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The Role of TEC Family Kinases in Innate T Cell Development and Function: a Dissertation

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THE ROLE OF TEC FAMILY KINASES IN INNATE T CELL DEVELOPMENT AND FUNCTION

A Dissertation Presented

By

Martin Felices

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Abstract

The Tec family kinases Itk and Rlk have been previously shown to have an important role in signaling downstream of the T cell receptor [TCR]. Almost all of the work done in the past on these two kinases looked at their role in conventional αβ T cells, specifically $CD4^+$ T cells. These studies demonstrated functions for Itk [primarily] and Rlk in T cell development, activation, and differentiation. However, despite the wealth of knowledge on conventional $CD4^+$ T cells, prior to the work presented here little to no studies addressed the role of Tec family kinases on $CD8^+$ or innate T cell development. My studies show a clear role for Itk [and in some cases Rlk] in innate T cell development; whether it be deprecating, in the case of innate $CD8^+$ T cells or some subsets of γδ T cells, or beneficial, in the case of NKT cells. I show that Itk has a crucial role in conventional $CD8^+$ T cell development, as absence of Itk [or Itk and Rlk] causes strongly reduced numbers of conventional $CD8^+$ T cells and a vigorous enhancement of an innate-like $CD8^+$ T cell population. In NKT cells, my work demonstrates that Itk [and to a lesser extent Rlk] is required for terminal maturation, survival, and cytokine secretion. Finally, on γδ T cells Itk is important in maintaining the Th1 cytokine secretion profile usually associated with these cells, and regulating the development of $CD4^+$ or $NK1.1^+$ γδ T cells. Taken

together, this work clearly illustrates an important role for Tec family kinases in innate T cell development and function.

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This Thesis is dedicated to my grandmother, Luisa Garcia Novelli [Guagua]. Espero que donde sea que estes esto ponga una sonrisa en tu cara. Te quiero y te estra~o.

CHAPTER I: Intro

Chapter I Attributions and Copyright information

Some of the material in the intro [particularly in the Tec family kinase section] was derived from the Felices et al. 2007 *Advances in Immunology* review (1). All of the material derived was from sections originally written by me.

Felices, M., M. Falk, Y. Kosaka, and L. J. Berg. 2007. Tec kinases in T cell and mast cell signaling. Advances in immunology 93:145-184.

Innate versus adaptive immunity

The immune system has been classically divided into two arms, the innate immune response and the adaptive immune response. The innate immune response provides rapid, non-specific responses to invading pathogens without conferring protective immunity. A large group of cells with varied functions and tissue distributions make up the innate immune system. Amongst these are mast cells, phagocytes [including macrophages, neutrophils, and dendritic cells], basophils and eosinophils, NK cells, NKT cells, γδ T cells, CD8αα T cells, non-classical MHC class Ib restricted $CD8^+$ T cells, B1 B cells and marginal zone B cells. On the other hand, the adaptive immune response provides delayed, specific responses to pathogens which will eventually engender memory and long lasting protective immunity to that pathogen. The adaptive immune response is composed of "conventional" αβ T cells [$CD4^+$ or $CD8^+$] and B cells.

The distinction between innate and adaptive immunity is less clear cut than the original dogma dictated, with several subsets of cells containing characteristics of both arms (2). For instance γδ T cells, NKT cells, m2B cells, and B1 B cells all require RAG mediated rearrangement in order to develop, a criteria usually associated with adaptive immunity. Likewise, NK cells and neutrophils can posses both variable and memory immune receptors, also associated with the adaptive arm (3, 4). Human γδ T cells have been shown to

be capable of upregulating costimulatory molecules typically involved in B cell help, a hallmark previously thought to be restricted to conventional $CD4^+$ T cells (5). This and other data seems to indicate that certain subsets of cells cannot easily be placed in the innate arm or the adaptive arm, but rather belong to both. Perhaps the best way to think of the immune response is not as two separate branches of cells responding with different kinetics and functions, but rather as a continuum of signals leading to resolution of a pathogen and protective immunity. Of particular interest to the work presented here is the development and function of certain subsets of innate T cells, specifically innate $CD8^+$ T cells, NKT cells, and γδ T cells.

Innate T cell subsets

Although there are several unconventional T cell subsets, including CD8αα T cells, MR1 restricted $CD8^+$ T cells and $FOXP3^+CD4^+CD25^+$ regulatory T cells, this thesis will focus solely on three innate T cell subsets: innate $CD8^+$ T cells [specifically H2-M3 restricted], NKT cells, and γδ T cells. Other than their ability to respond quickly and their expression of NK1.1 [only in some subsets when looking at the γδ cells], these innate groups of T cells are quite different. One common factor between these three subsets is their involvement in responses to bacterial pathogens (6-8).

$CD8^+$ T cells can be selected by the classical MHC class Ia, which gives rise to conventional $CD8^+$ T cells, or by the non-classical MHC class Ib, which can select subsets of non-conventional $CD8^+$ T cells capable of rapidly responding to conserved pathogenic antigens (9-13). H2-M3 is one of the class Ib molecules, and it presents *N*-formylated peptides to MHC class Ib restricted $CD8^+$ T cells [addressed as H2-M3 restricted T cells henceforth] (14). H2-M3 restricted T cell response to *Listeria monocytogenes* peaks much quicker than the conventional [class Ia restricted] $CD8^+$ T cell response (6). In accordance to the speedy kinetics, these cells also have characteristics of innate immune subsets such as selection on hematopoietic cells, $CD44^{hi}$ expression [even in uninfected mice], and quick capability to secrete IFNγ and promote Th1 priming (13, 15-17). Despite all these functional studies, data on H2-M3 restricted T cells is lacking.

NKT cells have been studied to a much greater extent than H2-M3 restricted T cells. The majority of mouse NKT cells express an invariant T cell receptor [TCR] containing the Vα14-Jα18 pairing, with a limited Vβ pairing, and recognize lipid antigens in the context of CD1d (18, 19). The NKT cells referred to and studied throughout this thesis are indeed this invariant group of NKT cells. NKT cells are positively selected on CD1d and SLAM molecules and go on to acquire several “memory-like” markers during their development (20-22). NKT cell maturation is split up into three stages denoted by the expression of CD44 and NK1.1: in stage 1 NKT cells express low levels of CD44 and NK1.1, in stage

2 they express high levels of CD44, and stage 3 they express high levels of CD44 and NK1.1 [as well as several other NK markers] (21). From early phases of their development NKT cells are capable of quickly secreting IL-4 and they acquire the capability to secrete IFNγ later on, once they have upregulated NK1.1 marking their terminal maturation (23-25). The ability to secrete cytokines quickly makes NKT cells great immune modulators involved in diseases ranging from viral and bacterial infections, to autoimmunity and tumor responses (26).

The third group of innate T cells discussed in this thesis, γδ T cells, is not restricted by MHC and can recognize soluble proteins and non-protein antigens of endogenous origin (27). They constitute a small fraction of the lymphocytes in the blood and peripheral organs, but can compose up to half of the lymphocytes in the epithelial rich tissues, such as the skin, reproductive track, and the intestine. γδ T cells are the first lymphocytes lineages generated during fetal development and originate through a coordinated temporal program which will give rise to different Vγ subsets, populating different tissues [such as the skin, uterus, lung, etc.] (28-31). Function of the γδ T cells seems to be dictated by their tissue distribution and Vγ usage. γδ T cells have a biased Th1 [IFNγ] secretion profile, but they are capable of secreting Th2 cytokines such as IL-4 (27). Besides functioning as a first line of defense at the epithelial rich tissues, γδ cells can be involved in modulation of innate and adaptive immune responses, can aid in viral and bacterial infections, can regulate epithelial integrity and

turnover, might have a role in elimination of stressed or transformed cells, and have the potential of functioning as antigen presenting cells (32-45).

