to NKT cells that are selected by CD1d-expressing thymocytes. In this study, we investigated signaling requirements for the development and effector function of T-CD4 T cells. By analyzing several molecules known to be associated with NKT cell ontology, we showed that SAP, which is crucial for the NKT cell development in both humans and mice, is also required for the generation of T-CD4 T cells. Fyn and PKCθ were also involved in the development of both lineages. We observed that T-CD4 T cell development was more severely impaired by the deficiency of SAP than Fyn. SAP is known to regulate CD4 T cell-mediated help for humoral immunity through a Fyn-independent pathway (Cannons et al., 2006; McCausland et al., 2007). Perhaps, SAP could also activate Fyn-independent pathways that facilitate T-CD4 T cell development. Nonetheless, the SAP/Fyn/PKCθ signaling pathway plays an important role in the development of both thymocyte-selected NKT cells and T-CD4 T cells. On the other hand, T-bet and IL-15Rα necessary for maturation, expansion, and survival of NKT cells, were dispensable for E- or T-CD4 T cell development. Therefore, our data indicate that development of T-CD4 T cells and NKT cells share the requirement for some of the same molecules presumably due to the common selection pathway by thymocytes. Yet, the two distinct lineages of T cells seem to diverge at a certain point during their development and then require different sets of signaling molecules for further maturation (Figure 6).

The SLAM/SAP/Fyn/PKCθ signaling pathway is considered to be specific for NKT cell generation. However, our current study implies that the same cascade is important for thymocyte-mediated CD4 T cell development. Positive selection of NKT and T-CD4 T cells requires the engagement of their TCR with the corresponding CD1d and MHC class II complexes on selecting thymocytes through homotypic T-T interactions. In contrast, E-CD4 T cell development is mediated by heterotypic cellular interactions between developing thymocytes and cTEC. Consequently, differences in the surface receptors and/or cytokines expressed by cTEC and thymocytes are likely to determine the activation of distinct signaling pathways that contribute to the development and function of different T cell subsets and NKT cells. In this regard, the co-stimulatory SLAM related family molecules expressed on thymocytes but not on cTEC could play an essential role in thymocyte-mediated positive

selection but have a minimal effect on TEC-selected T cells. It is well established that SLAM-initiated signals are relayed by the SH2 domain-containing adaptor protein SAP to the Fyn kinase. Activated Fyn subsequently can enhance TCR-mediated PKCθ-dependent activation of NF-κB activity. Our study showed that SAP, Fyn and PKCθ, critical for NKT cell ontology, are the three of the important molecular requirements for the T-CD4 T cell lineage. Perhaps, H2-M3-restricted CD8 T cells that are selected on non-classic MHC class Ib expressing-hematopoietic cells (Urdahl et al., 2002) may also depend on the same signaling components.

Ly108, a member of the family of SALM related receptors had a modest role in both NKT and T-CD4 T cell development. Since several SLAM related receptors are expressed on thymocytes, it is conceivable that there may be a redundant role in NKT and T-CD4 cell development among these receptors. Indeed, mice with the deficiency in individual SLAM members such as CD229 do not show a defect in NKT cell generation (Graham et al., 2006). In the accompanying manuscript, however, homotypic self-interactions of both CD150 and Ly108 on thymocytes are shown to provide essential costimulatory signals to TCR signaling to drive NKT cell differentiation. In addition, a recent report also linked NKT cell defect in NOD mice to the CD150 and Ly108 loci (Jordan et al., 2007). Whether the same homotypic signals play a role during T-CD4 T cell development needs a further investigation.

Signaling mediated by the IL-15Rα chain was not required for T-CD4 development. The pleiotropic cytokine IL-15 acting through its receptor IL-15R can enhance survival, induce proliferation, and promote differentiation, which depend on cell types and their maturation status. After positive selection, immature NKT cells have to undergo a maturation process, which includes the up-regulation of CD122 expression, conferring their IL-15 responsiveness. Hence, NKT cells proliferation and homeostasis highly depend on IL-15 signaling (Matsuda et al., 2002; Ranson et al., 2003). IL-15, IL-15Rα, and IL-2/IL-15Rβ deficient mice all have reduced numbers of NKT cells (Kennedy et al., 2000; Lodolce et al., 1998; Ohteki et al., 1997; Ranson et al., 2003; Schluns et al., 2004). Recent studies have shown that IL-15Rα that binds IL-15 with high affinity can present IL-15 *in trans* to bystander IL-15Rα$^{-/-}$ cells (Dubois et al., 2002). Both parenchymal and BM-derived cells can trans-present IL-15 to support NKT cell development, although the later seem to be more important for NKT cell recovery within BM and the spleen (Schluns et al., 2004). However, as we have shown here (Figure 3F), IL-15Rα deficiency seems to affect NKT cell development. It is not clear at the moment what the mechanisms behind the reduction of NKT cells are. Nevertheless, signaling mediated by the IL-15Rα chain is not critical for T-CD4 development. As the majority of resting T-CD4 T cells do not express CD122, the nonessential role of IL-15Rα in T-CD4 generation may not be surprising. Similarly, the reason why T-bet is not required for T-CD4 T cell development could also be due to lack of CD122 expression on those cells. T-bet$^{-/-}$ NKT cells have a defect in the terminal maturation and homeostasis, which is associated with decreased expression of CD122 and several other genes normally expressed in mature NKT cells (Matsuda et al., 2006; Townsend et al., 2004).

