γδ TCR recruits the Syk/PI3K axis to drive proinflammatory differentiation program

Ryunosuke Muro,1,2 Takeshi Nitta,2 Kent Nakano,3 Tadashi Okamura,3,4 Hiroshi Takayanagi,2 and Harumi Suzuki1

Introduction

γδ T cells have recently attracted considerable attention because of their inflammatory cytokine-producing potential and their contribution to various pathophysiological states. A subset of γδ T cells that produces IL-17 (termed γδ T17 cells) plays a pivotal role not only in protection against bacterial and fungal infection (1) but also in the progression of inflammatory disorders (2–4), tumor growth and metastasis (5–8), and tissue regeneration (9). The cytokine-producing potential of γδ T cells is programmed during their development in the thymus (10, 11).

Unlike αβ T cells, the development of which is dependent on positive and negative selections upon the interaction between αβ T cell receptor (αβ TCR) and peptide-MHC complexes, γδ T cells do not require γδ TCR recognition of ligands for their development in the thymus. Self-oligomerization of γδ TCR at the cell surface of precursor thymocytes induces their differentiation into mature γδ T cells (12, 13). It has been proposed that γδ TCR-ligand interaction determines the effector function of γδ T cells (14). γδ T cells that receive the ligand-dependent strong or ligand-independent weak γδ TCR signals are induced to differentiate into IFN-γ–producing or IL-17–producing subsets, respectively (12, 15). However, the molecular basis for this remains unclear.

Rearranged TCR chains (α/β or γ/δ), together with CD3 subunits (ζ, δ, ε, and ζ), form TCR-CD3 complexes, which initiate the sequential phosphorylation of proximal tyrosine kinases and activation of downstream signaling pathways in response to ligand-dependent or ligand-independent oligomerization (16). A series of studies have indicated the differences between αβ T and γδ T cells in terms of the TCR-CD3 complex structure and downstream signals. Although the CD3ζ subunit is contained in αβ TCR, it is barely detectable in γδ TCR complexes (17). CD3ζ subunit–deficient mice exhibit a developmental arrest of αβ T cells but not γδ T cells (18). The mutation of CD3ε (C80G) that prevents conformational changes in TCR-CD3 complexes completely inhibits αβ T cell development at an early stage. However, it does not impair the development of certain γδ T cell subsets (19). A recent report showed that mice haploinsufficient for CD3γ and CD3δ (CD3γ+/– CD3δ+/–) have reduced TCR signaling and abnormal differentiation in γδ T cells but not αβ T cells (20). αβ T and γδ T cells also show differential requirements of the Src family kinases in their development. In Lck/Fyn–doubly deficient mice, αβ T cell development is completely inhibited, whereas γδ T cell development is partially impaired (21, 22). Moreover, γδ T cells require Blk, an Src family kinase primarily expressed in B cells, for the induction of the γδ T17 subset (23). These findings indicate that the TCR-CD3 complexes and TCR proximal signaling modules in γδ T cells are distinct from those in αβ T cells.

In the present study, we investigated the molecular mechanism underlying γδ TCR signaling that determines the development and effector function of γδ T cells. We found that Syk, a tyrosine kinase known to associate with the B cell receptor (BCR) and Fc receptor, was pivotal for γδ TCR signal transduction and γδ T17 development. Our present results revealed that the deficiency of Syk, but not Zap70, completely abolished the development of γδ T17 and that Zap70 failed to functionally substitute Syk in γδ T17 development. We also showed that Syk distinctively induced the development of γδ T17 through activation of the PI3K/Akt pathway. These results provide a mechanistic insight into the Syk-mediated TCR signal transduction in the determination of γδ T cell fate.

Results

Preferential requirement of Syk rather than Zap70 in γδ TCR signals and γδ T cell development. To characterize the intracellular signal
transduction downstream of γδ TCR, we examined protein tyrosine phosphorylation in ex vivo γδ T cells isolated from the mouse thymus. In response to γδ TCR engagement with anti-CD3ε antibody, we detected tyrosine phosphorylation of signaling proteins such as Zap70 and Lat in γδ T cells (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI95837DS1). Interestingly, we noticed that γδ T cells exhibited TCR-induced tyrosine phosphorylation of Syk, a kinase primarily responsible for signal transduction of the BCR, the Fc receptor, and certain innate receptors (24). Syk is a tyrosine kinase primarily responsible for signal transduction downstream of γδ TCR, the Fc receptor, and certain innate receptors (24). Syk and Zap70, which belong to the Syk family kinases, preferentially associate with certain immune receptors in a cell-specific manner and phosphorylate downstream signaling proteins such as Lat and BLNK. It has been recognized that Zap70, but not Syk, is an essential ζβ TCR proximal kinase required for positive and negative selection of ζβ T cells (25–27). In the early stage of ζβ T cell development, Zap70 and Syk play a redundant role (28).