Molecules involved in innate T cell selection/generation

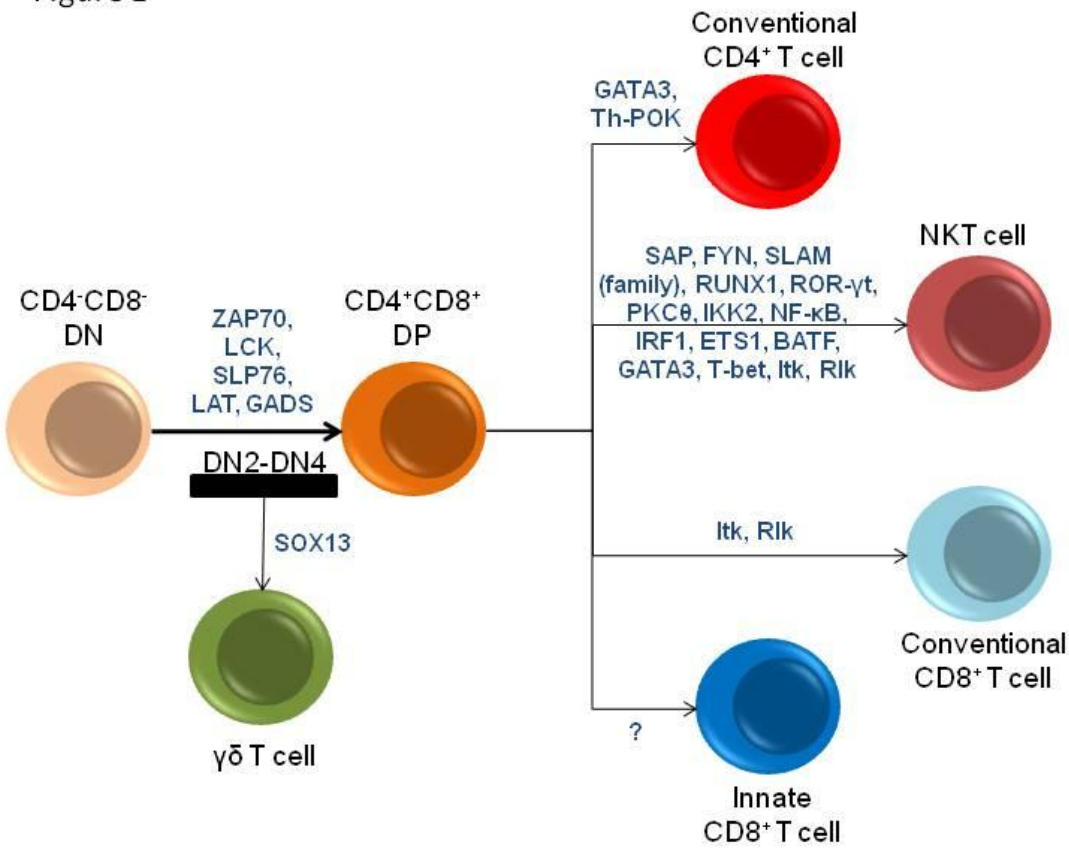
Innate and adaptive T cell subsets all generate from thymic lymphoid progenitors, although their lineage bifurcation may occur at different stages of development. Where commitment to the γδ T cell subset occurs early on in development in the DN stage during the DN2-DN3 transition [albeit there is some controversy about the specific divergence point (46)], NKT cells and innate $CD8^+$ T cells arise from DP thymocytes, much like mature "conventional" αβ T cells. One key difference between innate and conventional T cells is that the latter is dependent on classical MHC class I and class II molecules presented on cortical epithelial cells (47) while the former is mostly dependent on non-classical MHC molecules presented on hematopoietic cells (10, 12, 13, 27, 48-50).

Although advances have been made, the molecular circuitry that dictates innate versus conventional T cell bifurcation remains quite complicated, and in some cases, like H2-M3 restricted T cells, a complete mystery [Figure 1]. For γδ T cells only one gene has thus far been described to be involved in γδ specific bifurcation, the transcription factor SOX13 (51). Interestingly a slew of signaling

Figure 1: molecular signature involved in innate versus adaptive T cell development

Innate and adaptive T cells develop from double negative [DN] thymocytes. Transition to the double positive [DP] state requires several signaling components including ZAP70, LCK, SLP76, LAT, and GADS. γδ T cells develop prior to entry in the DP stage and are dependent on SOX13 expression for their lineage decision. Conventional $CD4^+$ T cells depend on expression of GATA3 and Th-POK for their differentiation from DPs. NKT cells have been shown to depend on several signaling molecules to differentiate from DPs including: SAP, FYN, SLAM family members, RUNX1, ROR-γt, PKCθ, IKK2, NF-κb, IFR1, ETS1, BATF [BATF actually mimics inhibition of AP-1], GATA3, T-bet, and as will be shown later on, Itk and Rlk. As will be described in chapter 2, development of conventional $CD8^+$ T cells is also dependent on Itk and Rlk. It is not yet known what factors are required for the development of innate $CD8^+$ T cells like the H2-M3 restricted cells.

Figure 1


Conventional
CD4+ T cell
GATA3,
Th-POK
NKT cell
SAP, FYN, SLAM
(family), RUNX1, ROR-γt,
PKCθ, IKK2, NF-κB,
IRF1, ETS1, BATF,
GATA3, T-bet, Itk, Rlk
CD4-CD8-
DN
ZAP70,
LCK,
SLP76,
LAT, GADS
CD4+CD8+
DP
DN2-DN4
SOX13
Itk, Rlk
Conventional
CD8+ T cell
γδ T cell
?
Innate
CD8+ T cell

components found to be dispensable for conventional αβ T cell development have been shown to have important roles in the development of NKT cells. For instance the Fyn-SAP-SLAM family axis has been shown to be indispensible for initial NKT cell development, while it does not seem to have a role in conventional αβ T cell development (20, 52-56). Much like with the aforementioned axis, a deficiency in the transcription factors Runx1 and RORγt seems to cause a specific defect in early NKT cell selection/development (57, 58). Several other molecules have also been specifically associated with early and later stages of NKT cell development including: PKCθ (59, 60), Vav-1 (61), IKK2 (59) and members of the NF-κB family (62), IRF1 (63), ETS1 (64), BATF [in the context of AP-1 inhibition] (65, 66), GATA-3 [particularly in generation of $CD4^+$ NKTs] (67), and T-bet [necessary for terminal maturation] (68). Interestingly, until recent work from our lab and others, no signaling molecules had been identified that were required for generation of conventional T cells, but Itk and Rlk seem to have a role in the linage decision of conventional versus innate $CD8^+$ T cells (69-74). This issue as well as Tec family kinases will be described in much greater detail later on.

TCR signaling in development of innate T cell subsets

The subject of TCR signal strength and its role in the development of innate T cell subsets is still poorly understood and in some cases controversial. Regardless of the cell type, one possibility is that conventional versus innate T cell lineage bifurcation is dependent on strength of signals transduced by the TCR. H2-M3 restricted T cells and NKT cells are both selected on non-classical MHC-Ib expressed on hematopoietic cells while conventional αβ T cells are selected on classical MHC expressed on the thymic epithelia. Differences in expression of selecting molecules for conventional and innate T cells could result in differences in the robustness of signaling leading up to a lineage decision [innate versus conventional]. In fact cell-surface expression of non-conventional MHC class Ib molecules on thymic cortical cells has been shown to be lower than expression of classical MHC molecules on the thymic epithelia (9, 75). In the case of NKT cells, maintaining low levels of expression might be required in order to keep the cells from undergoing negative selection due to [most likely] excessive signaling (76). Selection of NKT cells on thymic epithelial cells [mimicking classical MHC selection] is also inefficient (77). This is probably due to two factors, increased expression of CD1d, leading to negative selection, and lack of expression of SLAM family members on the cortical epithelia, which as previously mentioned is critical for NKT development (20). Differences in

strength of interactions between classical and non-classical MHCs and the cells they select could also have a very important role in the selection process, but no studies have been done thus far to address this question.

Selection of γδ T cells versus conventional αβ T cells has been studied to a much greater extent, but the field still remains controversial with two models for lineage bifurcation, selection versus instruction. The selection model dictates that αβ and γδ lineage commitment occurs independent of TCR signaling prior to the onset of the TCR while the instruction model dictates that commitment is determined by the TCR isotype [i.e.: pre-TCR instructs DN thymocytes to become αβ while γδTCR instructs them to become γδ cells] (78, 79). There is evidence available for both model. For instance, expression of rearranged TCRβ, TCRγ and TCRδ together in thymic precursors does not seem to have much effect on the developmental outcome, favoring the selective model (80). On the other hand, in a study where γδTCR signal strength was altered, it was shown that increasing the strength of the γδTCR favored γδ T cell development while decreasing it favored αβ T cell development, supporting the instructive model (81). There are several more studies supporting either model, but due to caveats existing for the studies supporting both sides the question has yet to be resolved. This question is complicated by the fact that a γδ T cell selecting ligand and cell type has yet to be determined for the most part. Two exceptions to this are G8-TCRγδ^{+} T cells, which are selected directly by class Ib molecules [T10^{b} and T22^{b}], and epidermal γδ T cells, which have recently been shown to be

selected by an immuloglobulin-like protein encoded by *Skint1* (82, 83). One more component required for proper γδ T cell development is double positive [DP] thymocytes. Although independent of TCR signaling, these cells provide signaling through lymphotoxin β receptor pathways (84). Absence of these signals during development results later on in defective activation of γδ cells in the spleen.