We have reported the increase and the decrease of CD8 T cells in the thymus and the periphery of CIITA^{Tg} mice, respectively, which is influenced by IL-4 production potential of T-CD4 T cells (Patel et al., 2005). In agreement of our observations, IL-4 transgenic mice also showed a similar pattern of CD8 T cell compartments (Lewis et al., 1991; Tepper et al., 1990). In several mixed BM chimeras presented here, we also observed differential reconstitutions of CD8 T cells originated from CIITA^{Tg} BM. In addition, co-transferred BM cells deficient in each individual signaling molecule yielded a varying degree of thymic and peripheral CD8 T cells in the chimeras. One explanation could be that the thymic environment is influenced by the mixed population of thymocytes developing from the two BM sources, which could potentially affect the generation of CD8 SP thymocytes.

Besides the role in NKT cell development, the SLAM pathway also regulates Th2 cytokine production. In particular, SAP, Fyn, and PKCθ have been shown to affect TCR-mediated IL-4 production in E-CD4 T cells (Cannons et al., 2004; Graham et al., 2006; Howie et al., 2005; Marsland et al., 2004; Wang et al., 2004; Wu et al., 2001). The relative significance of this pathway in Th2 differentiation of T-CD4 T cells is not the same as we demonstrated here. We showed that T-CD4 T cells lacking SAP but not PKCθ have a defect in IL-4 production under Th2-skewing conditions, which is in contrast to the role of SAP or PKCθ in E-CD4 T cells. Therefore, CD4 T cells require a different set of signaling molecules for their development as well as function depending on the selection pathway in the thymus. Although molecular mechanisms governing the differences in shaping the effector function between E- and T-CD4 T cells are not yet clear, T-T interaction seems to be responsible for chromatin remodeling of the IL-4 locus in the absence of Th1 or Th2 differentiation signal (Li et al., 2007). We have demonstrated that histone acetylation of the IL-4 locus in CD4 SP and naïve CD4 T cells is increased when they are selected by thymocytes (Li et al., 2007). Perhaps, signals from TCR together with the SLAM/SAP/Fyn pathway set the intracellular environment that facilitates to activate and to maintain IL-4 gene transcription. If so, Stat6 would have no role in Th2 cytokine production in T-CD4 T cells or NKT cells. Moreover, when CD4 T cells are developed on cTEC, they may not receive the SLAM/SAP/Fyn-mediated signal necessary to enhance the accessibility of transcriptional machinery to the IL-4 locus. These CD4 T cells would require Th2 inducing factors, such as IL-4 and Stat6, to differentiate to IL-4 producing effector cells. Further investigations are warranted to have a better understanding of how the IL-4 locus is regulated differently in developing T-CD4 T cells upon receiving a signal from another thymocyte.

The similarities and differences among E-CD4 T, T-CD4 T, and NKT cells are summarized in Table 1. Notably, T-CD4 T cells are similar to NKT cells in that both are positively selected by thymocytes and they can promptly produce IL-4 in a Stat6-independent manner (Bendelac, 1995;Coles and Raulet, 2000;Kaplan et al., 1999;Li et al., 2007;Patel et al., 2005;Stetson et al., 2003). However, T-CD4 T cells are also distinct from NKT cells as they require MHC class II but not CD1d to develop; they do not express the NK1.1 marker; and they have a diverse TCR repertoire (Choi et al., 2005;Li et al., 2005). Our current study adds additional similarities and differences, demonstrating a unique T cell population bearing distinct developmental requirements and effector function.

The clinical significance of SAP has been illustrated in the inherited immunodeficiency X-linked lymphoproliferative syndrome (XLP). Many of XLP patients carry mutations in the SH2D1A locus that encodes SAP. They suffer from dysfunctional immune responses to the Epstein-Barr Virus (EBV) infection (the uncontrolled expansion of B cells and other leukocytes the production of inflammatory cytokines), hypogammaglobulinmia and lymphomas (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998). The pathogenesis has been linked to defective cell-mediated and humoral immunity to EBV infection (Ma et al., 2007). Given the multiple roles of NKT cells, the clinical manifestation in these patients could be primarily attributed to NKT cell deficiency. However, our study presented here suggests that XLP patients likely have the impairment in development and function of T-CD4 T cells as well. If so, defects in both NKT and T-CD4 T cells could contribute to the compound immune dysfunction in XLP patients.

