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The Tec kinases Itk and Rlk regulate conventional versus innate T-cell development

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Summary

Tec family kinases are important components of antigen receptor signaling pathways in B cells, T cells, and mast cells. In T cells, three members of this family, Itk, Rlk, and Tec, are expressed. In the absence of Itk and Rlk, T-cell receptor signaling is impaired, with defects in mitogen-activated protein kinase activation, Ca^{2+} mobilization, and actin polymerization. During T-cell development in the thymus, no role has been found for these kinases in the $CD4^+$ versus $CD8^+$ T-cell lineage decision; however, several studies indicate that Itk and Rlk contribute to the signaling leading to positive and negative selection. In addition, we and others have recently described an important role for Itk and Rlk in the development of conventional as opposed to innate $CD4^+$ and $CD8^+$ T cells. Natural killer T and γδ T-cell populations are also altered in Itk- and Rlk/Itk-deficient mice. These findings strongly suggest that the strength of T-cell receptor signaling during development determines whether T cells mature into conventional versus innate lymphocyte lineages. This lineage decision is also influenced by signaling via SLAM (signaling lymphocytic activation molecule) family receptors. Here we discuss these two signaling pathways that each contribute to conventional versus innate T-cell lineage commitment.

Keywords

kinases; signal transduction; T-cell development

Introduction

T cells are a vital component of the immune system, enhancing both humoral and cellular immunity. In the absence of the T-cell response, immunodeficiencies occur. Conventionally, T cells have an polyclonal repertoire and express αβ chains as components of their T-cell receptors (TCRs) together with the coreceptors CD8 or CD4; more recently, unconventional, innate-like subsets of T cells (hereafter referred to as 'innate T cells') have been described that generally have a more restricted TCR repertoire. Examples of innate T cells include γδ T cells, natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, CD8αα intraepithelial T cells, and H2-M3 restricted T cells (reviewed in 1, 2). Other characteristics that distinguish conventional versus innate T cells include surface marker expression, exertion of effector function, and signaling pathway requirements during development (reviewed in 1, 2).

Conventional αβ T cells exhibit a naive phenotype, characterized by low expression of CD44 and little to no expression of CD122 or NK cell markers. Innate T cells differ in this regard, expressing high levels of CD44, CD122, and NK cell markers, all of which are indicative of an activated or memory phenotype (reviewed in 1). In addition to cell surface

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phenotype, conventional αβ T cells require differentiation following antigen-mediated activation to exert their effector functions such as cytokine secretion or cytotoxicity; in contrast, innate T cells are able to exert their effector functions immediately upon stimulation (reviewed in 1). These differences in phenotype and function may result from differential signaling requirements during development. Most subsets of innate T cells require interleukin-15 (IL-15) and/or the signaling lymphocytic activation molecule (SLAM) signaling pathways for their development, function, and/or survival, whereas these pathways are dispensable for the development of conventional αβ T cells (3, 4).

Role of T-cell receptor signaling in T-cell development

Despite these characteristic differences between conventional and innate T cells, innate T cells undergo similar developmental processes as conventional αβ T cells. Precursors of both the conventional and innate T-cell lineages enter the thymus upon migration from the bone marrow and are characterized as double negative (DN) cells that lack expression of CD4, CD8, and CD3. The DN subset is divided into stages one through four based on expression of CD44 and CD25, where DN1 cells are $CD44^{high}CD25^{-}$, DN2 cells are $CD44^{high}CD25^{+}$, DN3 cells are $CD44^{low}CD25^{+}$, and DN4 cells are $CD44^{low}CD25^{-}$. The bifurcation of αβ and γδ T-cell lineages occurs around the DN2 stage of T-cell development and is discussed in detail later (5, 6). During the DN3 to DN4 transition, αβ T cells undergo selection of the their β chain via signaling of the pre-TCR complex, which consists of the TCRβ chain, the pre-Tα chain, CD3ε, CD3γ, and TCRζ. These signals induce allelic exclusion of the β chain and promote the survival, proliferation, and differentiation of DN thymocytes to the double positive (DP) stage of development, where thymocytes express both CD4 and CD8 coreceptors (7).

Once αβ T cells have reached the DP stage of development, these cells undergo positive and negative selection, where the strength of the TCR signal is critical for the decision of a cell to continue maturation or to die (8). Thymocytes that do not receive TCR signals at this juncture die from neglect, and T cells that react too strongly are induced to undergo cell death to prevent autoreactive cells from entering the periphery. However, some of the unconventional T cell types require strong TCR signals in order to develop, such as $CD4^{+}CD25^{+}$forkhead box protein 3 $(FOXP3)^{+}$ natural T-regulatory (nTreg) and NKT cells. Further, $CD4^{+}$ T cells are thought to also develop from stronger signaling than is required for $CD8^{+}$ T-cell development. For example, inhibition of extracellular signaling kinase (Erk) downstream of TCR signaling, promotes $CD8^{+}$ T-cell development while diminishing the development of $CD4^{+}$ T cells (9–11). In addition, peptide studies have determined that only the weakest agonists can induce the positive selection of H2-M3-restricted T cells while strong agonists induce cell death (12, 13). Further, similar results have been seen with conventional $CD4^{+}$ and $CD8^{+}$ αβ T cells where agonist peptides induce $CD4^{+}$ T cell development and antagonist peptides induce $CD8^{+}$ T-cell development (reviewed in 14). Thus, the strength of the TCR signal plays a major role in T-cell development and lineage differentiation, and this role of TCR signaling appears to be important for the development of both conventional and innate T cells.

Role of Tec kinases in TCR signaling and T-cell development

Inducible T-cell kinase (Itk) and resting lymphocyte kinase (Rlk) are downstream of TCR signaling (Fig. 1). They are members of the Tec family of non-receptor protein tyrosine kinases that is predominantly found in hematopoietic cells. This family consists of five members, Bruton's tyrosine kinase (Btk), Itk, Rlk, Tec, and Bmx. These kinases are structurally similar and contain a kinase domain, a Src homology 2 (SH2) domain, an SH3 domain, a Tec homology domain consisting of a Btk homology domain and a proline-rich

region, and a pleckstrin homology domain (Fig. 2). As an exception to this structure, Rlk has a cysteine-string motif in place of the pleckstrin homology domain, which results in Rlk being constitutively localized at the cell membrane (reviewed in 15) (Fig. 2).

Btk is a major component downstream of B-cell receptor (BCR) signaling, and humans deficient in Btk have defective B-cell development resulting in X-linked agammaglobulinemia (XLA). Similar, although lesser, effects are seen in *xid* mice that contain mutations within Btk (reviewed in 16). This kinase is also expressed in mast cells and is a positive regulator of FcεR1 signaling. Mast cells deficient in Btk have defects in cytokine production and activation (reviewed in 16). In addition to Btk, mast cells also express Itk, Rlk, and Tec. Data from our laboratory indicate a negative role for Itk in mast cells signaling, in that Itk-deficient mast cells secrete increased amounts of cytokine upon stimulation and appear to have increased phospholipase Cγ1 (PLCγ1) phosphorylation (17). These results are intriguing, since Itk has a positive role in αβ TCR signaling.

Itk, Rlk, and Tec are expressed in αβ T cells, and all three kinases appear to have a positive role in TCR signaling. In the absence of Itk or both Rlk and Itk, PLCγ activation is decreased, leading to diminished Ca^{2+} flux and reduced mitogen-associated protein kinase (MAPK) activation (reviewed in 15). This, in turn, results in defective nuclear factor for activated T cells (NFAT) and activator protein-1 (AP-1) activation downstream of TCR signaling. As a consequence, Itk- and Rlk/Itk-deficient T cells produce little IL-2 and have a reduced proliferative response following activation (reviewed in 15). Interestingly, there appears to be a hierarchy in the importance of these kinases in T cells as follows: Itk > Rlk > Tec.