The phosphorylation of both Zap70 and Syk after γδ TCR stimulation led us to investigate their role in γδ TCR signals. We analyzed γδ T cells in Zap70-deficient (Zap70−/−), Syk-deficient (Syk−/−), and Zap70/Syk–doubly deficient (Zap70−/−Syk−/−) mice. Because the deletion of Syk results in neonatal lethality, we examined γδ T cells isolated from the thymus of these mice at E15.5 or at birth (day 0).

In the E15.5 thymus, we found that the number of CD3ε−TCRε− γδ T cells was comparable between WT and all mutant mice (Figure 1, A and B). On day 0, Zap70−/− mice had a normal number of γδ T cells in the thymus, whereas Sykb−/− and Zap70−/−Sykb−/− mice showed a drastic reduction in the number of thymic γδ T cells, suggesting a critical contribution of Syk to the development of γδ T cells.

To assess the effect of Syk and/or Zap70 deficiency on γδ TCR signaling pathways, we examined the phosphorylation of the MAP kinases ERK1 and ERK2 upon anti-CD3ε stimulation (Figure 1, C and D). In Zap70−/− γδ T cells, ERK phosphorylation was mildly decreased (1 minute after stimulation, 16% reduction of mean fluorescence intensity [MFI]) compared with that detected in WT γδ T cells. Sykb−/− γδ T cells showed a substantial reduction in ERK phosphorylation (79% reduction of MFI), whereas it was undetectable in Zap70−/−/Sykb−/− γδ T cells. These results indicate a dominant role for Syk, but not Zap70, in γδ TCR signaling, despite their functional redundancy. Indeed, the surface expression of CD5, an indicator of in vivo TCR signal strength, was markedly reduced in Sykb−/− γδ T cells and was nearly undetectable in Zap70−/−Sykb−/− γδ T cells, whereas it remained unaffected in Zap70−/− γδ T cells (Figure 1E). Taken together, our results demonstrate that Syk is the major γδ TCR proximal tyrosine kinase in γδ TCR signaling and γδ T cell development in the thymus, whereas Zap70 has only a partial contribution.
Syk, but not Zap70, is required for γδT17 development. Subsequently, we examined the functional differentiation of γδT cells in mice at birth, as γδT17 preferentially develops during the late embryonic stage. In the thymus of WT mice on day 0, a substantial fraction (nearly 20%) of γδT cells produced IL-17 upon stimulation with PMA and ionomycin (Figure 2A). The number of γδT17 cells was reduced by approximately 50% in Zap70−/− mice (Figure 2A). The number of γδT cells expressing RORγt, a transcription factor mandatory for IL-17 production, was reduced in Zap70−/− (Figure 2, A and B). Consistent with these observations, the frequency of γδT cells expressing RORγt, a transcription factor mandatory for IL-17 production, was reduced in Zap70−/− and Sykb−/− mice (Figure 2C). These results indicate that Syk is essential for γδT17 differentiation and that Zap70 is solely required for the Vγ6+ subset of γδT17 cells.

We detected the expression of all Vγ chains (Vγ1, Vγ4, Vγ5, and Vγ6) in E15.5 fetal thymi from WT, Zap70−/−, and Sykb−/− mice (Figure 2D). Analysis of day-0 neonatal thymus revealed that Zap70 was dispensable for the development of most γδT cells, including Vγ1−, Vγ4−, and Vγ5− cells, with the exception of Vγ6− cells. The number of Vγ1−, Vγ4−, and Vγ6− cells, but not Vγ5− cells, was significantly reduced in Sykb−/− neonatal mice. These Vγ cell subsets were further reduced in number or were nearly absent in Zap70−/− Sykb−/− mice (Figure 2D). These results indicate the redundant and nonredundant roles of Zap70 and Syk in the development of different neonatal γδT cell subsets: Vγ1− and Vγ4− cells require Syk, Vγ5− cells require either Zap70 or Syk, and Vγ6− cells require both.