Taken together all this data paint a confusing picture, but it does seem clear that in NKT cells and γδ T cells modulation of TCR signal strength can influence the outcome of development and function of innate subsets. This thesis focuses on how ablation of Tec family kinases, which as explained in the next section enhance TCR signaling, alters the development and function of innate T cell subsets, and in the case of $CD8^+$ T cells, shifts their development to an innate H2-M3-like $CD8^+$ T cell.

Tec family kinases structure

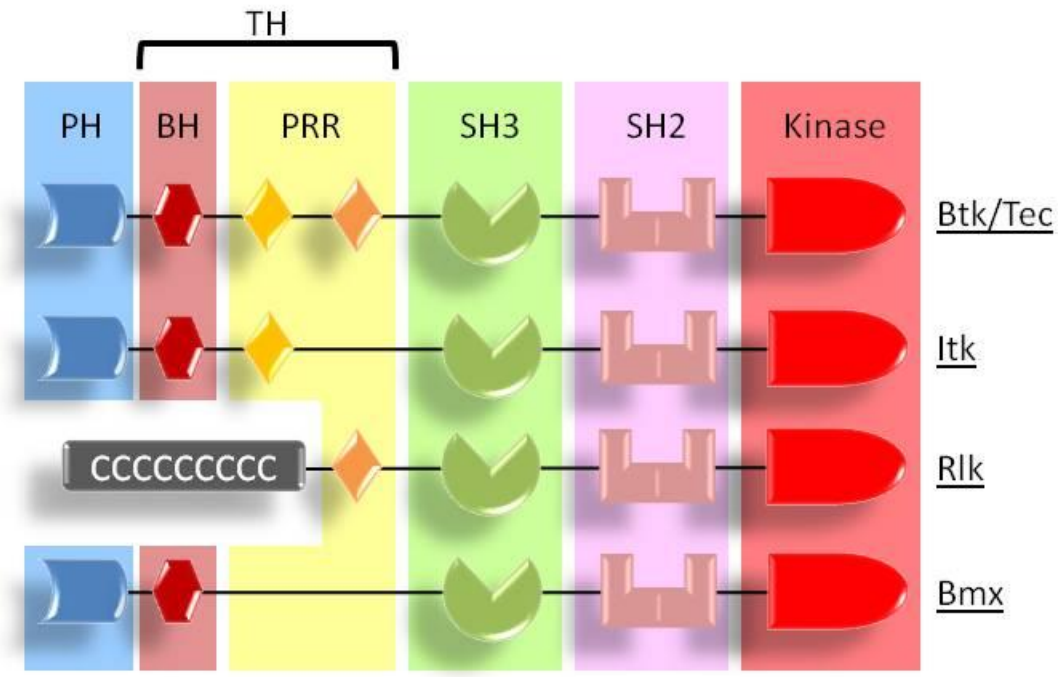
Five Tec kinase family members have been described in mammalian cells. These kinases are highly expressed in hematopoietic cells, including B cells (85-88), T cells (85, 89-93), and mast cells (94-96). The Tec kinase family members share a number of structural features [Figure 2]. Each has a C-terminal kinase

domain, preceded by a Src homology [SH]-2 domain and an SH3 domain, much like Src family PTKs (97). However, unlike the Src kinases, Tec family kinases, with the exception of Bmx, possess a proline rich region [PRR] at the N-terminal side of the SH3 domain; interestingly, Btk and Tec have two of these regions, whereas Itk and Rlk have only one. At the N-terminal side of the PRR, in Itk, Btk, and Tec, there is a Zn^{2+}-binding region known as the Btk homology [BH] motif. The combination of the BH and the PRR has been labeled the Tec homology [TH] domain. Finally, at the N-terminal end, all Tec family kinases, with the exception of Rlk, possess a pleckstrin homology [PH] domain; in Rlk, the N-terminal region contains a cysteine-string motif (98). These structural differences, specifically the difference in PRRs and N-terminal domains, may contribute to the distinct functions of each Tec kinase.

Figure 2: structure of Tec family kinases

Tec family kinases share several structural similarities including their catalytic C terminal kinase domain, and two Src homology domains [SH2 and SH3]. While Btk and Tec contain two proline rich repeat [PRR] regions, Itk and Rlk only contain one. The only Tec family kinase which does not contain a Btk homology domain [BH] or a N-terminal plextrin homology [PH] region is Rlk, which instead possesses a string of N-terminal cysteine repeats.

Figure 2



Tec family kinase expression

Tec family kinases are predominantly expressed in hematopoietic cells; however, each individual Tec kinase has a distinct cell type-specific pattern of expression. In addition, each cell type has a hierarchy of expression levels and functions for the Tec kinases expressed in that cell. Of the three Tec kinases expressed in T cells, Itk, Rlk, and Tec, Itk appears to have the predominant role in TCR signaling. Itk is expressed in thymocytes and mature T cells, and is found at maximal levels in the mature adult thymus (90, 92, 93, 99, 100). Similar to Itk, Rlk is expressed in thymocytes and mature resting T cells; however, Rlk mRNA levels are 3-10 fold lower than the levels of Itk mRNA in resting T cells (91, 96, 101, 102). Furthermore, unlike Itk, Rlk is dramatically downregulated at both the mRNA and protein levels upon TCR stimulation (91, 96). The third Tec kinase expressed in T cells, Tec, is expressed at much lower levels in resting T cells, with mRNA levels ~100-fold lower than that of Itk (98). Interestingly, Tec is upregulated in T cells 2-3 days following their stimulation, suggesting a more important role for Tec in T cell effector function and restimulation, rather than in T cell development or initial activation (103).

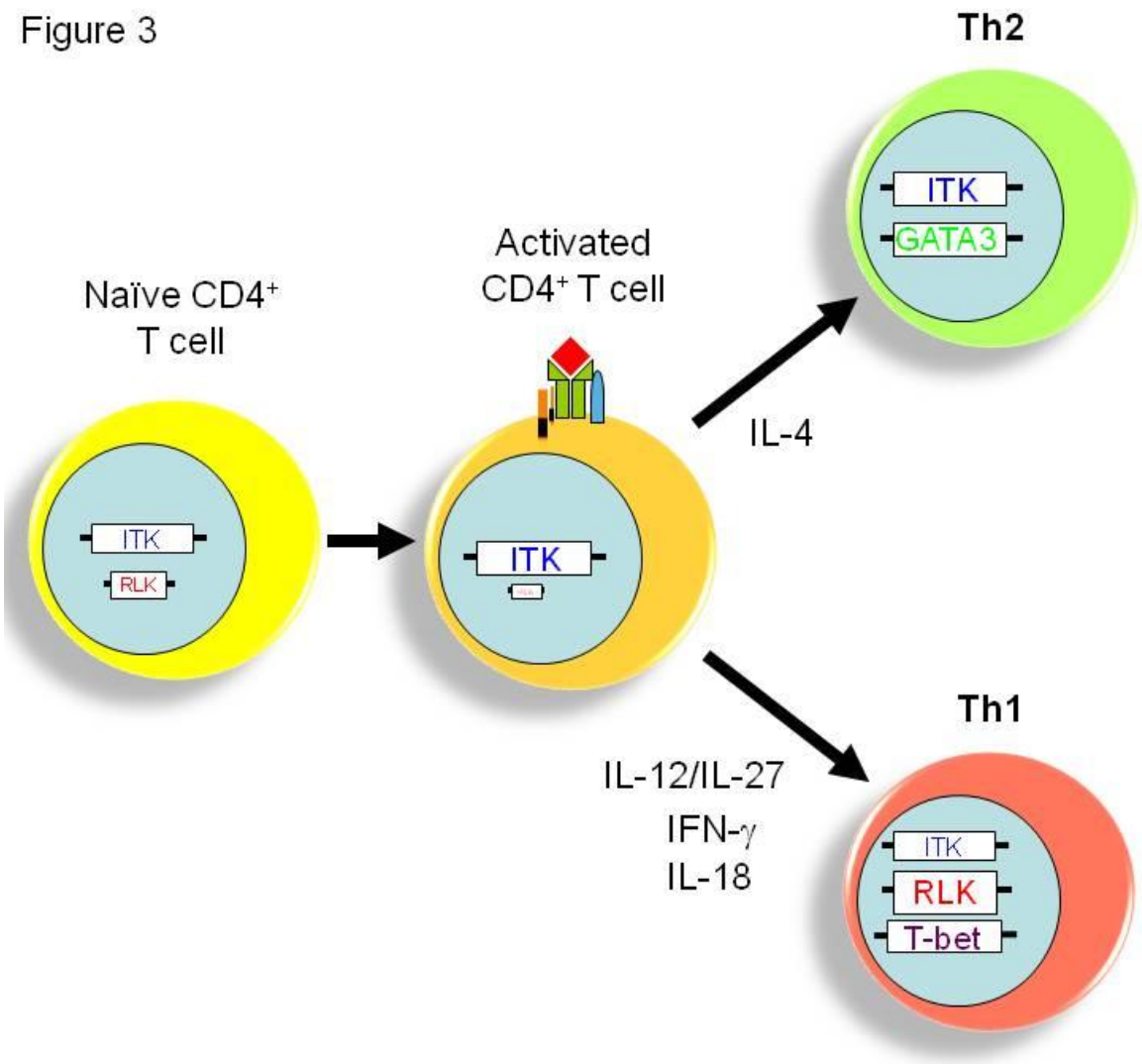
Tec kinase levels are also individually regulated during T helper cell differentiation [Figure 3]. When naïve $CD4^+$ T cells differentiate into T helper [Th] cells, Itk levels increase ~2-3-fold in Th2 cells, which express the master