Due to the defects in TCR signaling in Tec kinase-deficient mice, it was surprising that T cells developed in the absence of Itk. To determine if Itk had any role in T-cell development, we and others examined this issue in more detail. The two main stages of T-cell development where Itk might potentially have a role are β selection and positive and negative selection after α chain rearrangement. Our laboratory has recently described that the absence of Itk and Rlk/Itk affects β selection during T-cell development. We found that Itk, Rlk, and Tec are all expressed during the DN stages of T-cell development, although Itk has the highest expression during all four stages. Whereas no block during the DN1 and DN2 stages of development was observed, a modest proliferative defect during the DN to DP transition of development was seen in the absence of Itk or Rlk/Itk (18). After β selection, thymocytes undergo multiple rounds of proliferation as they transition to the DP stage of development (7). In Itk- and Rlk/Itk-deficient thymocytes, this proliferative burst is somewhat blunted (18). Further, Itk-deficient thymocytes were not as effective as wildtype thymocytes at repopulating the DP and SP subsets during competitive repopulation assays (18). Thus, although defects in the development of Itk- and Rlk/Itk-deficient T cells are more substantial at later stages, Tec kinases do appear to have a role in the transition of thymocytes from the DN to the DP stage of maturation.

The effects of the Itk and Rlk/Itk deficiency are more apparent at the DP stage of development. Although lineage commitment of conventional αβ T cells into the $CD4^+$ or $CD8^+$ subsets appears unaltered in the absence of Itk, there are alterations in positive and negative selection. For example, when the major histocompatibility complex (MHC) class I-restricted HY TCR transgenic line was crossed with Itk-, Rlk- or Rlk/Itk-deficient T cells, the positive selection HY^+ $CD8^+$ T cells in transgenic female mice was inhibited in the absence of Itk or Rlk/Itk (19). Further, when HY male mice were examined, $CD8^+$ T cells survived negative selection in the absence of Itk and Rlk/Itk due to impaired death-inducing signals (19). We also examined positive selection in the absence of Itk using the MHC class II-restricted TCR transgenic lines, AND, 5C.C7, and 2B4. These TCRs are specific for same

MHC/peptide antigen but have different avidities for their selecting ligand and thus have different efficiencies of positive selection. Our laboratory found that 2B4 transgenic T cells, with the weakest avidity, had a reduced T-cell population in Itk-deficient mice when compared to wildtype mice (20). However, the AND transgenic mice, which have the highest avidity TCR, had comparable T-cell populations when comparing Itk-deficient and wildtype mice (20). Further, when examining the stages of positive selection via CD69, TCR, and heat stable antigen (HSA) expression, these studies indicated that Itk-deficient thymocytes have a delay in positive selection (20). In addition, the marker for strength of TCR engagement, CD5, was not as highly expressed on Itk-deficient T cells (20). Thus, it appears that a lack of Tec kinases in the TCR signaling cascade can alter the outcome of positive and negative selection processes.

In this review, we examine how Tec kinases might function in the selection of conventional αβ T cells versus innate T cells. As Itk and Rlk have been shown to influence positive and negative selection, it seemed likely that these kinases would also have a role in the lineage decisions between innate versus conventional T-cell subsets. Previously, our laboratory and others (3, 4, 21, 22) showed that Tec kinase-deficient $CD8^+$ T cells have an innate phenotype; in addition, a small $CD4^+$ T-cell population with similar innate characteristics has recently been described in Itk-deficient mice (23). Our laboratory and others (24, 25) have also reported defects in NKT cell development and function, and more recently, we have observed that the γδ T-cell population is affected by the lack of Itk and Rlk/Itk (authors' unpublished data). Several studies have demonstrated a role for SLAM family signaling in the development of many innate T-cell subsets (26–29, reviewed in 2). Thus, it appears that the signals controlling lineage selection and fate may be more complicated than previously thought, with a combination of different signals affecting the outcome of T-cell development. Here we describe how TCR signaling contributes to the conventional versus innate T-cell lineage decision.

The role of Tec kinases in $CD8^+$ T cells

Conventional $CD8^+$ T cells develop in the thymus and require the expression of MHC class I on thymic epithelial cells for their selection. Upon thymic exit, the differentiation of $CD8^+$ T cells continues following antigen encounter. $CD8^+$ T cells are crucial for clearance of intracellular pathogens, and once a cell encounters its specific antigen bound to the MHC class I molecule on an infected cell, $CD8^+$ T cells further mature by downregulating the expression of CD62L and the IL-7 receptor, both of which are found at high levels on the surface of naive cells. After antigen encounter, $CD8^+$ T cells also gain functions to become cytotoxic effector cells, including the expression of perforinand granzyme and the capacity for interferon-γ (IFNγ) production and release (30).

Several phenotypic criteria define naive conventional versus innate $CD8^+$ T cells. Although both cell subsets express an αβ TCR and are selected in the thymus, innate $CD8^+$ T cells express markers found on recently activated and memory T cells and can produce cytokines immediately upon *ex vivo* stimulation. The activation and memory markers these cells express include CD44, the NK cell marker NK1.1, and the IL-15 receptor α chain, CD122. Further, innate $CD8^+$ T cells are dependent on IL-15 signaling for their development and survival and do not require classical MHC class I expressed on the thymic epithelium for their development. Instead, innate $CD8^+$ T cells are selected by non-classical MHC class I molecules expressed on hematopoietic cells during development. Interestingly, Itk-deficient mice have significantly higher numbers of innate $CD8^+$ T cells than their wildtype counterparts; in fact, these innate T cells are the majority of $CD8^+$ T cells found in Itk-deficient mice (reviewed in 1).

Altered CD8+ T-cell development in Tec kinase-deficient mice

Early studies investigating T-cell development in Itk-deficient mice demonstrated the presence of a higher frequency of $CD8^+$ T cells expressing memory markers in the thymus (20). Initially, it was hypothesized that the increased number of $CD8^+$ T cells in the thymuses of Itk- as well as Rlk/Itk-deficient mice was due to defective $CD4^+$ T-cell selection, causing cells to be rerouted to the $CD8^+$ lineage. After lengthy investigation, one early study concluded that the absence of Itk inhibited the positive selection efficiency of $CD4^+$ T cells but that this decrease did not affect the frequency of $CD8^+$ T-cell selection in the Itk-deficient thymuses (20). Later it was found that the $CD8^+$ T cells that develop in Itk- and Rlk/Itk-deficient thymuses are not normal naive T cells; instead, they express a memory-like phenotype and release effector cytokines rapidly upon TCR engagement. These $CD8^+$ innate T cells found in the thymus appeared to develop there and were not peripheral memory T cells that had migrated back into the thymus. This latter point was demonstrated using adoptive transfer of $CD8^+$ T cells and showed that the transferred $Itk^{-/-}CD8^+$ T cells were found solely in peripheral organs and not in the thymus (3). Additionally, to show that these cells developed in the thymus and had not been activated in the periphery and trafficked back into the thymus, fetal thymic organ cultures (FTOC) were analyzed. The results of these experiments also indicated that the innate T cells did arise in the thymus and further indicated that their memory phenotype could not be due to peripheral T-cell activation (4).

The Itk or Rlk/Itk deficiency accounts for the increase in innate $CD8^+$ T cells, but the mechanism or mechanisms that induce the development of these cells are largely unknown. Consistent with conventional $CD8^+$ T cells, the innate T cells appear to be selected in the thymus. However, unlike conventional cells, which are selected on thymic epithelial cells, the innate T cells appear to be selected predominantly on hematopoietic cells. This was demonstrated when Itk- and Rlk/Itk-deficient bone marrow was used to repopulate β2-microglobulin (β2M)-deficient mice (4). Under these conditions, the host did not express MHC class I on the thymic epithelium. The only MHC class I available to the developing thymocytes was found on the donor-derived hematopoietic cells. This experiment yielded somewhat reduced but still substantial numbers of $CD8^+$ T cells, indicating that these cells were selected on other hematopoietic cells in the thymus. Interestingly, Rlk/Itk-sufficient mice that lack the classical MHC class I molecules H2-K^b and H2-D^b ($K^bD^{b-/-}$) did not develop $CD8^+$ T cells, due to the lack of positive selection. However, when K^bD^b-deficient mice also lack Itk, a significant number of $CD8^+$ T cells develop and exhibit the characteristics of innate lymphocytes (4, 26). As an additional control, Itk/K^bD^b-deficient bone marrow was used to repopulate β2M-deficient or wildtype mice. In this experiment, classical MHC class I was not present on either the host or donor cells, and non-classical MHC class I molecules were solely expressed on the donor-derived cells. Despite these deficiencies in classical MHC class I, $Itk^{-/-}CD8^+$ T cells develop, albeit at a reduced frequency (26). However, development was impaired when the host is devoid of all MHC class I molecules, as was demonstrated when Itk/β2M-deficient bone marrow was used to repopulate β2M-deficient hosts. Altogether, these data support the idea that innate $CD8^+$ T cells deficient in Itk develop in the thymus and utilize both classical and non-classical MHC class I molecules for their selection, since $CD8^+$ T cells develop in the absence of classical MHC class I, albeit at a reduced frequency.