Furthermore, to examine γδT17 development in adult mice, hematopoietic progenitor cells from fetal liver were transplanted into T cell–deficient (Terb−/− Terc−/−) mice. We observed that WT progenitor cells differentiated into γδT17 cells in the thymus, spleen, and lungs of the reconstituted mice (Figure 2E and Supplementary Figure 2, A and B). However, in mice reconstituted with...
γδ T cells. Fetal liver T progenitor cells from Sykb–/– mice were infected with retroviruses expressing Syk or Zap70 along with EGFP and seeded into a fetal thymus organ culture (FTOC) (Figure 3A). Compared with WT cells, the retrovirus-infected Sykb–/– cells in the FTOC expressed approximately 10-fold higher levels of Syk or Zap70 proteins (Figure 3B). Syk expression clearly recovered γδ T cell development from Sykb–/– T progenitor cells (Figure 3, C and G). We found that expression of CD5 in γδ T cells was also completely restored by Syk expression (Figure 3, D and H). Most important, the differentiation of γδ T17 cells was fully restored to WT cell levels (Figure 3, E and I). The overexpression of Zap70 in Sykb–/– progenitors also restored the frequency of γδ T cells (116% of WT and 84% of Syk expression). However, this overexpression failed to fully induce CD5 expression (63% of WT and 59% of Syk expression levels) and γδ T17 development (48% of WT and 44% of Syk expression levels). The frequency of IFN-γ–producing γδ T cells, γδT17 was completely undetectable. These results, along with those in neonatal mice, showed that Syk is required for γδT17 development throughout life.

In contrast, we found that IFN-γ–producing γδT cells were detectable in Zap70–/–, Sykb–/–, and Zap70–/– Sykb–/– mice (Supplemental Figure 3). In agreement with a previous report (29), the IFN-γ–producing potential was detectable in CD4/CD8 double-negative (DN) thymocytes from Rag2–/– mice, indicating that immature thymocytes possess an IFN-γ–producing potential in response to certain extracellular stimuli. Given that Sykb–/– γδT cell development was arrested at the immature stage, we could not investigate the requirement of Syk in γδ TCR-mediated IFN-γ production in mature γδ T cells upon agonistic ligand stimulation.

Zap70 fails to functionally substitute Syk in γδT17 development. To clarify the functional difference between Syk and Zap70, we examined whether Syk can be replaced by Zap70 in developing γδT cells. Fetal liver T progenitor cells from Sykb–/– mice were infected with retroviruses expressing Syk or Zap70 along with EGFP and seeded into a fetal thymus organ culture (FTOC) (Figure 3A). Compared with WT cells, the retrovirus-infected Sykb–/– cells in the FTOC expressed approximately 10-fold higher levels of Syk or Zap70 proteins (Figure 3B). Syk expression clearly recovered γδ T cell development from Sykb–/– T progenitor cells (Figure 3, C and G). We found that expression of CD5 in γδT cells was also completely restored by Syk expression (Figure 3, D and H). Most important, the differentiation of γδT17 cells was fully restored to WT cell levels (Figure 3, E and I). The overexpression of Zap70 in Sykb–/– progenitors also restored the frequency of γδT cells (116% of WT and 84% of Syk expression). However, this overexpression failed to fully induce CD5 expression (63% of WT and 59% of Syk expression levels) and γδT17 development (48% of WT and 44% of Syk expression levels). The frequency of IFN-γ–producing γδT cells.
cells was significantly increased in the absence of Syk but was restored to WT levels by the overexpression of Syk or Zap70 (Figure 3, F and J). These results indicate that, although the expression levels of Zap70 are 10-fold higher than normal levels, Zap70 cannot be a substitute for Syk in γδ TCR signal transduction and induction of γδT17 development in Sykb−/− γδ T cells, suggesting a nonredundant role of Syk in γδ TCR signaling. Requirement of Zap70 in γδ T cells. Despite the critical role of Zap70 in αβ T cell development, our results showed that its requirement in γδ T cell development in the thymus was limited to the Vγ6+ cell subset. We focused on Vγ6+ cells in Zap70−/− mice and observed that CD5 expression levels were normal at E15.5. However, these levels were significantly reduced on day 0, suggesting that Zap70 is not essential for initial γδ TCR signaling but rather is required for the thymic maturation of Vγ6+ γδ T cells, probably via continuous γδ TCR signaling (Supplemental Figure 4, A and B). This idea is supported by the fact that the expression levels of Zap70 protein and mRNA were the highest in the Vγ6+ sub-
set among γδT cells (Supplemental Figure 4C). These results are in agreement with previous findings that Vγ6+ cell development is impaired in mutant mice harboring a hypomorphic Zap70 mutation (30). Zap70 is also required for peripheral Vγ4+ cells, including the γδT17 subset, as well as for IFN-γ-producing γδT cells in the spleen and lungs (Supplemental Figure 4, D and E). In contrast, the Vγ1+ cell subset was normal or increased in the periphery of Zap70–/– mice. Although it still remained unclear why peripheral Vγ4+γδT cells were reduced in Zap70-deficient mice, it is possible that Zap70-dependent TCR signals support the survival and/or migration of these cells. Thus, the requirement of Zap70 in γδT cell development is limited to the thymic maturation of Vγ6+ cells and peripheral maintenance of Vγ4+ cells.