transcription factor GATA3, versus Th1 cells, which express the master transcription factor T-bet, consistent with the known role of Itk in Th2 responses (101, 102). Similar to Itk, Tec is expressed at two-fold higher levels in Th2 cells than in Th1 cells; however, in this latter case, the functional significance of this differential expression is not known, as Tec-deficient mice have no reported T cell signaling defects (103). In contrast to Itk and Tec, Rlk is down-regulated following naïve CD4+ T cell activation, and is re-expressed in Th1 cells, but not Th2 cells; these data have suggested a specific role for Rlk in Th1 responses (91, 102, 104).

Figure 3: Itk and Rlk expression in $CD4^+$ T cells upon differentiation

Itk and Rlk are both expressed in naïve $CD4^+$ T cells, albeit Itk is expressed at higher levels. Upon activation Itk is upregulated and while Rlk is strongly downregulated. Th2 differentiation, mediated by IL-4, causes ablation of Rlk expression while Itk levels remain high [2-3 fold higher than basal levels]. It should also be mentioned that Tec expression is also upregulated 2-fold on Th2 cells when compared to Th1 cells. These cells also express the Th2 master transcription factor GATA3. In contrast Th1 differentiation, mediated by IL-12/IL-27, IFNγ and IL-18, results in upregulation of Rlk while Itk goes back to being expressed at basal [naïve] levels. These cells also express the Th1 master transcription factor T-bet.

Figure 3


Th2
ITK
GATA3
Activated
CD4+ T cell
Naïve CD4+
T cell
IL-4
ITK
RLK
ITK
Th1
IL-12/IL-27
IFN-γ
IL-18
ITK
RLK
T-bet

Subcellular localization of Tec family kinases: membrane recruitment

Each Tec family kinase shows a distinct pattern of subcellular localization [Figure 4]. At steady state levels Itk and Tec are found in the cytoplasm; following activation of Phosphoinositide-3-kinase [PI3K] and the generation of phosphatidylinositol 3,4,5-triphosphate [PIP_3] at the plasma membrane, Itk and Tec are recruited to the membrane via their PH domains (105-107). In contrast, Rlk, which lacks a PH domain, is constitutively associated with the plasma membrane via its palmitoylated cysteine-string motif. Thus, while Itk and Tec both require PI3K activity for plasma membrane association, Rlk lipid raft association is PI3K-independent (108).

Following TCR stimulation and the activation of PI3K, Itk recruitment to the membrane requires its PH domain, and is independent of other domain interactions (105, 109). Deletion of the Itk PH domain abolishes TCR activation-induced colocalization of Itk with the TCR complex at the plasma membrane and also prevents the subsequent tyrosine phosphorylation and activation of Itk. Substitution of the PH domain of Itk with a membrane localization [e.g., myristylation] sequence from Lck restores Itk membrane localization, but does not allow TCR signal-induced tyrosine phosphorylation of Itk, indicating a more complex role for the PH domain than simple plasma membrane association

(105). Possible roles for the PH domain include recruitment to the immediate vicinity of the activated receptor, or a more structural role in Itk activation.

Figure 4: Membrane localization of Tec family kinases

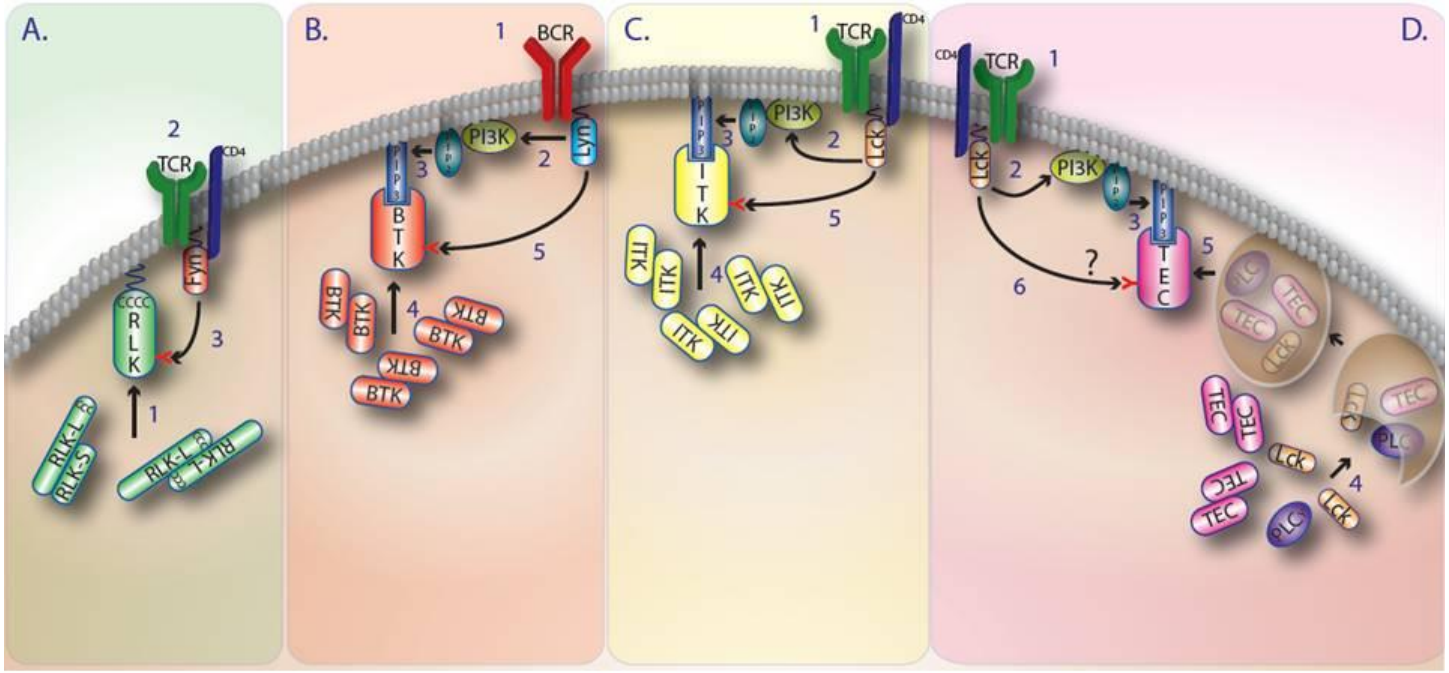
A] The larger isoform of Rlk [RLK-L] is constitutively localized to the plasma membrane through palmitoylation of the cysteine string motif at the amino terminus [1]. Upon T cell receptor [TCR] engagement [2], Rlk proteins in the vicinity of the TCR are activated by Fyn through phosphorylation of the Rlk kinase domain.