Signaling pathways critical for the development of Itk-deficient innate CD8+ T cells

Cell surface molecules that promote the induction and/or survival of innate $CD8^+$ T cells deficient in Itk have been investigated. These studies identified IL-15/IL-15R signaling as

important for the development and survival of these cells. In both the thymus and peripheral lymphoid organs of IL-15- and IL-15/Itk-deficient mice, reduced numbers of $CD8^+$ T cells with an innate phenotype are seen (3). Additional data indicating that IL-15 signaling is necessary for the survival of innate T cells *in vivo* was derived from studies in which IL-15 transpresentation was inhibited by injection of a blocking antibody into Itk-deficient or wildtype mice. After one week, there was a significant reduction in the percentage of innate $CD8^+$ T cells in circulation (21).

In addition to cytokine receptors, signaling via cell surface receptors of the SLAM family have been implicated in the development and survival of innate T cells. Initial studies showed the importance of SLAM family receptors for NKT cell differentiation in the thymus (28, 29, 31). SLAM receptors, most of which engage in homotypic interactions, are found on differentiating thymocytes (reviewed in 2). Since the selection of NKT cells and Itk-deficient innate $CD8^+$ T cells occurs via interactions between thymocytes and other hematopoietic cells, SLAM receptors are optimally positioned to provide signals that would promote the innate versus conventional lineage choice. Interestingly, mice deficient for the intracellular SLAM family receptor signaling molecule, SLAM-associated protein (SAP), do not have a noticeably altered thymic phenotype (26). However, when SAP-deficient mice were crossed with Itk-deficient mice, the resulting thymi had a phenotype strikingly similar to wildtype mice (26). The CD4:CD8 ratio returned to normal, and the $CD8^+$ T cells did not produce cytokines rapidly upon TCR engagement, nor did they express the innate T-cell markers CD44 and CD122 (26). The specific roles for SLAM molecules during the differentiation of innate $CD8^+$ T cells are still under investigation.

Once molecules like SLAM family receptors or the IL-15 receptor have signaled in the absence of Itk, a host of currently undefined signaling cascades must be initiated. One specific factor important for innate T-cell development that has been identified is the transcription factor eomesodermin (Eomes). Eomes was initially discovered in lymphocytes as a complementary transcription factor to T-bet, since T-bet-deficient $CD8^+$ T cells maintained nearly normal effector function (32). Eomes was further shown to regulate both IFNγ and CD122 expression in $CD8^+$ T cells and NK cells (33). Recently, T cells lacking Eomes have been found to be defective in their ability to clear LCMV infections, because their $CD8^+$ T cells cannot differentiate into cytotoxic effector cells (34); these data confirm the role of Eomes in directing $CD8^+$ T-cell effector function. Interestingly, Eomes expression is significantly increased in Itk- and Rlk/Itk-deficient $CD8^+$ innate T cells, which could account for their rapid release of IFNγ upon TCR engagement, as well as their constitutive expression of CD122 (3). However, Eomes is barely detectible in Itk-deficient NKT cells and is not detectable in wildtype NKT cells (24), indicating that this factor is unlikely to be the master transcriptional regulator of all innate-like lymphocytes. Recently, the transcription factor promyelocytic leukemia zinc finger (PLZF) has been described as a key regulator of NKT cell development (35, 36). Nonetheless, it remains possible that Eomes is important in other innate $CD8^+$ T-cell subsets. To date, the regulation of Eomes expression is not well characterized; in Itk- and Rlk/Itk-deficient $CD8^+$ T cells, it is not known whether upregulation of Eomes results from decreased TCR signal strength, from SLAM family signaling, or from a combination of these two signaling pathways.

The role of Itk-deficient innate $CD8^+$ T cells in infection

A major defining attribute of innate T cells is their ability to exert effector function rapidly following their initial stimulation. Thus, it is no surprise that some subsets of innate T cells are important during the early stages of infection. For example, H2-M3-restricted $CD8^+$ T cells help control *Listeria monocytogenes* during the first several days post-infection (37). Few infectious models have probed the responses of Itk-deficient $CD8^+$ T cells, and these

models have centered on viral infections. However, a recent study demonstrated that Itk-deficient mice have a significantly lower bacterial burden at day three post-infection with *L. monocytogenes* when compared to wildtype mice (22). Itk-deficient $CD8^+$ T cells were also able to decrease the bacterial burden of IFNγ-deficient mice upon adoptive transfer of these cells prior to infection (22). Thus, Itk-deficient $CD8^+$ T cells appear to function similarly to H2-M3-restricted $CD8^+$ T cells.

Despite a positive role for Itk-deficient $CD8^+$ T cells during *L. monocytogenes* infection, the absence of Itk appears to have a negative affect during viral infection. Cytotoxic responses of $CD8^+$ T cells deficient in Itk are impaired in response to vaccinia virus (VV) and vesicular stomatitis virus (VSV), and slightly impaired in response to lymphocytic choriomeningitis virus (LCMV) strain WE (38). A more recent study using the Armstrong strain of LCMV found that Itk-deficient mice also had reduced $CD8^+$ T-cell proliferation, IFNγ production, and tumor necrosis factor α (TNFα) production compared to wildtype mice (39). Nonetheless, these defects were all modest, as Itk-deficient mice are able to clear infections of each of these viruses (38, 39).

One unresolved issue is whether innate $CD8^+$ T cells are able to generate memory responses to viral infections. We have recently investigated this issue for Itk-deficient $CD8^+$ T cells. We find that when LCMV(Armstrong)-immune splenocytes from wildtype or Itk-deficient mice were transferred into a congenic host prior to challenge with LCMV CL13, mice receiving LCMV-immune Itk-deficient splenocytes had a significant increase in viral titer in comparison to mice that received wildtype LCMV-immune splenocytes (authors' unpublished data). In addition, similar defects in IFNγ and TNFα production from Itk-deficient 'memory' $CD8^+$ T cells were observed during the secondary response, as seen during the primary response (authors' unpublished data). There are several possible explanations for the impaired memory responses of Itk-deficient $CD8^+$ T cells. For instance, IL-2 signaling is critical for the induction and maintenance of memory $CD8^+$ T cells (40), and IL-2 is not produced in large quantities by Itk-deficient $CD4^+$ or $CD8^+$ T cells. Also, the TCR repertoire of Itk-deficient $CD8^+$ T cells has been shown to be diverse by Vβ staining (unpublished data), but it remains possible that the precursor frequency of LCMV-specific T cells in $Itk^{-/-}$mice is reduced compared to wildtype mice. It is also possible that the Itk-deficient $CD8^+$ T cells, which are innate T cells, are terminally differentiated prior to the infection and thus unable to respond differently to secondary challenge. Current studies are underway to distinguish between these possibilities.