The PI3K/Akt pathway controls γδT17 development. These results prompted us to examine the unique function of Syk in γδTCR signaling and γδT17 development. Previous studies showed that Syk is recruited to the BCR and Fcε receptor for the activation of PI3K in B cells and mast cells, respectively (31). The activated PI3K produces phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which in turn activates downstream protein kinases including Akt, PDK1, and Tec. The level of PIP3 is negatively regulated through its hydrolysis catalyzed by PTEN, a phosphoinositide phosphatase. In αβT cells, αβTCR signal–induced activation of the PI3K/Akt pathway requires CD28 costimulation (32), whereas in γδT cells, the γδTCR signal can induce Akt phosphorylation in the absence of costimulatory signals (19).

Although Akt phosphorylation induced by γδTCR stimulation was not altered in Zap70–/– γδT cells, it was significantly reduced in Sykb–/– γδT cells compared with WT γδT cells (Figure 4, A and B). We found that γδTCR-induced Akt phosphorylation, but not ERK phosphorylation, was completely inhibited by treatment with IC87114, an inhibitor of the p110δ catalytic subunit of PI3K (Supplemental Figure 5, A and B). This finding indicates that Akt activation depends on PI3K in γδTCR signaling. These results demonstrate that Syk plays a critical role in γδTCR-induced activation of the PI3K/Akt pathway.

To examine the roles of PI3K and PTEN in γδT cell development, we cultured E15.5 fetal thymus from WT mice with IC87114 or SF1670, an inhibitor of PTEN. After 7 days of FTOC, the develop-
ment of γδT17 cells and RORγt-expressing γδT cells was drastically impaired by treatment with IC87114, although the total number of γδT cells and IFN-γ–producing γδT cells was not decreased (Figure 4, C–F). We also found that IC87114 reduced the mRNA expression of Rorc, Sox13, and Sox4, the transcription factors essential for γδT17 induction, in γδT cells (Figure 4G). In contrast, we found that the number of γδT17 cells and RORγt-expressing γδT cells was increased by SF1670 treatment, whereas the number of IFN-γ–producing γδT cells was normal (Figure 4, D–F). Vγ6+ cell numbers were significantly reduced by IC87114 and increased by SF1670 treatment, while Vγ4+ cell numbers were increased by SF1670 (Figure 4H).

We further investigated the in vivo role of the PI3K pathway in γδT cell development, using mice doubly deficient in the PI3K catalytic subunits p110γ and p110δ (Pik3cg–/– Pik3cd–/–). The Pik3cg–/– Pik3cd–/– mice had a normal total number of γδT cells and CD5 expression in neonatal thymus (Figure 5, A and B), indicating that PI3K is not required for γδTCR signaling or thymic γδT cell development. ERK phosphorylation occurred in response to γδTCR stimulation (Figure 5C), although γδTCR-induced Akt phosphorylation was severely impaired in Pik3cg–/– Pik3cd–/– γδT cells (Figure 5D). These γδT cells showed a complete loss of IL-17–producing capacity (Figure 5E) and a significant reduction in Vγ4+ and Vγ6+ subsets (Figure 5F). Notably, we found that the development of IFN-γ–producing γδT cells was not impaired in Pik3cg–/– Pik3cd–/– mice (Figure 5E), indicating the specific requirement of PI3K in γδT17 development.

Figure 6. Syk mediates the Lat-independent TCR signal to the PI3K/Akt pathway. (A) Flow cytometric profiles for CD3ε and TCRδ in total thymocytes from 5-week-old WT and Lat–/– mice. The total number of thymocytes is shown above each flow cytometric plot (n = 3). (C) TCR-induced ERK phosphorylation in thymic γδT cells. Graph indicates the MFI relative to the nonstimulated control (n = 3). (D) TCR-induced Akt phosphorylation in thymic γδT cells pretreated or not with IC87114 (10 μM). Graph shows the MFI relative to the nonstimulated control (n = 3). (E) Flow cytometric analysis of Zap70 and Syk expression in thymic γδT cells from 5-week-old WT and Lat–/– mice (n = 3). (E) TCR-induced Akt phosphorylation in Lat–/– γδT cells pretreated or not with BAY61-3606 (10 μM). Graph shows the MFI relative to the nonstimulated control (n = 3). (G) Intracellular staining for IL-17A production in neonatal thymic γδT cells from WT mice (n = 3) and Lat–/– mice (n = 5) after stimulation with PMA and ionomycin. The number of IL-17A+ γδT cells (per mouse) is shown. All data represent the mean ± SEM. *P < 0.05 and **P < 0.01, by 2-way ANOVA (C, D, and F) and unpaired t test (G). Data represent 2 independent experiments (A, B, D, F, and G) or a single experiment (C and E).
Previous studies have shown that the expression of Zap70 and Syk is inversely regulated during αβ T cell development in the thymus; Zap70 is hardly detectable at the DN1–3 stages, increases thereafter, and reaches the maximum level in mature αβ T cells, while Syk is robustly expressed at the DN1–3 stages, gradually decreases thereafter, and reaches an undetectable level at the mature stage (34). In γδ T cells, Syk protein expression is detectable even at the mature stage (35). In agreement with these previous data, we found that Zap70 expression was almost undetectable in Lat−/− γδ T cells, which showed developmental arrest at the DN3 stage. Syk expression was almost comparable between WT and Lat−/− γδ T cells, indicating that γδ TCR signals solely depended on Syk at this stage (Figure 6E). Notably, pretreatment with BAY61-3606, a specific inhibitor of Syk, significantly reduced γδ TCR-induced Akt phosphorylation in Lat−/− γδ T cells (Figure 6F). These results indicate that γδ TCR-induced PI3K/Akt activation depends on Syk but not Lat. Although the PI3K/Akt pathway was intact,