Experimental Procedures

Mice

Mice carrying the human type III CIITA transgene (Tg) were described previously (Patel et al., 2005). CIITA^{Tg} mice were bred to carry both the CD45.1 and CD45.2 congenic markers (CD45.1/2). Non-CIITA^{Tg} littermate from heterozygous CIITA^{Tg} breeding was used as wild

type (WT) control. T-bet$^{-/-}$ mice on the B6 background were obtained from the Jackson Laboratory (Bar Harbor, ME). SAP$^{-/-}$, Fyn$^{-/-}$, PKCθ$^{-/-}$, and IL-15Rα$^{-/-}$ mice on the B6 background mice were described previously (Eberl et al., 1999; Lodolce et al., 1998; Sun et al., 2000; Wu et al., 2001). Ly108$^{-/-}$ mice were generated by targeting exon 2 and 3 in Bruce 4 (C57BL6) stem cells and are described in detail elsewhere (Wang, Rietdijk and Terhorst, manuscript in preparation). CD45.1^{+} C57BL/6.SJL (B6) mice and the MHC class II Aβ deficient mice on the C57BL/6 or C57BL/6.SJL background (Aβ$^{-/-}$) carrying the CD45.2 and CD45.1 congenic marker, respectively, were purchased from Taconic (Germantown, NY). All mice were housed in the animal facility at the Indiana University School of Medicine (IUSM) or The University of Michigan Medical School under SPF conditions and used at 6-12 weeks of age. All animal experiments were performed under protocols approved by the institutions.

Bone Marrow Chimeric Mice

For bone marrow transfer experiments, the recipient B6 or Aβ$^{-/-}$ mice were lethally irradiated with 950 rads 24 hr before receiving BM transfers. Total BM cells were harvested from the femurs and tibias of donor mice (2-3 months of age) and depleted of mature T cells, B cells, and MHC class II positive lymphocytes by using a cocktail of antibodies containing anti-CD4 (RL172) and anti-CD8 (TIB105, TIB210), anti-CD19 (1D3), and anti-MHC class II (M5/114), followed by complement-mediated lysis. T-depleted BM cells from two different types of donor mice were mixed at a ratio of 1:1 and each recipient mouse received 2-5×10^{5} cells in 500 μl of 1× PBS via tail vein injection. All BM chimeras were reconstituted for at least 8 weeks before analysis of T and NKT cell development and function.

Flow Cytometry

All antibodies used for flow cytometry were purchased from BD Pharmingen (San Diego, CA). Cells were pre-incubated with the anti-FcγR mAb 2.4G2 to block non-specific antibody binding before stained with the following FITC-, PE-, PerCP-, CyChrome-, APC-or biotin-conjugated antibodies: TCRβ (H57), CD4 (L3T4), CD8 (53-6.7), NK1.1 (PK136), CD45.1 (A20), CD45.2 (104), anti-IL-4 (11B11), and anti-IFN-γ (XMG1.2). Flourochrome-conjugated streptavidin was used to visualize staining by biotinylated primary antibodies. Events were acquired on FACSCalibur, LSRII, or FACSCanto (Becton Dickinson) flow cytometer and the data were analyzed using the CELLQuest Pro or FlowJo software.

CD4 T Cell Preparation and Differentiation

CD4 cells were purified from single cell suspensions of splenocytes from chimeric mice with anti-mouse CD4 microbeads (Miltenyi Biotec, Auburn, CA). To induce Th differentiation, 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 of IL-2 (Roche, Indianapolis, IN) for 5-7 days. For Th1 differentiation, 3.5 ng/ml of IL-12 and 10 μg/ml of anti-IL-4 (11B11) were added. Th2 cultures were supplemented with 10 ng/ml of IL-4 and 10 μg/ml of anti-IFN-γ (R4-6A2).

Cytokine Intracellular Staining

Freshly-isolated splenocytes depleted of red blood cells or differentiated Th1 and Th2 CD4 T cells were stimulated with 50 ng/ml phorbol myristyl acetate and 1.5 μM ionomycin (Calbiochem, San Diego, CA) for 5 hours. Monensin (Sigma, St. Louis, MO) at 3 μM was added during the last three hours of stimulation. Activated splenic cells were first stained with flurochrome-conjugated anti-CD45.1, anti-CD45.2, anti-CD4, and anti-NK1.1. Activated Th1 and Th2 cells were stained with anti-CD45.1, anti-CD45.2 antibodies. Cells were then fixed in 2-4% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.2% saponin (Sigma), followed by staining with anti-IL4 (11B11) and anti-IFN-γ (XMG1.2) for flow cytometry.