The role of Tec kinases in $CD4^+$ T cells

T cells expressing TCRs with an appropriate affinity for MHC class II molecules during development are positively selected to differentiate into conventional naive $CD4^+$ T cells (9–11, 14). These cells are a major component of the immune system, contributing to both cellular and humoral immunity; in addition, $CD4^+$ T cells are required for memory formation of $CD8^+$ T cells (reviewed in 41). Following antigen recognition, $CD4^+$ T cells have the ability to differentiate into T helper 1 (Th1) and T helper 2 (Th2) cells that are characterized by their ability to secrete IFNγ and IL-4, respectively. Th1 cells also secrete IL-2 and lymphotoxin-α (LTα), differentiate under the control the transcription factor T-bet, and are required for the clearance of intracellular pathogens, such as *Listeria monocytogenes*, *Toxoplasma gondii*, and *Leishmania major* (reviewed in 42). Th2 cells secrete IL-4, IL-5, IL-13, and IL-10 in addition to IL-4. These cells are controlled by the transcription factor GATA-3 and are required for the clearance of extracellular pathogens, such as *Shcistosoma mansoni* and *Nippostrongylus brasiliensis* (reviewed in 42). In addition, Th2 cells have been implicated in allergy and asthma disorders, which highlight the importance of controlling the regulation of Th2 cells (reviewed in 42).

Recently, two other subsets of $CD4^+$ T helper cells have been described. Inducible T-regulatory cells (iTregs) are thought to differentiate in the periphery rather than during T-cell development in the thymus. These cells secrete TGFβ and IL-10, similarly to nTregs that develop in the thymus, and have been implicated in the maintenance of peripheral self-tolerance (reviewed in 42). In addition to iTregs, Th17 cells have been described, and these cells are key components in the pathologies associated with autoimmune and inflammatory diseases. These cells secrete IL-17A, IL-17F, and IL-21 and are characterized by the transcription factor RORγT (reviewed in 42). Although Th1 and Th2 cells differentiate preferentially compared to Th17 cells, these latter Th cells are involved in controlling fungal and extracellular bacterial infections. TGFβ plays a major role in the differentiation of iTregs and Th17 cells: the addition of IL-6 leads to Th17 differentiation, and the absence of IL-6 leads to iTreg differentiation (reviewed in 42). With the discovery of these new subsets of Th cells, the role of Tec family kinases in the differentiation pathways promoting these lineages will be an interesting area of future study.

The role of Tec kinases in Th cell differentiation

The role of Tec kinases has been examined in the differentiation of Th1 and Th2 cells. Although Itk-deficient mice succumb to infection with *T. gondii* (43), *in vitro* studies showed that Itk-deficient $CD4^+$ T cells preferentially differentiate into Th1 cells (44). This preference is due to dysregulated expression of the transcription factor T-bet (44). However, Itk-deficient $CD4^+$ T cells can be forced into the Th2 lineage *in vitro*, but defects in IL-4 production upon secondary stimulation are still observed (45). Thus, Itk may be required for Th2 differentiation as well as for the proper regulation of T-bet expression and, in addition, for Th2 effector function upon secondary stimulation. Supporting this notion, Itk-deficient mice are unable to mount the appropriate Th2 response to parasites such as *S. mansoni*, *N. brasiliensis*, and fail to make the characteristic Th2 response against *L. major* in Balb/c mice (46, 47). Recently, evidence supporting the regulation of T-bet activity by Itk has been reported, suggesting an additional aspect to the function of Itk in Th cell differentiation (48). Itk has also been shown to promote NFATc translocation (47), thereby contributing to the regulation of IL-4 production.

In striking contrast to Itk, Rlk appears to have a role in Th1 differentiation. This function of Rlk may in part explain the strong preference of Itk-deficient $CD4^+$ T cells to differentiate into the Th1 lineage. Rlk is expressed in Th1 cells as early as 24 h post-stimulation (44). Further, when Rlk/Itk-deficient mice are infected with *S. mansoni*, an appropriate Th2 response occurs, rather than the nonprotective Th1 response seen in Itk-deficient mice to this pathogen (46). Thus, with the current data available, the role of Itk and Rlk in the differentiation of Th cells involves a complex network, and how Itk regulates T-bet and GATA-3 in an *in vivo* system requires further investigation.

The role of Itk and Rlk/Itk in the development of $CD4^+$ innate T cells

In depth analysis of thymocytes from Itk- and Rlk/Itk-deficient mice indicates the presence of a subset of innate $CD4^+$ T cells in addition to those expressing CD8. These $CD4^+$ T cells have an activated phenotype, including the expression of high levels of CD44 and CD122, and can secrete IL-4 and IFNγ immediately upon *ex vivo* stimulation (23); however, unlike the innate $CD8^+$ T cells described in these mice, these innate $CD4^+$ T cells require MHC class II expression on the thymic epithelium for their development (authors' unpublished data). Further, these Itk- and Rlk/Itk-deficient innate $CD4^+$ T cells express high levels of Eomes, similarly to the phenotype seen for the Itk- and Rlk/Itk-deficient $CD8^+$ T cells (unpublished data). T-bet has also been found to be upregulated in Itk-deficient $CD4^+$ T cells with this innate phenotype (23). However, in Rlk/Itk-deficient mice, there are fewer

innate T cells expressing CD4 than in the single Itk-deficient mice (unpublished data). This may be related to the differences seen with Th cell differentiation, in which Rlk/Itk-deficient $CD4^+$ T cells can mount Th2 responses, while Itk-deficient $CD4^+$ T cells cannot (46).

The innate phenotype of $CD4^+$ T cells is not seen in Itk-deficient mice crossed to the 5C.C7 TCR transgenic line (authors' unpublished data). This finding is again similar to that observed with innate $Itk^{-/-}CD8^+$ T cells. In the latter case, OT-I TCR transgenic $Itk^{-/-}CD8^+$ T cells also develop into the conventional T-cell lineage (3). These data suggest that expression of these transgenic TCRs induces stronger signaling during development, thus overcoming the defects leading to innate T-cell lineage commitment. This hypothesis is supported by data showing that expression of a hyperactive allele of the ERK MAPK restores conventional $CD8^+$ T-cell development in $Itk^{-/-}$mice (4). In addition, expression of a kinase-dead mutant form of Itk in wildtype $CD4^+$ T cells promotes the maturation of innate $CD4^+$ T cells (23). Thus, signaling by Itk inhibits the development of innate T cells in the thymus.

Signaling pathways involved in the development of innate $CD4^+$ T cells deficient in Itk

To ensure that Itk-deficient innate $CD4^+$ T cells develop in the thymus, FTOCs were generated, and the results confirmed that innate T cells lacking Itk develop in the thymus (23). Further, the development of innate $CD4^+$ T cells was shown to be due to a cell-intrinsic defect (23). An additional similarity to innate $CD8^+$ T cells and NKT cells is the finding that Itk-deficient innate $CD4^+$ T cells require SLAM family signals, since SAP/Itk-deficient mice do not develop these innate $CD4^+$ T cells (Mueller and Schwartzberg, personal communication). In contrast to innate $CD8^+$ T cells in Itk-deficient mice, additional data from our laboratory demonstrates that IL-15 is not required for the development of Itk-deficient innate $CD4^+$ T cells (unpublished data). In fact, IL-15 deficiency, which results in the absence of innate $CD8^+$ T cells, appears to promote the development of Itk-deficient $CD4^+$ T cells (unpublished data). It may be that, in the absence of innate $CD8^+$ T cells, competition for resources such as IL-7 are reduced, allowing innate $CD4^+$ T cells to thrive.

A subset of innate $CD4^+$ T cells, called 'thymocyte-selected $CD4^+$ cells' (T-CD4), has been described recently; these cells closely resemble the innate $CD4^+$ T cells in Itk-and Rlk/Itk-deficient mice (49, 50). These T-CD4 cells are selected by MHC class II expression on thymocytes, which is accomplished via transgene-driven expression of MHC class II transactivator (CIITA) in DP thymocytes. T-CD4 cells also require SAP for development, which further implicates SLAM family signaling in the development of innate $CD4^+$ T cells (27). However, unlike T-CD4 cells, Itk-deficient $CD4^+$ T cells require MHC class II expression on the thymic epithelium for their selection (unpublished data). This was demonstrated by our laboratory using bone marrow chimeras; in these experiments, bone marrow from wildtype, Itk-deficient, or Rlk/Itk-deficient mice was transferred into lethally irradiated MHC class II-deficient ($H2\text{-}A^{b-/-}$) hosts. In these recipients, all $CD4^+$ T-cell development was severely impaired (unpublished data). Thus, the innate $CD4^+$ T cells seen in Itk- and Rlk/Itk-deficient mice appear to require interactions with the thymic epithelium as well as with SLAM family receptors present on hematopoietic cells during selection in the thymus. Understanding the details of these interactions will require further investigation.