B] Prior to B cell receptor [BCR] engagement, Btk is cytosolic. Following BCR stimulation [1], Lyn activates PI3K [2], leading to the production of PIP_3 [3]. Btk is then recruited to the plasma membrane through interaction of its PH domain with PIP_3 [4]. Lyn phosphorylates and activates Btk [5].

C] Prior to TCR engagement, Itk is cytosolic. Following TCR stimulation [1], Lck activates PI3K [2], leading to the production of PIP_3 [3]. Itk is recruited to the plasma membrane through interaction of its PH domain with PIP_3 [4]. Lck then phosphorylates and activates Itk [5].

D] Following TCR engagement [1], Lck activates PI3K [2], leading to the production of PIP_3 [3]. Tec is then recruited into vesicles at the plasma membrane that contain signaling components such as Lck and PLC-□ [4]. Tec is then recruited to PIP_3 through its PH domain [5] where it can be activated [presumably by Lck in T cells and Lyn in B cells] [6].

Figure 4



Felices et al. 2007

The regulation of Tec recruitment to the plasma membrane following TCR stimulation has some distinct features compared to Itk. Like Itk, Tec can be recruited to the membrane through the interaction of its PH domain with PIP_3. However, the interaction of the Tec PH domain with PIP_3 must be different from that of the other Tec kinases, as illustrated by the behavior of PH domain substitution mutants. For instance, substitution of the glutamic acid residue at position 41 with lysine in the Btk PH domain has been shown to increase Btk binding to PIP_3; in contrast, the comparable mutation in the PH domain of Tec [E42K] reduces Tec binding to PIP_3 (107). In addition, while the Tec PH domain is required for tyrosine phosphorylation of Tec, membrane recruitment could also be mediated by the Tec SH2 domain (107). Finally, one recent study found that the PH domain of Tec is dispensable for Tec accumulation at the plasma membrane, and instead identified the SH3 domain as essential for accumulation of Tec at the immunological synapse (110). Interestingly, these authors also found that a functional Tec PH domain is required for proper activation of Tec following TCR stimulation, but suggest that membrane accumulation of Tec is not PI3K-dependent.

An interesting report indicates that in B cells, Btk can promote its own sustained activation by a positive feedback loop (111). Btk binds to PI5-kinase, transporting it to the membrane following activation; at the membrane, Phosphotidylinositol 5 kinase [PI5K] converts $PI_{[4]}P$ into $PI_{[4,5]}P_2$, thereby

providing a renewable source of substrate for PI3K, and prolonging the activation signal.

Since membrane localization is a prerequisite for Tec kinase function in antigen receptor signaling pathways, these signals can be terminated by inhibition of membrane recruitment. For Itk, recruitment to the plasma membrane is negatively-regulated by the lipid phosphatase, PTEN, which removes phosphates from the D3 position of phosphoinositides, and thereby reduces the levels of PIP_3 at the membrane (106). In PTEN-deficient cells, such as the Jurkat T cell tumor line, Itk is constitutively localized to the plasma membrane and hyperresponsive to TCR stimulation (106). This mechanism for negative regulation sets Itk apart from Rlk, which has no dependence on phosphoinositides for membrane localization. Membrane localization of Tec, in contrast, is regulated by the SHIP family of inositol phosphatases (112). The proposed mechanism for this regulation is similar to that of PTEN, involving dephosphorylation of PIP_3 leading to decreased PH domain-mediated recruitment of Tec to the plasma membrane. However, unlike the indirect regulation of Itk by PTEN, Tec also seems to directly interact with SHIP1 and SHIP2, an interaction that is dependent on the Tec SH3 domain (112). Based on this observation, it is possible that in conditions of PI3K-independent recruitment of Tec to the membrane, interaction of SHIP with the Tec SH3 domain might be sufficient to preclude Tec membrane localization. One final feature that sets Tec apart from other family members is its unique subcellular localization upon TCR-

induced activation. Whereas Itk appears diffusely localized at the immunological synapse, Tec has a more punctuate localization pattern at the T-cell-APC interface, indicative of its presence in vesicular structures (103). Formation and maintenance of these Tec-containing vesicles requires TCR signaling through Src-family kinases and PI3K (113). These vesicles also contain the early endosomal antigen 1 [EEA1] marker as well as signaling components such as Lck and the Tec substrate PLC-γ1. In theory, this packaging of signaling components facilitates signaling through Tec. No such assembly of signaling components has been described for any of the other Tec family kinases.

Subcellular localization of Tec family kinases: nucleus

Several reports have also indicated that Tec kinases may have an important role in the nucleus of T cells [Figure 5]. In this regard, the data for Rlk is the most compelling. Two isoforms of Rlk, that arise from alternative sites of translation initiation on the same mRNA have been described (114). The larger 58 kDa isoform is cytoplasmic, and localizes to lipid rafts through the cysteine-string motif upon palmitoylation. The shorter 52 kDa isoform lacks the cysteine-string motif and localizes to the nucleus when expressed in the absence of the larger form. Consistent with these data, a mutation that abolishes palmitoylation in the cysteine-string motif of the larger isoform allows this protein to migrate to the

nucleus. However, in spite of the fact that both isoforms contain a nuclear localization sequence [residues 57-71], both proteins are found only in the cytoplasm when coexpressed, suggesting a direct physical interaction between the two isoforms. While the data demonstrating the ability of Rlk to traffic to the nucleus are compelling, little is known about the function of nuclear Rlk. One report indicated that Rlk could bind to the IFN-γ promoter region. Combined with its upregulation in Th1 cells, these findings suggest an important function for Rlk in Th1 cell development and signaling (104).

Some data also indicate that Itk may traffic to the nucleus. In CD3-stimulated Jurkat T cells, a small proportion of the total Itk protein was found in the nucleus. In this case, the nuclear localization was mediated by karyopherin alpha [Rch 1α], a nuclear transporter binding to the Itk SH3 domain via its proline-rich region [PRR] (115). This finding is somewhat surprising, as Rch1α is generally required for the nuclear import of proteins containing a basic-type [classical] nuclear localization signal, which is lacking in Itk. Nonetheless, expression of a PRR mutant of Rch1α in Jurkat cells prevented nuclear translocation of Itk as well as mitogen-induced IL-2 production by these T cells. These data suggest that the nuclear fraction of Itk may play a role during T cell activation. Consistent with this notion, one report indicates that Itk might directly interact with and phosphorylate T-bet, a constitutively nuclear transcription factor that regulates IFNγ transcription (116). This report also mentions that this phosphorylation might be critical for direct cross-regulation of T-bet to GATA3.

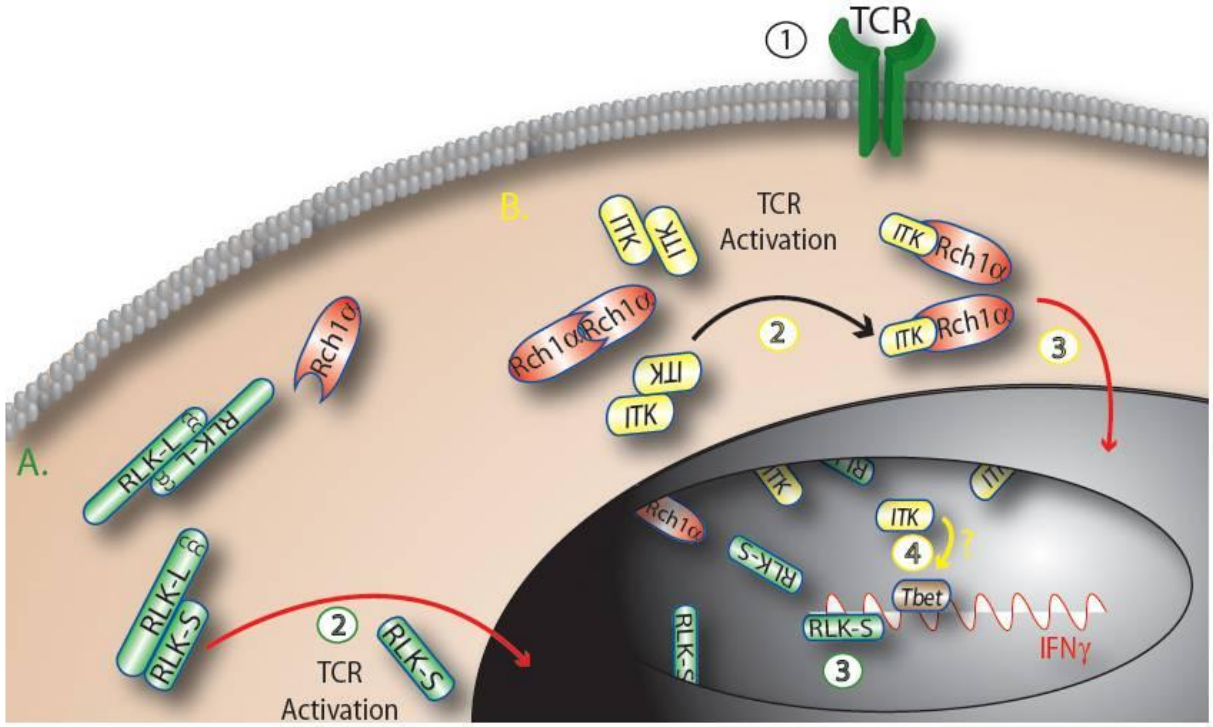
Absence of T-bet phosphorylation, or absence of Itk, lead to an abolishment of T-bet co-immunoprecipitation with GATA3, thus decreasing the ability of T-bet to directly inhibit GATA3 binding to the DNA. Btk has also been shown to migrate to the nucleus in B cells, but due to the subject of this thesis this will not be elaborated in further detail (117-122).