Statistic Analysis

The two-tailed Student's t-test was used to analyze the statistic significance of the difference in percentage of T cells between different groups. P value smaller than 0.05 was considered statistically significant. *= $p<0.05$, **= $p<0.01$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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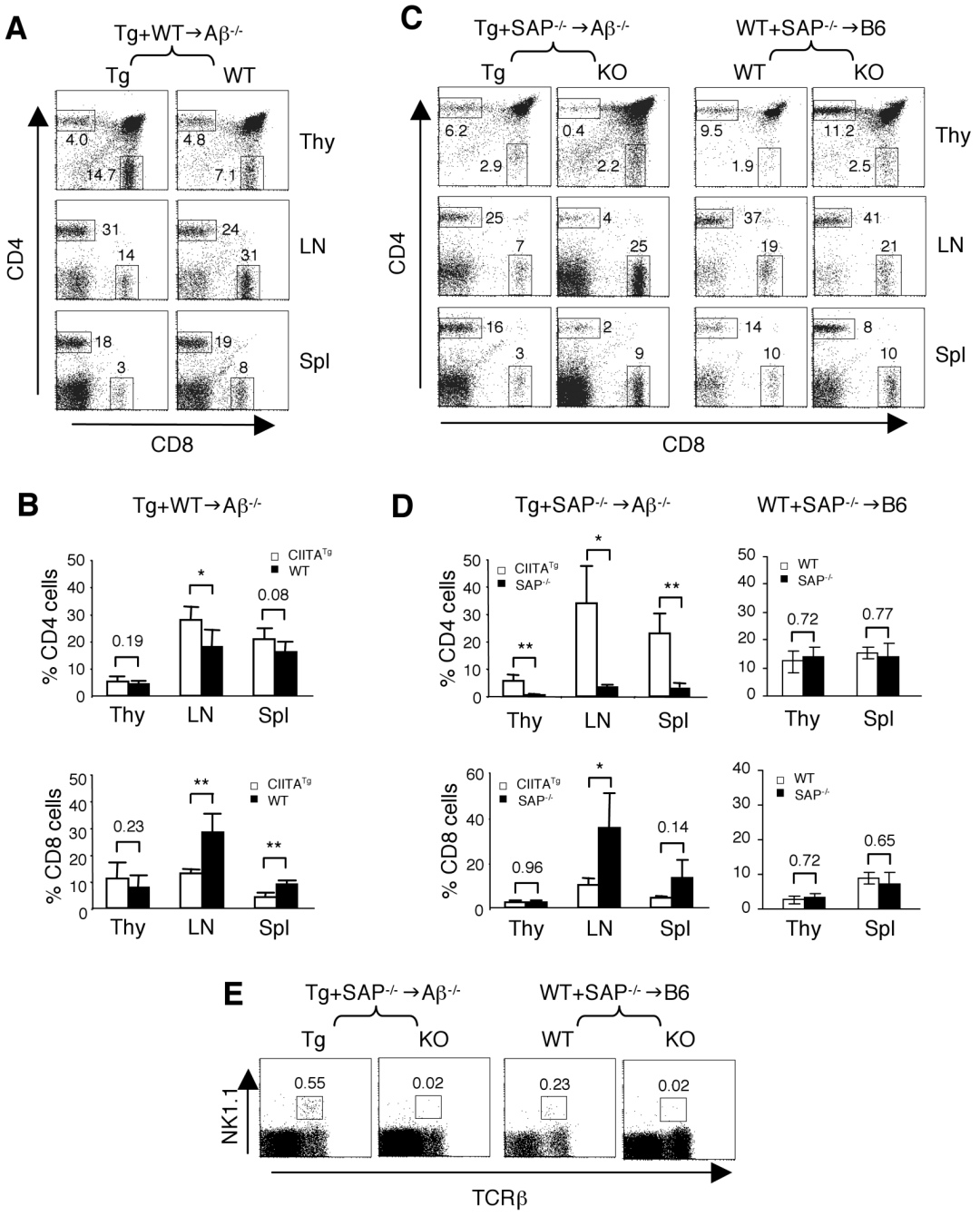

Figure 1. SAP plays a critical role in T-CD4 T cell development

(A) CIITA-expressing thymocytes can efficiently select WT CD4 T cells. CIITA^{Tg} (Tg) BM (CD45.1) were mixed with WT BM (CD45.1/2) and co-transferred to the MHC class II deficient Aβ$^{-/-}$ hosts (CD45.2). Thymocytes, LN and splenic cells from chimeric mice were analyzed for T cell repopulation by Tg and WT BM. The numbers in the dot plots indicate the percentages of gated CD4 and CD8 T cells of each BM driven cells. Data are representative of 5 mice.

(B) Percentages of CD4 (top graph) and CD8 (bottom graph) in Tg+WT$^{-/-}$→Aβ$^{-/-}$ chimeric mice. Data are shown as mean ± SD from 5 mice. * and ** indicates $p<0.05$ and $p<0.01$, respectively.

(C) SAP is necessary for T-CD4 T cell selection. Tg (CD45.1/2) and $SAP^{-/-}$ (CD45.2) BM were co-transferred to $A\beta^{-/-}$ (CD45.1) recipients (left group). As a control, WT (CD45.1/2) and $SAP^{-/-}$ (CD45.2) BM were used to reconstitute B6 (CD45.1) hosts (right group). CD4 and CD8 populations in thymi, LN, and spleens by cells derived from the indicated BM source were shown. The numbers in the dot plots are the percentages of gated CD4 and CD8 cells of each BM driven cells.

(D) Percentages of CD4 (top panels) and CD8 (bottom panels) cells in $Tg+SAP^{-/-}\rightarrow A\beta^{-/-}$ (right groups) and $WT+SAP^{-/-}\rightarrow WT$ (left groups) chimeric mice. Data are shown as mean ± SD from 4 $Tg+SAP^{-/-}\rightarrow A\beta^{-/-}$ mice and 2 $WT+SAP^{-/-}\rightarrow WT$ mice.