The role of Itk in invariant NKT cells

The canonical NKT cells are innate lymphocytes characterized by their invariant TCRα chain and the ability to secrete both the Th2 cytokine IL-4 and the Th1 cytokine IFNγ upon stimulation. In addition, NKT cells have the ability to produce TNF, IL-2, IL-4, IL-13,

IL-10, IL-5, IL-9, IL-12p70, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (reviewed in 51, 52). The invariant TCR expressed by NKT cells uses Vα14-Jα18 paired with either Vβ8.2, Vβ7, or Vβ2 in mice, or Vα24-Jα18 paired with Vβ11 in humans (reviewed in 51, 52). In mice, this invariant TCR is specific for glycolipids, including the self-glycosphingolipid iGB3, bound to the MHC class Ib molecule, CD1d. These lymphocytes reside predominantly in the thymus, spleen, liver, and bone marrow and are rarely detected in the lymph nodes or intestine (reviewed in 51, 52).

During development, NKT cell precursors undergo three distinct stages characterized by cell surface markers and cytokine production. Stage one of NKT cell development consists of thymocytes that are $HSA^{high}CD4^{high}CD8^{-}$ but lack expression of NK1.1 or CD44 (reviewed in 51, 52). In addition, these cells are capable of producing IL-4 upon stimulation. Stage two of NKT cell development is marked by upregulation of CD44 and downregulation of HSA, and stage three NKT cells upregulate other surface markers characteristic of NKT cells, *i.e.* NK1.1 and CD69 (reviewed in 51, 52). Further, as NKT cells mature, they undergo a switch in cytokine production; immature NKT cells produce high levels of IL-4, whereas more mature NKT cells produce high levels of IFNγ but less IL-4 (reviewed in 51, 52). Additionally, $NK1.1^{-}$ thymocytes expressing the invariant Vα14-Jα18 TCR are able to migrate into the periphery, where they later mature into $NK1.1^{+}$ NKT cells (reviewed in 51, 52).

Role of TCR and SLAM family signaling in NKT cell development

The strength of TCR signaling is thought to regulate the development of NKT cells, with strong signaling promoting NKT cell selection. Interestingly, TCR signaling requirements differ dramatically between NKT and conventional T-cell development. For instance, the Src kinase Fyn is required for NKT cell development but is dispensable for conventional T-cell development (reviewed in 51). Further, Fyn may have a role in NKT cell development prior to TCR expression on developing NKT cells, as was suggested by studies in which Vα14 TCR transgenic mice were crossed to Fyn-deficient mice. Although some NKT cells developed in these mice, NKT cell numbers are significantly diminished (reviewed in 51). Interestingly, Fyn interacts with SAP downstream of SLAM family receptors; of these receptors, SLAMF1 and SLAMF6 have been implicated in the development of NKT cells (28). SAP is also required, as indicated by the defective development of NKT cells in mice and humans deficient in SAP (29, 31). Thus, the role of Fyn in NKT cell development may be associated more with SLAM signaling than with a role downstream of the TCR.

PLZF has been identified recently as a key regulator of NKT cell development (35, 36). This transcription factor is highly expressed during stage one and two of NKT cell development, but expression of PLZF decreases as NKT cells mature (35, 36). Therefore, although Fyn-deficient liver NKT cells express similar levels of PLZF as wildtype NKT cells (35), it remains possible that PLZF expression may be decreased in immature thymic NKT cells in Fyn-deficient mice. However, NKT cells deficient in SAP arrest at an early stage of development, and these arrested cells express PLZF (36). Thus, expression of PLZF is not dependent upon SLAM family receptor signaling.

The role of Itk in NKT cell development and function

Our laboratory and others (24, 25, 53) have determined recently that Itk is also important for the development of NKT cells. This finding is interesting, since Itk is required for the efficient development of conventional $CD4^{+}$ and $CD8^{+}\alpha\beta$ T cells (3, 4, 23, authors' unpublished data). The overall NKT cell percentages and numbers are significantly decreased in Itk-deficient mice in comparison to wildtype mice (24, 25). This decrease has been seen at both HSA^{high} and HSA^{low} stages of development, indicating that Itk may affect

NKT cell development prior to TCR expression (24). One possibility is that Itk interacts with Fyn downstream of SLAM family receptor signaling prior to TCR expression. To date, Itk-deficient mice have not been crossed to Vα14 TCR transgenic mice to determine if the development of NKT cells can be rescued by expression of the canonical TCR, as is seen in Fyn-deficient mice. Further, it does not appear that the transcription factor PLZF is expressed in innate T cells from Itk-deficient mice (35), although Itk-deficient NKT cells have not been directly tested for PLZF expression. Thus, Itk may be required to promote PLZF expression downstream of SLAM family receptor signaling during NKT cell development.

Another possible explanation for the defect in NKT cells in Itk-deficient mice relates to our recent observation that CD1d expression is lower on TCRβ^{high} CD8 SP thymocytes in these mice compared to wildtype mice (unpublished data). Although it has been shown that expression of CD1d is required on DP thymocytes for selection of NKT cells, a role for CD1d expression on SP thymocytes has not been examined. Thus, it is possible that continuous TCR signaling is required for NKT cells to complete their maturation, and this ongoing signal is impaired in the absence of Itk. Together, these data suggest that a combination of reduced TCR signals and reduced CD1d expression may contribute to decreased NKT cell numbers and impaired maturation in Itk-deficient mice.

Upon stimulation through the TCR, Itk-deficient NKT cells produce virtually no IL-4 or IFNγ (24, 25). However, when the requirement for Itk is bypassed by stimulating cells with phorbol myristate acetate plus ionomycin, cytokine production by *Itk*$^{-/-}$NKT cells can be detected (24). In these studies, we found that a higher proportion of Itk-deficient NKT cells produce IL-4 than IFNγ, further demonstrating the role of Itk in NKT cell maturation (24). However, Itk does not appear to be involved in the basal transcription of cytokine genes by NKT cells, as cytokine mRNA levels appear to be similar between *Itk*$^{-/-}$and wildtype NKT cells (25), nor is it involved in the proliferation of these cells (24). Instead, the bulk of the data indicate a predominant role for Itk in the maturation, survival, and maintenance of NKT cells.

Role of Tec family kinases in γδ T cells

As described above, Itk has been shown to play a role in the differentiation of innate versus conventional αβ T cells. In addition to the increase in innate T cells, Itk-deficient mice have elevated levels of serum IgE and enriched germinal centers (GCs) (9, manuscript submitted). Previous data from our laboratory and others indicated that this surprising phenotype is unlikely to be caused by excess IL-4 production from CD4^{+} T cells or NKT cells, as Itk-deficient CD4^{+} T cells and NKT cells both have defects in IL-4 production following TCR stimulation. Instead, our recent data indicate that *Itk*$^{-/-}$γδ T cells are responsible for this phenotype.

γδ T cells, unlike conventional αβ T cells, participate in the early immune response by modulating humoral immunity (54). The majority of γδ T cells are not restricted by MHC and can recognize non-protein antigens of endogenous origin as well as recognizing soluble proteins (55). With a few exceptions, the selection signals regulating the development of γδ T cells is not well understood; however, G8-TCR γδ $^{+}$ T cells are selected by class 1b molecules, and epidermal γδ T cells are selected by the protein encoded by *Skint1* (56, 57). Similar to innate immune system components, γδ T cells constitute a small fraction of the lymphocytes in the blood and peripheral organs but represent over half of the lymphocytes in epithelial rich tissues, including the reproductive tract, skin, and intestine (58). Within these tissues, γδ T cells are the first line of defense; furthermore, they assist in wound repair and also modulate the innate and adaptive immune responses. Additionally, studies using

mice deficient in γδ T cells have shown that these cells aid in clearance of viral, bacterial, and parasitic infections (59–70). While a great deal is currently known about the function of Itk in innate versus conventionalαβ T-cell lineage decisions, little information is available about the role of Itk inγδ T cells.