Figure 5: proposed nuclear localization of Tec family kinases in T cells

A] Upon TCR engagement [1], the short form of Rlk [RLK-S], usually found in a complex with the long form [RLK-L], migrates to the nucleus [2-green]. Once in the nucleus RLK-S binds to the promoter region of the IFNγ gene [3-green].

B] Upon TCR engagement [1], the SH3 region of Itk interacts with the PRR region of the nuclear transporter Kayopherin alpha [Rch 1α] [2-yellow]. The Itk-Rch1α complex translocates into the nucleus [3-yellow]. In the nucleus, Itk may bind T-bet, a master regulator of IFNγ transcription [4-yellow], and perhaps phosphorylate it.

Figure 5



Adapted from Felices et al. 2007

Tec family kinase signaling downstream of the TCR

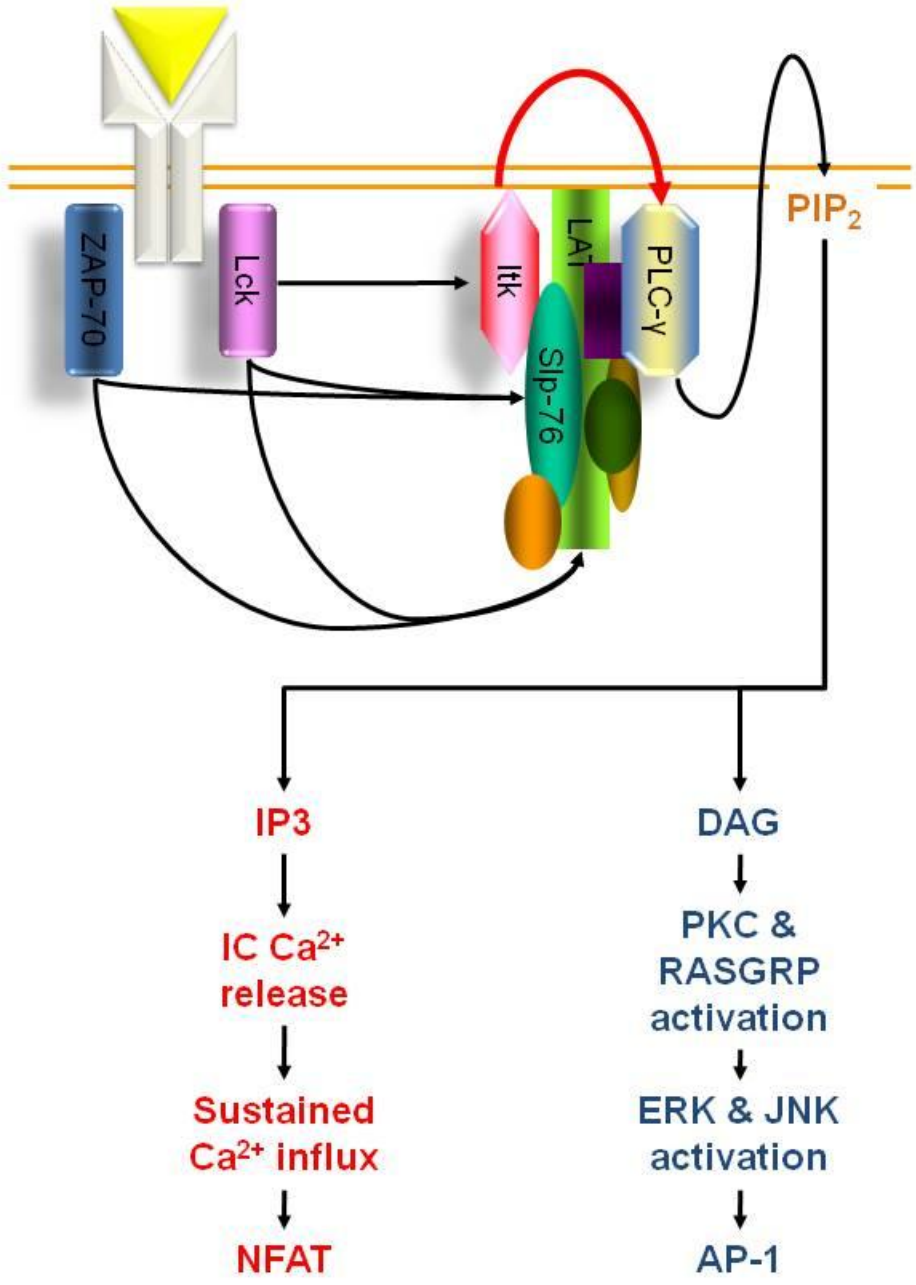
Although some of the details vary, the antigen receptor signaling pathways in T cells [Figure 6], B cells, and mast cells share a similar overall scheme. Briefly, following receptor engagement, a Src family tyrosine kinase [Lck in T cells] is activated and phosphorylates receptor subunits, leading to the recruitment and activation of a Syk family kinase [ZAP-70 in T cells] (123-126). Activated Tec family kinases are then recruited to the receptor signaling complexes through interactions with adapter proteins of the SLP-76 [SH2-domain-containing leukocyte protein of 76 kDa] and LAT [linker for activation of T cells] families (109, 127-129). Once at the membrane, Tec family kinases are activated through phosphorylation by Src family kinases [again Lck in T cells], and in turn, phosphorylate and activate PLC-γ (130-132). PLC-γ catalyzes catabolism of $PI_{[4,5]}P_2$ into inositol-1,4,5-triphosphate [IP_3] and diacylglycerol [DAG] (133). IP_3 is required for intracellular Ca^{2+} release, which triggers sustained calcium influx that activates downstream effectors like the NFAT transcription factors (134, 135). DAG activates members of the PKC [protein kinase C] family, as well as RASGRP [RAS guanyl-releasing protein], leading to the activation of JNK [JUN amino-terminal kinase] and ERK1/ERK2 [extracellular-signal-regulated kinase] and thereby regulating the transcription factor, AP-1 (126, 136). This model is supported by data from Tec kinase-deficient lymphocytes and mast cells, which

show defects in antigen-receptor mediated PLC-γ phosphorylation, IP_3 production, Ca^{2+} influx, ERK and JNK activation, and the downstream activation of transcription factors, NFAT and AP1 (123, 124, 131, 137-140). Very little work has been done on the role of Tec family kinases in innate T cell signaling, development, and function. What little work is out there will be highlighted in the introduction and discussion sections of each of the subsequent individual data chapters dealing with a specific innate T cell subset, starting with “innate-like” $CD8^+$ T cells.

Figure 6: role of Itk downstream of TCR engagement

Upon TCR engagement both Lck and ZAP-70 are quickly activated and phosphorylate Slp-76 and LAT, causing the formation of a signaling nucleating complex to which Itk binds. Itk is phosphorylated and activated by Lck, and goes on to phosphorylate and activate PLC-γ. Activated PLC-γ catalyzes PIP_2 into IP_3 and DAG giving rise to intracellular [IC] calcium fluxes, and the ensuing NFAT activation, and PKC and RASGRP activation, and the ensuing AP-1 activation.