(E) Percentage of thymic $NK1.1^{+}TCR\beta^{+}$ NKT cells from different BM in $Tg+SAP^{-/-}\rightarrow A\beta^{-/-}$ (left two panels) and $WT+SAP^{-/-}\rightarrow B6$ chimeras (right two panels). Numbers indicate the percentages of NKT cells among total thymocytes.

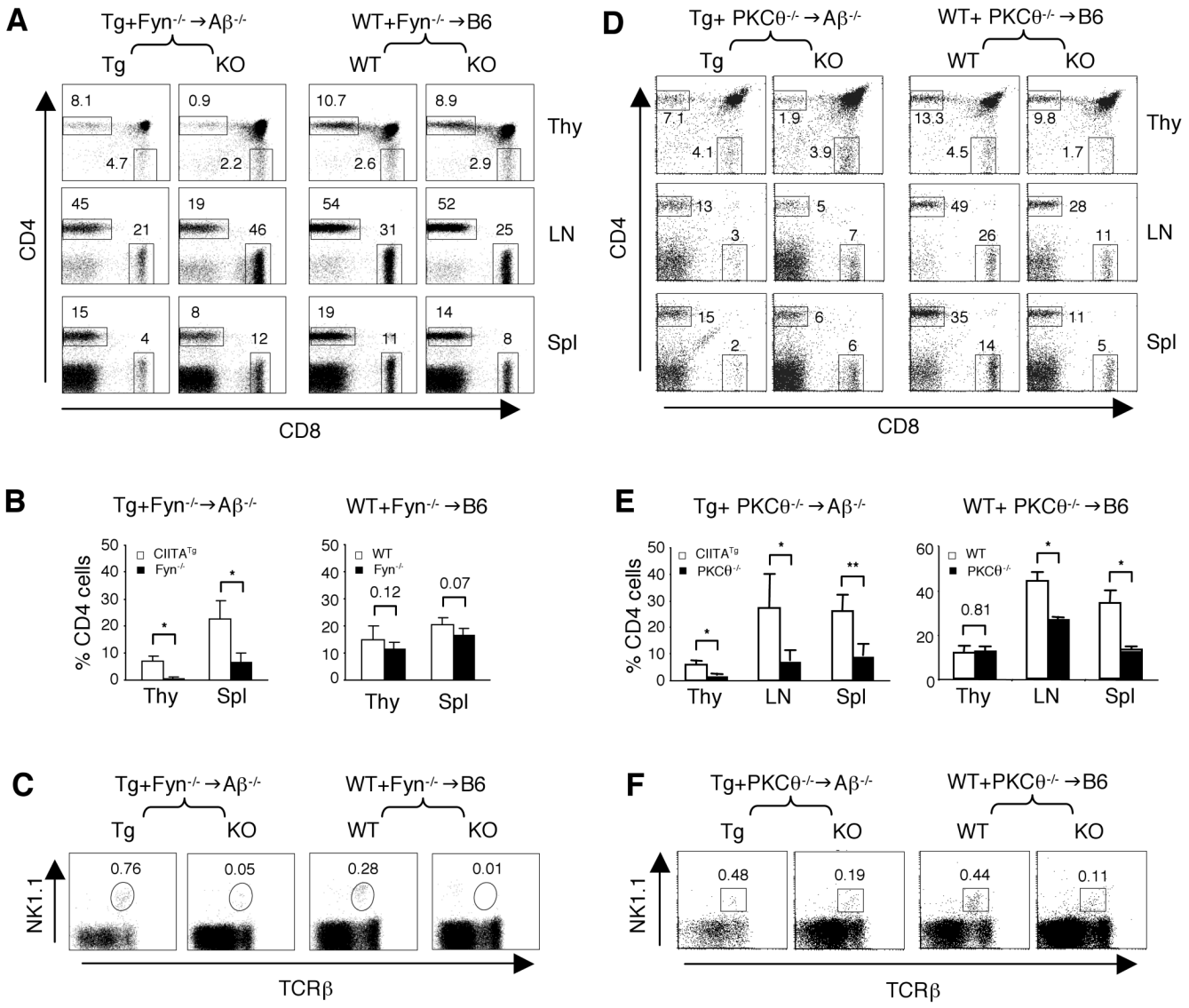

Figure 2. Important role of Fyn and PKCθ in T-CD4 T cell selection

(A) CD4 and CD8 cell profile in thymocytes, LN and splenic cells from Tg+Fyn$^{-/-}$→Aβ$^{-/-}$ (left group) and WT+Fyn$^{-/-}$→B6 (right group). The numbers in the dot plots show the percentages of gated CD4 and CD8 SP thymocytes.

(B) Percentage of CD4 SP thymocytes LN and splenic CD4 T cells in Tg+Fyn$^{-/-}$→Aβ$^{-/-}$ (left group) and WT+Fyn$^{-/-}$→B6 chimeras (right proup). Data are shown as mean ± SD from 3 and 4 mice, respectively.