Altered γδ T-cell development in Itk-deficient mice

The exact mechanism of lineage bifurcation between γδ T cells and αβ T cells is not well understood. Attempts to clarify this issue have led to two competing models. The selection model states that selection of γδ T cells is independent of TCR signaling, while the instruction model states that the TCR isotype (pre-TCR or γδ TCR) dictates lineage commitment (71–73). Recent data have been published to support both models. Evidence for the selection model includes data indicating that expression of rearranged TCRβ, TCRγ, and TCRδ genes in thymic precursors does not affect the developmental outcome (74). Recently, the transcription factor Sox13 has been shown to be critical in the specification of γδ T cells. This gene is required for γδ T-cell but not for αβ T-cell development. Ectopic expression of Sox13 in DN2 and DN3 precursors blocks αβ T cell development and induces a γδ lineage-specific molecular program (75). In contrast, evidence for the instruction model includes studies involving signal strength, in which increasing γδ TCR stimulus favored γδ TCR development over αβ TCR development (76). To date, there is no information regarding a role for Itk in γδ TCR-mediated signaling pathways.

While both innate and adaptive T-cell subsets arise from thymic lymphoid progenitors, lineage divergence occurs at different stages. γδ T cells, similar to the precursors of the αβ T-cell lineage, are derived from $CD4^{-}CD8^{-}$ precursors (5). Recent studies have attempted to elucidate the timing of lineage commitment through use of monolayers of OP9 bone marrow stromal cell that express the Notch ligand Delta-like-1. These data confirmed that by the DN3 stage, most thymocytes are committed to the αβ lineage, whereas the DN2 subset contained a developmentally heterogeneous population (6). Unlike the αβ T cells that transition from a DN to a $CD4^{+}CD8^{+}$ stage, the majority ofγδ T cells remain DN as they mature (77, 78).

While γδ T cells are predominantly DN, a small proportion of them are CD4 or CD8 single positive (79, 80). Further, it has been reported that a subset of γδ T cells express NK1.1 (81). We have found recently that Itk-deficient mice have increased numbers of γδ T cells, and that this increase is largely due to expansion of the $CD4^{+}$ and $NK1.1^{+}$γδ T-cell populations (manuscript submitted). In Rlk/Itk-deficient mice, there is an increase in the $CD4^{+}$γδ T-cell population in the thymus, spleen, and lymph nodes, similar to the increase seen in Itk-deficient mice (unpublished data). Interestingly, studies show that TCRβ/CD5 double deficient mice have an increased population of $CD4^{+}$γδ T cells (80). It is unclear whether expression of CD5, a molecule that negatively regulates differentiation of $CD4^{+}$αβ T cells and TCR-CD3 signaling, is affecting Itk-deficient γδ T cells (82). CD5 expression is increased in Itk-deficient $CD4^{+}$γδ T cells, which is in contrast to studies demonstrating that CD5 expression is low on $CD4^{+}$γδ T cells in wildtype mice (manuscript submitted). Overall, our data indicate that Itk plays a negative role in the development of $CD4^{+}$ and $NK1.1^{+}$γδ T cells (manuscript submitted).

γδ T-cell function is defective in Itk-deficient mice

In wildtype mice, γδ T cells account for 1–5% of the total lymphocytes in the blood and peripheral organs. However, in the absence of Itk, γδ T cells in the thymus, spleen, and mesenteric lymph nodes are increased in number. Mice lacking both Itk and Rlk have an increase in the proportion and number of γδ T cells in the thymus but not in the spleen or lymph nodes (Fig. 3). In contrast, $Itk^{-/-}$mice have no increase inγδ T cells in the intestine,

indicating that Itk does not affect the development of intestinal epithelial lymphocytes (authors' unpublished data).

The heterogeneity of γδ T cells allows them to play an essential role in protective immunity against infection. As mentioned previously, γδ T-cell-deficient mice are more susceptible to infection with viruses, bacteria, and parasites compared to wildtype mice (59, 63–70). The role γδ T cells during these types of infection depends upon environmental factors, the structure of the antigen receptor, and at what stage of the immune response they become activated (55). Based on our findings of altered γδ T-cell development in *Itk*$^{-/-}$mice, we evaluated the function of these γδ T cells. Interestingly, we first determined that the spontaneously high serum levels of immunoglobulin E (IgE) and enriched GCs seen in *Itk*$^{-/-}$ mice are abolished in *Itk*$^{-/-}$mice that also lacked γδ T cells (unpublished data).

To investigate this finding further, we examined cytokine production by Itk-deficient γδ T cells. We find an increase in both CD4^{+} as well as NK1.1^{+} γδ T-cell subsets in the spleens of Itk-deficient mice, and these populations secrete IL-4 (manuscript submitted). In addition, the Itk-deficient NK1.1^{+} population of γδ T cells also secretes higher levels of IL-10 and IL-13 than the wildtype population, and there was decreased production of IFNγ from this *Itk*$^{-/-}$γδ T-cell subset when compared to their wildtype counterparts (manuscript submitted). Thus, in the absence of Itk, γδ T-cell development is skewed towards populations that produce Th2-promoting cytokines.

In preliminary studies, we have also examined γδ T cells from mice lacking both Itk and Rlk. We find that the CD4^{+} γδ T-cell population is increased in these mice (Fig. 4), and this population is unable to secrete IL-4, IL-10, IL-13, or IFN γ (Fig. 5). These data suggest that TCR signaling in Itk-deficient γδ T cells may still function via compensation by Rlk. Further studies with Rlk-deficient γδ T cells will be required to assess the function of Rlk in γδ T-cell signaling.

The absence of Itk leads to aberrant development and function of γδ T cells, characterized by an excess of IL-4 and IL-13 production that presumably induces the increased levels of serum IgE and the systemic Th2 phenotype seen in Itk-deficient mice. A similar spontaneous enhancement of IgE production has also been reported in mice deficient in Itch (an E3-ubiquitin ligase), and further, γδ T cells have been reported to play a role in systemic IgE responses in allergic eosinophilic airway inflammation (85, 86). Interestingly, in mice expressing a mutant allele of the adapter protein linker for activation of T cells (LAT), where the three c-terminal tyrosines have been mutated to phenylalanine, an increased γδ T-cell population has also been seen. Like the Itk-deficient γδ T cells, these LAT-mutant γδ T are predominantly CD4^{+} and secrete IL-4 when stimulated (87). These similarities suggest that Itk and LAT are involved in a common signaling pathway, as is also the case in conventional αβ T cells.

Conclusions

In this review, we have described the role of the Tec family kinases Itk and Rlk in the development and function of a wide variety of T-cell subsets. Although Itk and Rlk have not been implicated in the lineage decision giving rise to CD4^{+} and CD8^{+}αβ T cells (19, 20), our laboratory and others (3, 4, 23) have demonstrated a role for Itk and Rlk in the lineage decision between conventional versus innate T cells. While we find that some cell types that require strong signaling thresholds for development, such as nTregs, are present in Itk- and Rlk/Itk-deficient mice, other subsets, such as NKT cells, are severely diminished (24, 25).