Figure 6


ZAP-70
Lck
Itk
LAT
PLC-γ
Slp-76
PIP2
IP3
IC Ca2+ release
Sustained Ca2+ influx
NFAT
DAG
PKC & RASGRP activation
ERK & JNK activation
AP-1

CHAPTER II: Tec family kinases and their role in the development of $CD8^+$ T cells

Chapter II Attributions and Copyright information

Most of the material in this chapter was done as a collaboration with Luana Atherly [LA] and Julie Lucas [JL] and presented in Atherly et al. 2006 (69). Likewise, much of it was derived from said publication. Specific contributions for each figure are as follows: figure 7 [MF, LA, and JL], figure 8 [LA and JL], figure 9 [LA and JL], figure 10 [LA and JL], figure 11 [LA], figure 12 [MF and JL], and figure 13 [MF].

Atherly, L. O., J. A. Lucas, M. Felices, C. C. Yin, S. L. Reiner, and L. J. Berg. 2006. The Tec family tyrosine kinases Itk and Rlk regulate the development of conventional CD8+ T cells. *Immunity* 25:79-91.

Intro

As alluded to in the general introduction T cell development in the thymus is regulated by multiple independent signals that derive from interactions between thymocytes and thymic stromal cells, in the case of conventional T cells, or thymocytes and cells of hematopoietic origin, in the case of innate T cells. These include signals mediated by the T cell antigen receptor [TCR] or pre-TCR, as well as cytokine signals. Signals mediated by SLAM family receptors also have a critical role in the development of some innate T cell subsets (20-22, 73). At each stage of thymocyte maturation, the appropriate magnitude and duration of these signals are critical for the proper progression of cells from one developmental stage to the next. The quality and strength of the signal will also be affected by the type of cell [stromal or hematopoietic] providing this signal. Signals through the TCR and pre-TCR are particularly critical for the development of αβTCR$^+$ T cells. In particular, as thymocytes progress from the CD4$^+$CD8$^+$ double positive [DP] stage to the CD4$^+$ or CD8$^+$ single positive [SP] stage, the specificity of the TCR, as well as the strength of signaling through this receptor, play a key role in determining the lineage of T cell that develops. Thus, conventional CD4$^+$ or CD8$^+$ cells, CD4$^+$ NKT cells, CD8$^+$ H2-M3-specific cells, and CD4$^+$CD25$^+$ regulatory T cells all arise from distinct types of TCR signaling (13, 141-145).

The signaling pathways that distinguish all of these alternative developmental lineages have not been well-described. For the maturation of conventional T cells, several signaling molecules proximal to the TCR have been implicated in the modulation of TCR signaling. Among them are the MAP-kinases Erk, Jnk, and p-38. Activation of the Erk/MAP-kinase signaling pathway is important for the positive selection of thymocytes, as impairment of Erk activation selectively affects positive, but not negative selection. The Jnk and p-38 pathways are seemingly more important for the regulation of thymocyte negative selection (146, 147). In addition, the activity of the tyrosine kinase p56Lck is critical for conventional T cell development, and also plays a role in determining CD4 versus CD8 lineage commitment (148). NKT cell development, on the other hand, is not dependent on Ras-MAP kinase signaling nor on Lck, but does required the src-family kinase, Fyn and the adapter protein SAP (52-56). It is not too clear however if Fyn is only required downstream of SLAM family signaling or if it's also critical downstream of the TCR in NKT cells, but in mature conventional T cells Fyn is activated upon TCR engagement. Interestingly, $CD4^+$ T cells selected on thymocytes [not stromal cells], that are not invariant NKT cells, also have the same requirements for Fyn and SAP (149). It should be mentioned that like NKT cells, these cells can produce cytokines, IL-4 and IFNγ, quickly without any kind of differentiation. These findings illustrate the role of distinct T cell signaling proteins in directing lineage decisions within

conventional T development, or in conventional versus innate T cell development.

Itk and Rlk have also been shown to be important during T cell development, and in particular, have been implicated in setting the thresholds of positive and negative selection. Specifically, positive selection of $CD4^+$ SP thymocytes is impaired in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice, a fact reflected by the lower percentages and total numbers of $CD4^+$ SP thymocytes and peripheral cells (150). Moreover, the absence of Itk lowers the positive selection efficiency of MHC Class II-specific TCR transgenic T cells as compared to wild-type littermate controls. Additional studies with MHC Class I restricted $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ H-Y TCR transgenic male mice demonstrated that there is a shift from negative selection to positive selection in these mice (151). These studies implicate Tec kinases in determining the magnitude of TCR signaling and thereby influencing the fate of conventional TCR $\alpha\beta^+$ thymocytes.

Prior to the work presented in the following results and discussion sections, the role of Itk and Rlk in $CD8^+$ T cell development was unclear. Contrary to the decrease in percentage and total numbers of $CD4^+$ SP thymocytes in the thymi of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice, there is an increase in both the percentage and total number of $CD8^+$ SP thymocytes in these mice. Given the importance of Itk and Rlk in the modulation of the signaling threshold for positive selection, it had been hypothesized that the generation of the increased numbers of $CD8^+$ SP thymocytes in the $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice may be due to a

switch in lineage commitment from the $CD4^+$ to the $CD8^+$ SP lineage (152). This was studied using MHC II restricted TCR transgenics on a variety of selecting backgrounds but never was an increase in either the percentages or total numbers of $CD8^+$ SP thymocytes observed in any of these mice in the absence of Itk, suggesting that lineage switching is not occurring in the $Itk^{-/-}$ mice (150).

The aforementioned studies did not resolve the role of Itk and Rlk in $CD8^+$ T cell differentiation, as they provided no explanation for the increased numbers of $CD8^+$ SP thymocytes in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice. These data suggested the possibility that Itk and/or Rlk play a distinct role in the $CD8^+$ T cell maturation process compared to their function in signaling pathways required for $CD4^+$ T cell differentiation. In accordance to this, our lab also observed that the $CD8^+$ SP thymocytes in $Itk^{-/-}$ (150) and $Itk^{-/-}Rlk^{-/-}$ mice have a mature and activated phenotype similar to that of homeostatically-expanded or memory T cells, and also similar to T cells selected on nonclassical MHC class I-b molecules (13). Due to this information we set out to further investigate the $CD8^+CD44^{hi}$ SP thymocytes in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice, particularly how they come about. Data presented in this chapter shows that the $CD8^+$ T cells in the Tec family deficient mice [$Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$] are phenotypically and functionally distinct from what are considered “conventional” $CD8^+$ T cells. Several of the differences mimic characteristics seen in innate T cells such as expression of CD122 and NK1.1, in addition to high levels of CD44, the capability to produce IFNγ directly *ex vivo*, as well as complete dependence on IL-15 for development in the thymus and

maintenance in the periphery. The $CD8^+$ SP thymocytes in these mice also express high levels of the T-box transcription factor, eomesodermin. In addition to this, another publication released at the same time also indicated that the $CD8^+$ SP thymocytes in the $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ deficient mice are selected by hematopoietic cells rather than the thymic stromal cells (71). Finally, a more recent article indicates that selection of $CD8^+$ SP cells in these mice is dependent on SAP (73). All these data illustrates a critical role for Itk and Rlk in the signaling pathway leading to conventional $CD8^+$ T cell differentiation.

Results

Memory cell phenotype of $CD8^+$ T cells in the thymus and periphery of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice

The Tec kinases Itk and Rlk have been implicated in the TCR signals leading to positive selection (150, 153). Therefore, it came as a surprise to discover that the proportions and total numbers of $CD8^+$ single positive [SP] thymocytes in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice are not reduced compared to that of wild-type mice [Figures 7, 8 and 12]. In addition, the fractions of $CD8^+$ T cells in the peripheries of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice are comparable to those seen in wild-type mice, and in the thymus, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ have significantly increased percentages and total numbers of $CD8^+$ SP cells [Figure 7 and ref. (152)]; %

$CD8^+$ SP thymocytes: wild-type, 3.5±0.3, $Itk^{-/-}$, 11.3±0.6, $p<0.0001$; absolute numbers $CD8^+$ SP thymocytes: wild-type, $5.6\pm0.8\times10^6$, $Itk^{-/-}$ $14.0\pm0.2\times10^6$, $p<0.0019$].