(C) Percentage of NK1.1^{+}TCRβ^{+} NKT cells in Tg+Fyn$^{-/-}$→Aβ$^{-/-}$ (left group) and WT +Fyn$^{-/-}$→B6 chimeras (right proup). Numbers indicate the percentages of NKT cells among total thymocytes.

(D) CD4 and CD8 profiles of thymocytes, LN and splenic cells from Tg+PKCθ$^{-/-}$→Aβ$^{-/-}$ (left group) and WT+PKCθ$^{-/-}$→B6 (right group). The numbers in the dot plots show the percentages of gated CD4 and CD8 SP thymocytes.

(E) Percentage of CD4 SP thymocytes, CD4 T cells in LN and spleen from Tg +PKCθ$^{-/-}$→Aβ$^{-/-}$ (left group) and WT+PKCθ$^{-/-}$→WT (right group) chimeric mice. Data are shown as mean ± SD from 4 and 3 mice, respectively.

(F) A partial defect in PKCθ$^{-/-}$ NKT cell generation. The percentage of thymic NK1.1^{+}TCRβ^{+}+ NKT cells in Tg+PKCθ$^{-/-}$→Aβ$^{-/-}$ (left two panels) and WT+PKCθ$^{-/-}$→B6 chimeras (right two panels).

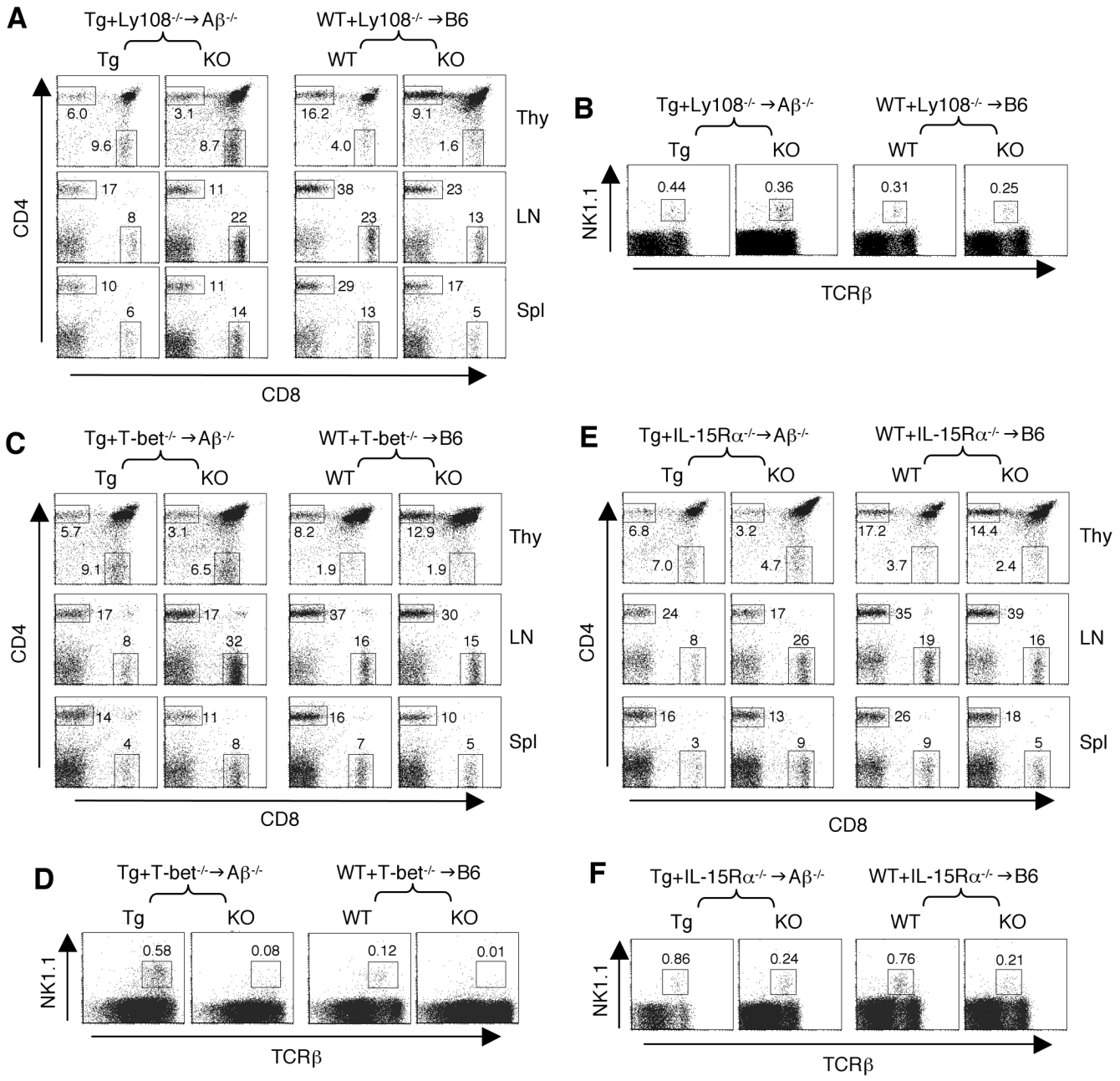

Figure 3. T-CD4 T cell generation in the absence of Ly108, T-bet or IL-15Rα

(A) CD4 and CD8 profiles of thymocytes, LN and splenic cells from Tg+Lyn$^{-/-}$→Aβ$^{-/-}$ (left group) and of WT+Ly108$^{-/-}$→B6 (right group). The numbers in the dot plots show the percentages of gated CD4 and CD8 T cells. The data shown here are one of three chimeras.