These results indicate that the PI3K/Akt pathway downstream of Syk-mediated γδ TCR signal controls γδ T17 development.

Syk mediates Lat-independent, noncanonical signaling to the PI3K/Akt pathway. How does Syk activate the PI3K/Akt pathway in γδ TCR signaling? Lat is known to be a direct substrate of Zap70 and Syk kinases, acting as a scaffold for downstream signaling molecules. We examined the γδ T cell signaling and developmental potential of Lat-deficient (Lat−/−) mice. As reported previously (33), Lat−/− mice showed a complete arrest of γδ T cell development at the precursor stage, as characterized by the extremely low number of γδ TCR+ cells and the absence of CD5 expression (Figure 6, A and B). Anti-CD3ε-induced ERK phosphorylation was also undetectable in Lat−/− γδ T cells (Figure 6C). Interestingly, we found that Akt phosphorylation levels were normal in anti-CD3ε-stimulated Lat−/− γδ T cells (Figure 6D). This Akt activation was inhibited by treatment with IC87114, indicating that the PI3K/Akt signaling axis is independent of the Lat-mediated pathway.

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Lat−/− γδT cells had no potential to produce IL-17, indicating that the Lat-independent PI3K/Akt pathway is not sufficient for γδT17 differentiation (Figure 6G).

Collectively, our results suggest that γδTCR-induced Syk activation stimulates the Lat-dependent canonical pathway, including the Ras/MAPK cascade, and the Lat-independent noncanonical pathway mediated by the PI3K/Akt axis. The former serves as a mainstream signal for γδT cell differentiation from precursor cells, whereas the latter induces the additional program toward γδT17 differentiation.

The adaptor protein RhoH mediates the γδTCR signaling required for γδT17 development. Previously, we and other groups have reported that Zap70 and Syk require the receptor proximal adaptor protein RhoH for their recruitment to the γδTCR in T cells and the Fce receptor in mast cells, respectively, and that RhoH is essential for the optimal activation of these receptor signals (36, 37). Subsequently, we investigated whether the γδTCR signals also require RhoH-mediated kinase recruitment. γδT cells from RhoH-deficient (Rhoh−/−) mice had a marked reduction in γδTCR stimulation–induced ERK and Akt phosphorylation, indicating that RhoH is required for γδTCR signal transduction (Figure 7, A and B). Indeed, the expression of CD5 in γδT cells was markedly reduced in Rhoh−/− mice (Figure 7C). We found that the neonatal development of Vγ4+ and Vγ6+ γδT17 cells was also significantly impaired in Rhoh−/− mice (Figure 7D). These γδT cell phenotypes recapitulated those of Sykb−/− mice, strongly suggesting that RhoH mediates the γδTCR/Syk signaling axis for the induction of γδT17 development.

Last, we assessed the in vivo significance of RhoH/Syk-mediated γδTCR signals using Rhoh−/− mice and found that Vγ4+ γδT cell numbers were markedly reduced, while Vγ6+ γδT cells were barely detectable in the thymus throughout ontogeny (Supplemental Figure 6, A and B). In adult Rhoh−/− mice, Vγ4+ and Vγ6+ γδT cell numbers were significantly reduced in peripheral tissues, whereas the total numbers of γδT cells and Vγ1+ (spleen and lung), Vγ5+ (skin), and Vγ7 (small intestine) cell subsets were comparable to those in WT mice (Supplemental Figure 6C). In particular, we did not detect γδT17 cells in the thymus or periphery in Rhoh−/− mice (Figure 7, E and F). On the other hand, we found that thymic development of IFN-γ-producing γδT cells was unimpaired in Rhoh−/− mice (Supplemental Figure 3). Previous studies have demonstrated that Vγ4+ γδT17 cells play a crucial role in psoriasis-like dermatitis induced by imiquimod (IMQ). Upon IMQ treatment, Vγ4+ γδT17 cells specifically expand in the draining lymph node and recirculate to inflamed skin (4, 38). We observed that IMQ-induced skin inflammation was significantly attenuated and that the induced increase in Vγ4+ γδT17 cells was completely undetectable in Rhoh−/− mice (Figure 7, G–I). A similar attenuation of inflammation was also observed in mice reconstituted with Syk-deficient fetal liver cells (Supplemental Figure 2C). These results indicate that a deficiency of RhoH-mediated γδTCR signals has a marked impact on the γδT17-mediated inflammatory response in vivo.