Although conventional CD4^{+} T cells also require a high threshold of signaling (9–11, 14) and are still found in Itk- and Rlk/Itk-deficient mice (23, unpublished data), the numbers

conventional $CD4^+$ T cells are reduced in the absence of these Tec kinases. Intriguingly, a novel subset of innate $CD4^+$ T cells develop in Itk- and Rlk/Itk-deficient mice, and these cells resemble the recently described T-CD4 cells. Since this innate $CD4^+$ T cell subset in *Itk*$^{-/-}$and *Rlk/Itk*$^{-/-}$mice requires MHC class II expression on the thymic epithelium for their development, these data highlight the importance of TCR signaling in the development of these innate T cells (unpublished data)(Fig. 7). Overall, we hypothesize that these cells develop due to a reduced signaling threshold in the absence of Itk. Further, the absence of both Itk and Rlk may diminish TCR signaling to the extent that these innate $CD4^+$ T cells die from neglect, leading to a reduced frequency of these cells in Rlk/Itk-deficient mice compared to Itk-deficient mice. It is also possible that Rlk has a unique role in innate $CD4^+$ T cells, for instance in the upregulation of T-bet and Eomes; thus, in the absence of the Rlk, these innate $CD4^+$ T cells may be unable to develop. Data from our laboratory indicate that Eomes expression is reduced in innate $CD4^+$ T cells deficient in Rlk/Itk when compared to the same cells deficient in Itk alone (unpublished data). Additionally, data indicating that Itk regulates T-bet may help to explain how these innate T cells develop in Itk-deficient mice (48). For instance, during conventional T-cell development, Itk may aid in the regulation of T-bet and/or Eomes to suppress premature T-cell differentiation into effector cells, and thereby to promote conventional T-cell development.

The most dramatic effect of Itk is on the $CD8^+$ innate T-cell lineage. In the absence of Itk or Itk and Rlk, virtually all $CD8^+$ T cells develop into this lineage (Fig. 6). One possibility is that the development of innate $CD8^+$ T cells is induced by a lower threshold of TCR signaling than is required for conventional $CD8^+$ T-cell development. These cells express high levels of Eomes. As discussed above, Itk may be required to downregulate Eomes expression during conventional $CD8^+$ T-cell development; thus, in the absence of Itk, Eomes may be induced thereby promoting the phenotype and function of $CD8^+$ memory T cells.

Both the $CD4^+$ and $CD8^+$ innate T-cell subsets deficient in Itk require SLAM family signals for their development. Thus, it is also possible that Itk works to inhibit these signals during conventional T-cell development (Fig. 7). SAP, the adapter required for SLAM family signaling, has been shown to interact with Fyn (reviewed in 2, 88), a Src family kinase. As Itk activation is dependent on phosphorylation by a Src kinase (reviewed in 15), it may be that SLAM signaling activates Itk. In this scenario, Itk would be expected to have a role in negatively regulating the SLAM signaling pathway. Alternatively, Itk may promote strong or prolonged TCR signaling that overrides SLAM family signaling to promote conventional versus innate T-cell development. Evidence in favor of this TCR override hypothesis includes the finding that conventional $CD4^+$ and $CD8^+$ T-cell development is restored in 5C.C7 and OT-I transgenic T cells lacking Itk, as well as in T cells expressing a hyperactive allele of ERK MAPK (3, 4, authors' unpublished data). Future studies dissecting the signals involved in the developmental regulation of conventional versus innate $CD4^+$ and $CD8^+$ T lineages will be required to resolve these issues.

γδ T cells are also increased in Itk- and Rlk/Itk-deficient mice (manuscript submitted). However, with γδ T cells, the role of the Tec family kinases is less clear due to the lack of biochemical data linking Itk and/or Rlk to γδ TCR signaling. Thus, Itk and Rlk may also play a role in other signaling pathways required for γδ T-cell development. Nonetheless, Itk and Rlk appear to contribute to the lineage differentiation of γδ T-cell subsets, as exemplified by the increase in $CD4^+$ and $NK1.1^+$ γδ T-cell populations in mice lacking these kinases (manuscript submitted) (Fig. 7). While genetic data demonstrate that *Itk*$^{-/-}$γδ T cells promote a systemic Th2 phenotype leading to elevated levels of serum IgE (manuscript submitted), there are as yet no data probing the function of theseγδ T cells during immune responses to pathogenic infections.

The work described in this review highlights the importance of TCR signaling in the development of innate and conventional lymphocytes (Figs 6 and 7). While the role of SLAM family signaling in the development of innate T cells has been elucidated recently, work from our laboratory and others demonstrates how the balance of TCR and SLAM family signaling pathways determines conventional versus innate T-cell lineage fate.

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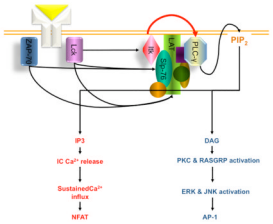

Fig. 1. Role of Tec kinases in TCR signaling

Upon stimulation of the TCR, Tec kinases (only Itk is shown for simplicity) are activated by the Src kinase, Lck, and bind to a complex involving LAT and SLP-76. Itk then phosphorylates PLCγ to induce Ca^{2+} mobilization and MAPK activation, resulting in the activation of transcription factors, such as NFAT and AP-1.

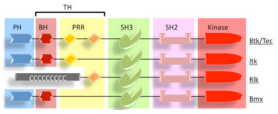

Fig. 2. Structures of the Tec family of non-receptor protein tyrosine kinases

The five members of the Tec family of kinases are depicted, indicating their domain structure. Each member contains C-terminal kinase domain, an SH2 domain for interaction with phosphorylated tyrosines, and an SH3 domain for interaction with proline-rich regions (PRRs). Other domains include a Tec homology (TH) domain that may contain a Btk homology domain plus one or two PRRs. With the exception of Rlk, each contains an N-terminal pleckstrin homology domain for localization to the cell membrane upon activation. Rlk instead contains an N-terminal cysteine-string motif that becomes palmitoylated to localize Rlk to the cell membrane.

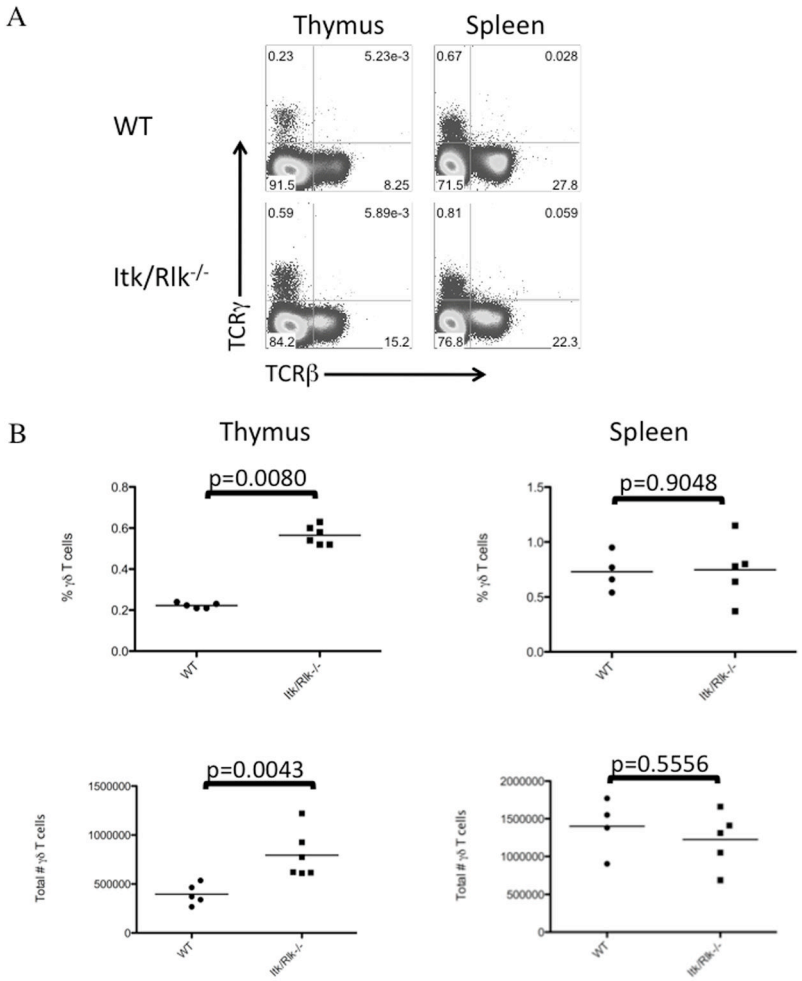

Fig. 3.