(B) A minor defect in Ly108$^{-/-}$ NKT cell generation. The percentage of thymic NK1.1^{+}TCRβ^{+} NKT cells in Tg+Ly108$^{-/-}$→Aβ$^{-/-}$ (left two panels) and WT+Ly108$^{-/-}$→B6 chimeras (right two panels). Numbers indicate the percentages of NKT cells among total thymocytes.

(C) CD4 and CD8 cell populations in thymocytes, LN and splenic cells from Tg+T-bet$^{-/-}$→Aβ$^{-/-}$ (left panels) and of WT+T-bet$^{-/-}$→B6 (right panels). The numbers in the dot plots

show the percentages of gated CD4 and CD8 T cells. The data shown here are one of three chimeras.

(D) A severe defect in T-bet$^{-/-}$ NKT cell generation in both Tg+T-bet$^{-/-}$→Aβ$^{-/-}$ and WT+T-bet$^{-/-}$→B6 chimeric animals. Numbers indicate the percentages of NKT cells among total thymocytes.

(E) CD4 and CD8 profiles from indicated organs in Tg+IL-15Rα$^{-/-}$→Aβ$^{-/-}$ and WT +IL-15Rα$^{-/-}$→B6 mice. The numbers in the dot plots show the percentages of gated CD4 and CD8 T cells driven from each BM source. The data shown here are one of three or four chimeras.

(F) IL-15Rα$^{-/-}$ BM had a reduction of the NKT cell population in the mixed BM chimeric mice. Data are representative of 4 individual chimeras. Numbers indicate the percentages of NKT cells among total thymocytes.

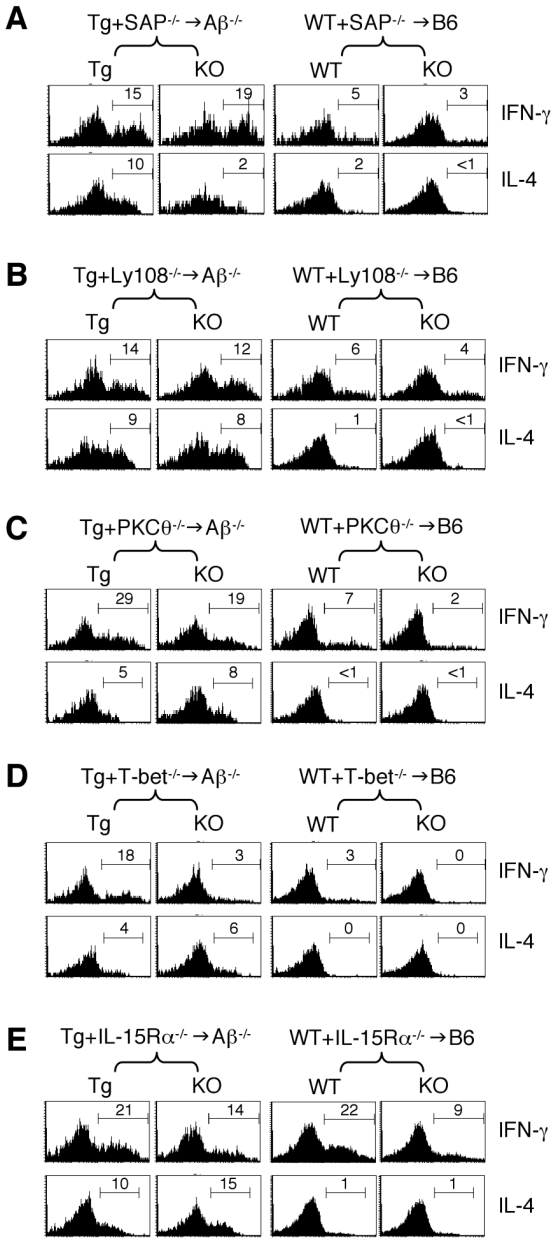

Figure 4. Effect of signaling molecules on the cytokine production potential of T-CD4 T cells

The same chimeric mice described in Figures 1-3 were used to prepare splenic CD4 T cells. Cells were then stimulated for 5 hours as described in Experimental Procedures in the absence of exogenous cytokines. IFN-γ (top row) and IL-4 (bottom row) production from $SAP^{-/-}$ (A), $Ly108^{-/-}$ (B), $PKC\theta^{-/-}$ (C), $T\text{-}bet^{-/-}$ (D), and $IL\text{-}15R\alpha^{-/-}$ (E) was assessed by ICS.