Discussion
In this study, we explored the molecular mechanisms of γδTCR signaling pathways for the development and effector fate decision of γδT cells during thymic development, focusing on the role of the receptor proximal tyrosine kinase Syk. Early pioneering studies demonstrated that Syk is required for the development of certain γδT cell subsets such as skin- or intestine-resident γδT cells (35, 39). However, the functional significance of Syk in the repertoire formation and effector function of γδT cells has not been addressed to date. The present study revealed that Syk-dependent γδTCR signals are indispensable for the thymic maturation and acquisition of the effector function of γδT cells.

It has been demonstrated that the TCR signaling machinery differs between γδT and γδT cells (17–23, 40), although these 2 cell types derive from common progenitors in the thymus. We showed here that in ex vivo γδT cells, engagement of the γδTCR-CD3 complex leads to the phosphorylation of Syk and Zap70. Experiments using gene-deficient mice proved that Syk plays a dominant role in γδTCR signaling. Unexpectedly, it was found that Zap70 has a limited contribution to γδTCR signaling and is dispensable for the thymic development of most γδT cells. This markedly contrasts γδTCR signaling and γδT cell development, both of which completely depend on Zap70 but not Syk (25–27). Taken together, these observations indicate that, unlike γδT cells that use the γδTCR/Lck/Zap70 axis, γδT cells use Syk as a dominant γδTCR proximal kinase to initiate downstream signaling cascades. This suggests a common feature of antigen receptor signaling machinery between γδT and B cells rather than γδT cells.

Why do γδT cells preferentially rely on Syk to drive γδTCR signals? It may be at least partly explained by the differential expression of Syk and Zap70 during T cell development (34). We found high Syk and low Zap70 expression in immature γδT cells (such as γδT cells from Lat−/− mice), which might account for the dominant role of Syk rather than Zap70 in the early phase of γδT cell development. While γδT cells lose Syk expression during development (34), γδT cells maintain Syk expression until the mature stage (35). In addition to the quantitative difference, the qualitative difference between Syk and Zap70 may be the cause of their differential requirement. Indeed, our experiments with FTOC demonstrated that the overexpression of Zap70 in Syk-deficient T progenitor cells did not fully restore γδTCR signaling and γδT17 development. A previous report also showed that the ectopic expression of Zap70 in Syk-deficient BM macrophages fails to restore the differentiation into normal osteoclasts (41). The functional incompetence of Zap70 compared with Syk may be explained by the differential behavior of these kinases. The kinase domain of Zap70 has been shown to exert lower catalytic activity than that of Syk (42). It is also possible that Syk and Zap70 have different specificities to target proteins, because the ectopic expression of Syk in human γδT cells can result in altered gene expression downstream of TCR signaling (43). The Src homology 2 (SH2) domains of Syk may be more structurally flexible than those of Zap70 (44), possibly leading to a higher accessibility of Syk to the immunoreceptor tyrosine–based activation motifs (ITAMs) in γδTCR-CD3 complexes. Furthermore, although the activation of Zap70 depends on Lck, Syk functions in an Lck-independent manner (45). These differential properties of the 2 kinases are likely to explain the preferential requirement of Syk in γδTCR signaling.

It is likely that the most important target of Syk in γδT cells is Lat, which forms a signalosome that provides docking sites for SH2-containing proteins such as PLCγ. This leads to activation of the
Ras/MAPK, Ca/NFAT, and PKC0/NF-κB pathways (16). Our results, along with those of a previous study (33), indicate that Lat\(^{-/-}\)γδ T cells show a complete loss of MAPK activation, no signs of maturation, and no γδT17 induction. However, our finding that Akt phosphorylation upon γδ TCR stimulation was not altered in Lat\(^{-/-}\)γδ T cells clearly indicates that the γδ TCR/PI3K/Akt axis is independent of the Lat signallingosome. A previous study using cell-free experiments showed that Syk directly binds to the p85α regulatory subunit of PI3K (46). In B cells, upon BCR stimulation, Syk phosphorylates BCAP, an adaptor protein that interacts with and activates PI3K (47). Activation of the PI3K/Akt pathway in αβ T cells is meditated by the binding of PI3K to the phosphorylated cytoplasmic domain of CD28 upon interaction with its ligands, CD80 or CD86 (48). This mechanism explains the requirement of costimulatory signals from antigen-presenting cells in αβ T cell activation. Our data indicate that γδ TCR signals can directly activate the PI3K/Akt pathway, probably through direct interaction between Syk and PI3K proteins or in an indirect manner, mediated by putative adaptor protein(s). Uncov-

ering the molecular links between Syk and PI3K in γδ T cells still remains a challenge.