***Rlk/Itk*$^{-/-}$ mice have an increased proportion of γδ T cells in the thymus.** Cells were prepared from thymus and spleen of wildtype (WT) and *Rlk/Itk*$^{-/-}$ mice. (A) Cells were stained with anti-TCRγ and anti-TCRβ antibodies and analyzed by flow cytometry. Numbers in quadrants indicate the percentage of cells in each subset. Data are representative of 2 independent experiments with 2–3 mice per group. (B) Percentages and absolute numbers of TCRγ^{+} cells, based on TCRγ^{+}TCRβ staining, are indicated for the thymus and spleen. Each data point represents a different animal, and the bars represent the mean. p values were determined using the Mann-Whitney test.

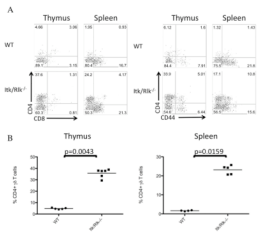

Fig. 4. Altered γδ T-cell subsets in the spleen and thymus of *Rlk/Itk*$^{-/-}$ mice

Cells were prepared from thymus and spleen of wildtype (WT) and *Rlk/Itk*$^{-/-}$ mice. (A) CD4 versus CD8 expression (left panels) and CD4 versus CD44 expression (right panels) on gated TCRγ^{+}TCRβ cells. (B) The percentages of CD4^{+}TCRγ^{+} cells in the thymus (n=5–6 mice) and spleen (n=4–5 mice) are shown. Each data point represents a different animal, and the bars represent the mean. Data are representative of 2 independent experiments with 2–3 mice per group. Statistically significant differences are shown; p values were determined using the Mann-Whitney test.

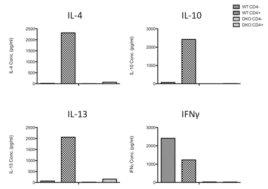

Fig. 5.

***Rlk/Itk*$^{-/-}$γδ T cells show impaired production of cytokines following *ex vivo* activation.**

Lymph node and spleen cells from WT and *Rlk/Itk*$^{-/-}$mice were pooled and TCRγ^{+} cells were isolated by cell sorting. 5× 10^{4} cells were stimulated with 10 μg/ml of anti-TCRγ antibody for 72 h, and supernatants were analyzed for the presence of IL-4, IL-10, IL-13, and IFNγ by cytometric bead array (CBA).

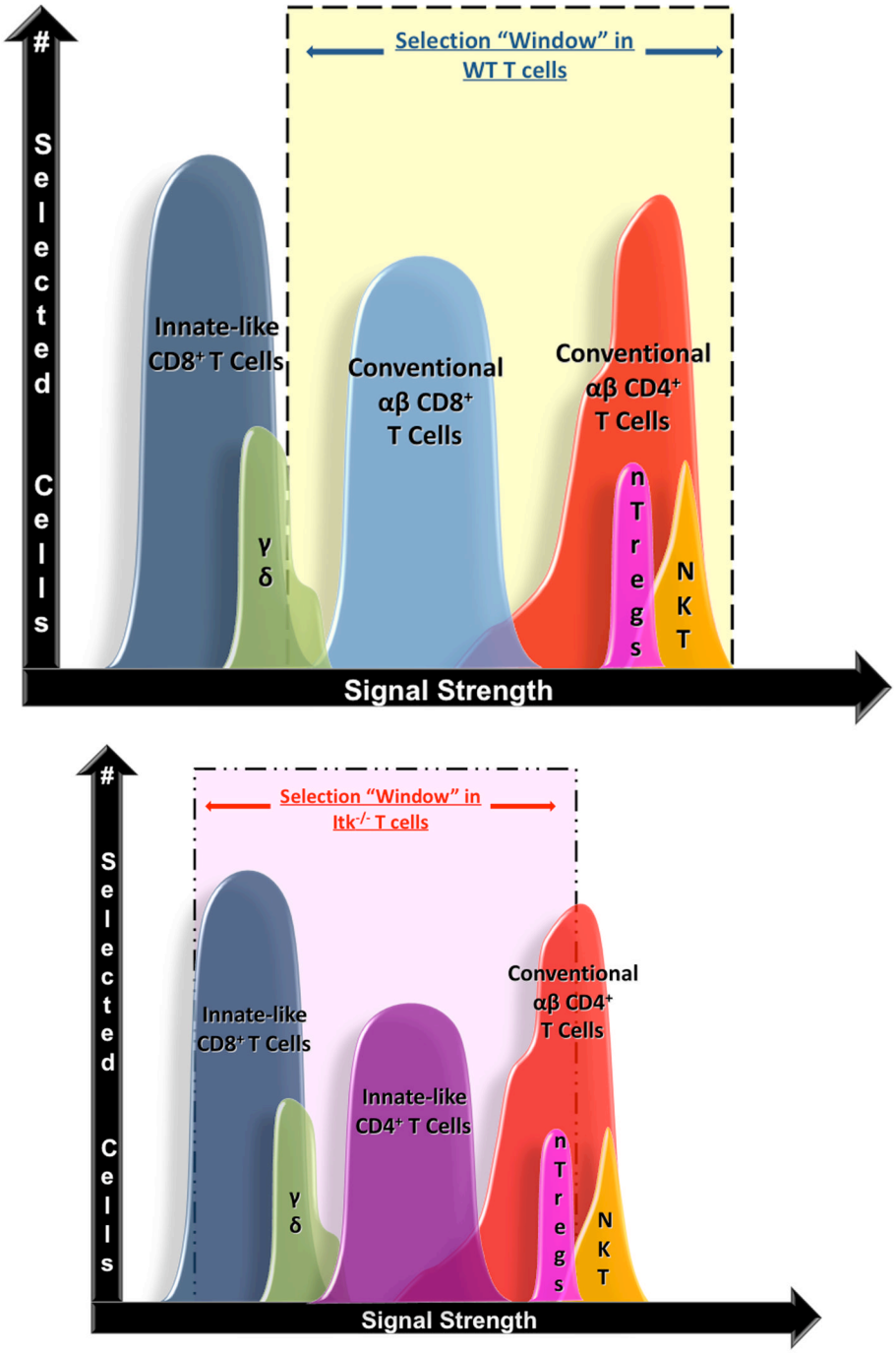

Fig. 6. Model depicting the role of TCR signaling in thymocyte lineage differentiation

(A). In the presence of Itk and Rlk, strong signaling thresholds promote the development of Tregs, NKT, and CD4$^+$ T cells, while weaker signaling promotes the development of conventional CD8$^+$ and γδ T cells. Few innate CD8$^+$ T cells develop under these conditions. (B) In the absence of Tec kinases, the signaling threshold for development in altered. Although a reduced population of conventional αβ CD4$^+$ T cells and Tregs remain, NKT and conventional αβ CD8$^+$ T cells are severely diminished. Populations of innate CD8$^+$ and CD4$^+$ T cells develop instead, and there is an increase in the γδ T-cell population. Thus, by

lowering the signaling threshold during development, innate-like lymphocytes are able to develop at the expense of conventional T cells.

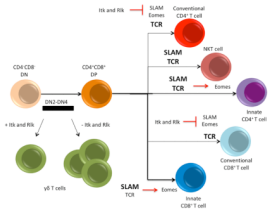

Fig. 7. TCR and SLAM family receptor signaling in conventional and innate T cell development

During the DN2 to DN3 stages of thymocytes development, γδ T cells are thought to differentiate from αβ T cells. In the absence of the Tec family kinases Itk and Rlk, the γδ T-cell population increases. In addition to the decision between γδ or αβ T-cell development, Itk and Rlk are also involved at the DP stage of development during the decision of an αβ T cell to become a conventional or innate T cell. Strong TCR signaling inhibits SLAM family signaling that potentially leads to the induction of the transcription factor Eomes. This inhibition leads to the development of conventional αβ $CD4^+$ and $CD8^+$ T cells. Interestingly, NKT cells appear to require both strong TCR signaling and SLAM family signals for their development. However, in conditions where TCR signaling is weak but SLAM family signaling is intact, innate-like $CD4^+$ and $CD8^+$ T cells develop. Thus, TCR signaling plays an important role in this developmental decision.