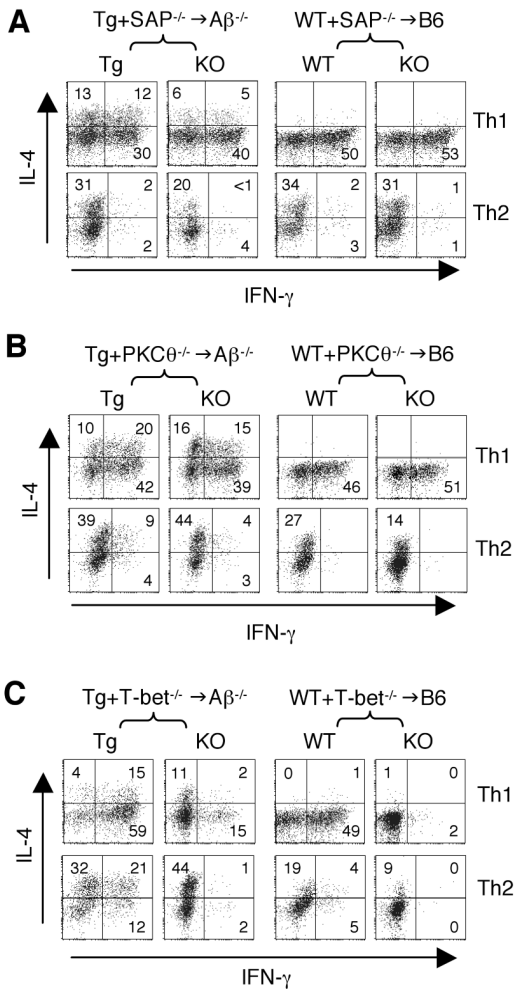

Figure 5. Cytokine production by Th1 or Th2 differentiated T-CD4 T cells deficient in signaling molecules

The same chimeric mice described in Figures 1-3 were used to prepare splenic CD4 T cells. Cells were cultured under the Th1 or Th2 skewing condition, restimulated for 5 hours, and analyzed for IFN-γ and IL-4 production as described in Experimental Procedures. Cytokine production by T-CD4 T cells deficient in SAP (A), PKCθ (B), and T-bet (C) are shown.

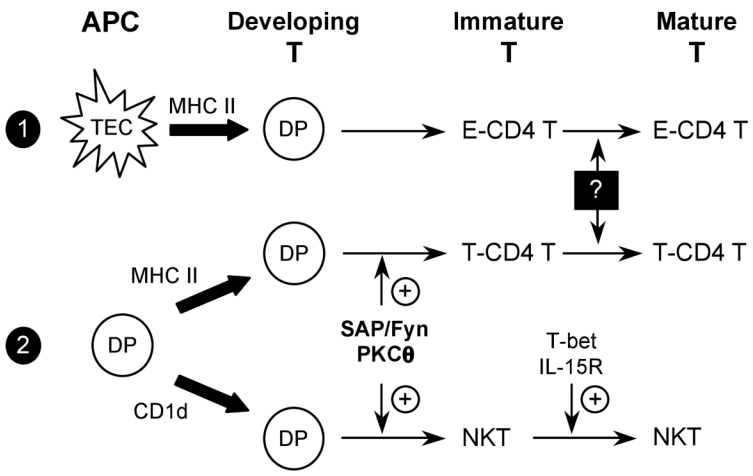

Figure 6. cTEC- and thymocyte-mediated T cell selection pathways

E-CD4 T cells are positively selected through heterotypic interactions between DP thymocytes and MHC class II-expressing cTEC (pathway 1). Thymocyte-mediated T cell selection pathway select T-CD4 T cells and NKT cells through homotypic interactions between TCR on developing DP thymocytes with MHC class II or CD1d on DP thymocytes, respectively (pathway 2). Our studies indicate that the SAP/Fyn/PKCθ signaling cascade is a common pathway required for the generation of both T-CD4 T cells and immature NKT cells, while T-bet and IL-15R are selectively involved in the maturation of NKT cells. Whether E-CD4 T cells and T-CD4 T cells share similar molecular requirements for their maturation is currently unknown.

Table 1

Comparison of two types of CD4 T cells and NKT cells

		CD4 T		NKT
		E-CD4 T	T-CD4 T	
Selecting cell type		Epithelial cells	Thymocytes	Thymocytes
Selecting element		MHC class II	MHC class II	CD1
Antigen		Peptides	?	Glycolipids
TCR repertoire		Diverse	Diverse[a]	Limited
NK1.1 expression		No	No[b]	Yes
Preformed IL-4 mRNA		No	Yes[c]	Yes
Stat6 requirement to express IL-4		Yes	No[d]	No
PKCθ requirement to express IL-4		Yes	No	Yes[e]
Developmental requirement	SAP	No	↓	↓
	Fyn	No	↓	↓
	PKCθ	No	↓	↓
	T-bet	No	NS	↓
	IL-15Rα	No	NS	↓
	Ly108	No	NS	NS

↓, reduced; NS, not significant

References for a, b, c, d, and e are Choi et al. (2005), Li et al. (2005), Li et al. (2007), Patel et al. (2005), and Stanic et al. (2004), respectively.