Collectively, Syk-mediated γδ TCR signals can activate the canonical pathway, in which the Lat signallingosome acts as a platform for the activation of downstream cascades, as well as the noncanonical accessory pathway mediated by the PI3K/Akt axis. We infer that the latter enables γδ T cells to efficiently respond to antigen recognition without costimulatory signals.

We further elucidated the essential role of the PI3K/Akt pathway in γδT17 development. The induction of γδT17 cells was significantly repressed by pharmacological inhibition or genetic ablation of PI3K and enhanced by PTEN inhibition, indicating that the production of PIP3 is a critical determinant of γδT17 cell differentiation during thymic development. Inhibition of PI3K reduced the expression of the transcription factors RORγt, Sox13, and Sox4 in the developing γδ T cells. Hence, the PI3K/Akt pathway plays a crucial role in the transcriptional program toward the γδT17 lineage. Similarly, the PI3K/Akt pathway directs the differentiation of IL-17–producing Th17 cells (49), suggesting that the PI3K/Akt pathway is a common regulatory system shared by αβ and γδ T cells to induce the differentiation program toward IL-17–producing subsets. A previous study showed that the genetic deficiency or pharmacological inhibition of PI3K attenuates γδT17-dependent inflammation (50), highlighting the physiological importance of this signalling pathway in properly mounting the inflammatory potentials.

In conclusion, we describe the significance of Syk-mediated TCR signalling in the physiological development and effector differentiation of γδ T cells. Syk acts as a dominant γδ TCR proximal tyrosine kinase that activates the canonical signalling cascades mediated by the Lat signallingosome as well as the Lat-independent noncanonical signal for activation of the PI3K/Akt pathway for γδT17 induction (Supplemental Figure 7). Elucidating the functional difference between Syk and Zap70 in terms of T cell function as well as their evolutionary history and contribution to pathogenesis would be intriguing for future studies.

Methods

Mice. C57BL/6 mice were purchased from Japan SLC. Zap70\(^{-/-}\) (25), Rag2\(^{-/-}\) (51), Rhok\(^{-/-}\) (37), Terb\(^{-/-}\) (52), and Tcdr\(^{-/-}\) (53) mice were described previously. Sykβ\(^{-/-}\), Lat\(^{-/-}\), Zap70\(^{-/-}\) Sykβ\(^{-/-}\), and Pik3cg\(^{-/-}\) Pik3cd\(^{-/-}\) mice were generated by the CRISPR/Cas9-mediated genome editing method. All mice were bred and maintained under specific pathogen–free conditions in our animal facility and were euthanized by overdose of inhalation anesthetics.

Antibodies. Monoclonal antibodies against CD4 (GK1.5), CD5 (53-7.3), CD8α (53-6.7), CD25 (PC61.5), TCRβ (H57-597), and RORγt (B2D) were purchased from eBioscience. Monoclonal antibodies against CD3ε (17A2), CD44 (IM7), CD11b (M1/70), CD11c (N418), B220 (RA3-6B2), CD49b (DX5), Gr-1 (RB6-8C5), TER-119 (TER-119), TCRδ (GL3), TCR-Vγ1 (2.11), TCR-Vγ4 (UC3A10A6), TCR-Vγ5 (clone 556), IL-17A (TC11-18H10.1), IFN-γ (XMG1.2), Zap70 (1E7.2), Syk (5F5), and Lat (11B.12) were purchased from BioLegend. Monoclonal antibodies against phosphorylated ERK (p-ERK) (197G2) and p-Akt (D9E) were purchased from Cell Signaling Technology. The monoclonal antibody 17D1, specific for TCR-Vγ6/Vδ1 and TCR-Vγ5/Vδ1, was provided by Robert E. Tigelaar (Yale University, New Haven, Connecticut, USA) and used as described previously (54). Anti-Vγ7 monoclonal antibody (GL1) was provided by the late Léo Lefrançois (University of Connecticut Health Center, Farmington, Connecticut, USA) (55). The γδ T cell subsets (according to the Heilig and Tonegawa nomenclature) examined in this study are listed in Supplemental Table 1.

Flow cytometry and cell sorting. Flow cytometric analysis and cell sorting were performed with FACSCanto II and FACSaria III systems (BD Biosciences). Prior to cell staining, the Fc blocker (anti-mouse CD16/CD32; clone 2.4G2; TONBO Biosciences) was used. Cells were stained with a mixture of the antibodies at a final concentration of 1 to 2 μg/ml 7-Aminoactinomycin D (7AAD) was used to exclude dead cells. For intracellular staining, cells were fixed with IC Fixation Buffer (eBioscience) for 30 minutes and stained with antibodies.

CRISPR/Cas9-mediated genome editing in mice. The preparation of single-guide RNA (sgRNA) and Cas9 mRNA was described previously (56). sgRNA and Cas9 mRNA were injected into the cytoplasm of pronuclear-stage eggs from C57BL/6 mice, and the eggs were transferred into the oviducts of pseudopregnant female ICR mice. The target sequences containing PAM sequences (underlined) were as follows: Zap70, GGCGAGTACGCAATCGCGGCGGAGG; Sykβ-1, CACACCACTACACCATCGAGAGG; Sykβ-2, GCCCAAGAACC-GACCCCTTPOAGG; Lat-1, ACTCAGGCGACGCGACCAGAGG; Lat-2, AGGAAAAACAGCGTTTTCCGGG; Pik3cg-1, GTACGTGTCGCCTGTTACCACTGGG; Pik3cg-2, GTACAAATGCTTGGACGTTGGG; Pik3cd-1, GTTCGGAAAGTCTGGTACTTCGGG; Pik3cd-2, TCTGCTATCGCAGCATACAGG.

Fetal liver chimera mice. E15 fetal liver cells were i.v. injected into x-ray–irradiated (4 Gy) Tcrδ\(^{-/-}\) mice. The cells were observed 8 weeks after the transplantation.

Retroviral infection. cDNA fragments encoding Zap70 or Syk were inserted into the retroviral vector pMSCV-ires-eGFP. Plat-E packaging cells were transfected with the retrovirus plasmid, and its supernatant was used for hematopoietic progenitor cell infection. To obtain hematopoietic progenitor cells, Gr-1\(^{-/-}\)TER119\(^{-/-}\) mice derived from E15.5 fetal livers were cultured in RPMI 1640 medium in the presence of IL-7 (25 ng/ml) and stem cell factor (SCF) (50 ng/ml) for 24 hours. The cells were then infected with retrovirus by the spin-infection method as described previously (57).

FTOC. Thymic lobes isolated from E15.5 fetuses were cultured as previously described (57). IC87114 (SYNkinase) or SF1670 (Milli-
poreSigma) were added into culture medium at 1 μM or 2.5 μM, respectively. For reconstitution with retrovirally transduced T progenitor cells, E15.5 thymic lobes were cultured with 1.5 mM deoxyguanosine (dGuo) for 5 days. The dGuo-treated thymic lobes were incubated with retrovirus-infected fetal liver cells in hanging-drop culture for 24 hours, rinsed with culture medium, and further cultured in normal FTOC.

Preparation of tissue-resident lymphocytes. To prepare lung- and skin-resident lymphocytes, lung or ear tissue from adult mice was minced into small pieces and digested with 0.2% collagenase D (Roche) and 0.01% DNase I (Roche) at 37°C for 30 minutes. The digested tissues were disrupted by using a syringe and 18-gauge needle, and cells were passed through a 100-μm nylon mesh to remove tissue debris. The enzymatic reaction was stopped by adding PBS containing 2 mM EDTA and 2% FCS. To prepare the small intestine cell suspension, gut fragments from which Payer’s patches were removed were cut open and incubated for 30 minutes at 4°C in PBS containing 30 mM EDTA. After incubation, the gut fragments were washed with PBS and then vigorously shaken to collect the small intestine epithelial fraction. Leukocytes from the intestine epithelial fraction were isolated with a 40%-80% Percoll gradient.

Quantitative mRNA analysis. Total RNA was extracted from isolated cells using the RNeasy Kit (QIAGEN) and reverse transcribed with SuperScript III (Invitrogen, Thermo Fisher Scientific). Quantitative PCR was performed with SYBR Premix ExTaq (TaKaRa) and the StepOne Real-Time PCR System (Life Technologies, Thermo Fisher). RM and TN performed most of the experiments, interpreted the results, and prepared the manuscript. HT provided advice on the project design and data interpretation and prepared the manuscript. HS supervised the project and wrote the manuscript.

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Address correspondence to: Takeshi Nitta, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan. Phone: 81.3.5841.3377; Email: nit-im@m.u-tokyo.ac.jp. Or to: Hiroshi Takayanagi, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan. Phone: 81.3.5841.3373; Email: takayana@m.u-tokyo.ac.jp. Or to: Harumi Suzuki, Department of Immunology and Pathology, Research Institute, National Center for Global Health and Medicine, 1-7-1, Kounodai, Ichikawa-shi, Chiba, 272-8516, Japan Phone: 81.47.375.4764; Email: lhbsuzuki@hospk.ncgm.go.jp.