In addition to the increased numbers of $CD8^+$ thymocytes in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice, analysis of activation marker profiles reveals that the majority of these cells have a mature and previously-activated phenotype [Figure 7A, C and ref. (150)]. These thymocytes are $CD44^{hi}$, $CD122^+$, HSA^{lo}, and $NK1.1^{int}$, but are also $CD69^{lo}$ and $CD25^{lo}$ [Figure 7A, C and data not shown], indicating their similarity to memory T cells that usually reside in the periphery. Like the $CD8^+$ SP thymocytes, approximately 85% of the $CD8^+$ T cells in the periphery of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice are also $CD44^{hi}$ and have increased expression of other memory markers such as CD122 and NK1.1, while still expressing high levels of CD62L [Figure 7B, D]. However, like the $CD8^+$ SP thymocytes, these peripheral $CD44^{hi}$ $CD8^+$ T cells do not express other markers of acute activation, such as CD25 or CD69 [data not shown].

Previously-activated/memory peripheral $CD8^+$ $CD44^{hi}$ T cells are characterized by their ability to secrete effector cytokines immediately ex vivo in response to stimulation. To determine whether the $CD8^+$ $CD44^{hi}$ SP cells in the thymus and spleen of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice are functionally, as well as phenotypically, similar to previously-activated peripheral T cells, we examined

Figure 7: $CD8^+$ T cells in Itk-/- and Itk-/-Rlk-/- mice resemble previously-activated T cells

[A, B] Dot-plots show CD4 vs. CD8 profiles of wild-type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ [A] thymocytes or [B] lymph node cells. Numbers in each quadrant indicate percentages of each subpopulation. The histograms below show [A] CD44, CD122 and HSA expression on gated $CD8^+$ TCR^{hi} SP thymocytes or [B] CD44, CD122 and CD62L expression on gated $CD8^+$ TCR^{hi} lymph node cells. Numbers indicate percentage of $CD44^{hi}$, $CD122^{hi}$, HSA^{lo}, or $CD62L^{hi}$ cells, respectively. Data are representative of one of two experiments.

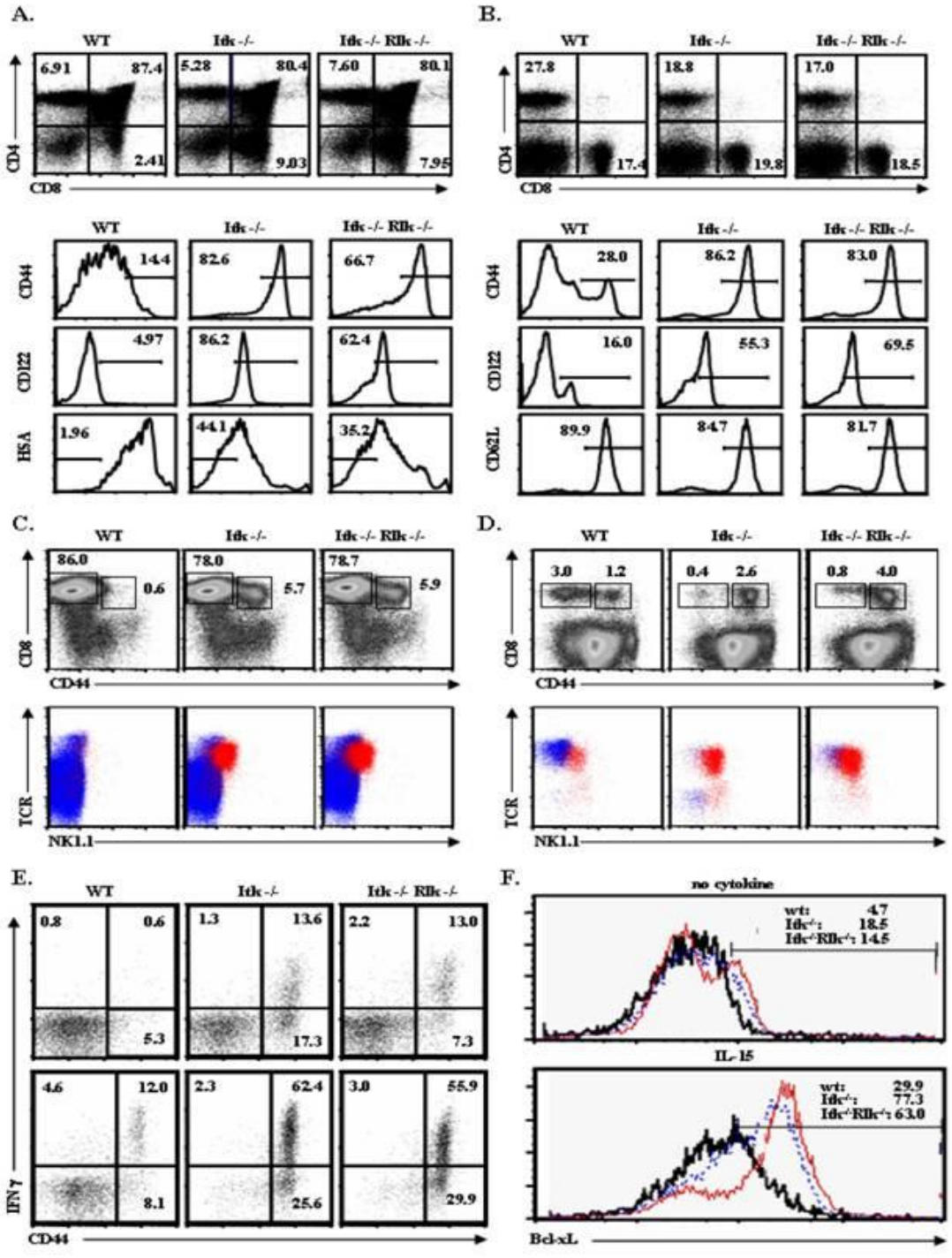
[C, D] Dot-plots show CD8 vs. CD44 profiles of five-week-old wild-type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ [C] thymocytes or [D] splenocytes. Numbers by each region indicate percentages of each subpopulation. The dot-plots below show TCRβ versus NK1.1 staining on gated $CD8^+CD44^{lo}$ [blue] or $CD8^+CD44^{hi}$ [red] populations. Data are representative of three independent experiments.

[E] Thymocytes [top row] and splenocytes [bottom row] from wild-type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice were stimulated with PMA and ionomycin for 5 h and IFNγ production was assessed by intracellular staining. Dot-plots show IFNγ vs. CD44 staining on gated $CD8^+$ cells. Numbers in each quadrant indicate percentages of each subpopulation.

[F] Thymocytes from wild-type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice were stimulated with IL-15 for 48 h and the levels of Bcl-xL were determined by intracellular staining.

Histograms show Bcl-xL expression on gated $CD8^+$ thymocytes. The wild-type sample is depicted by the thick black line, $Itk^{-/-}$ by the thin red line and $Itk^{-/-}Rlk^{-/-}$ by the dashed blue line. Data are representative of two independent experiments.

Figure 7


A.
WT
Itk -/-
Itk -/- Rlk -/-
6.91
87.4
2.41
5.28
80.4
9.03
7.60
80.1
7.95
CD4
CD8
CD44
14.4
82.6
66.7
CD122
4.97
86.2
62.4
HSA
1.96
44.1
35.2
B.
27.8
17.4
18.8
19.8
17.0
18.5
28.0
86.2
83.0
16.0
55.3
69.5
CD62L
89.9
84.7
81.7
C.
86.0
0.6
78.0
5.7
78.7
5.9
TCR
NK1.1
D.
3.0
1.2
0.4
2.6
0.8
4.0
E.
0.8
0.6
5.3
1.3
13.6
17.3
2.2
13.0
7.3
4.6
12.0
8.1
2.3
62.4
25.6
3.0
55.9
29.9
IFN γ
F.
no cytokine
wt: 4.7
Itk-/-: 18.5
Itk-/-Rlk-/-: 14.5
IL-15
wt: 29.9
Itk-/-: 77.3
Itk-/-Rlk-/-: 63.0
Bcl-xL

their ability to secrete the effector cytokine, IFNγ. In response to ex vivo stimulation with PMA and ionomycin, a large proportion of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ $CD8^{+}$ $CD44^{hi}$ SP thymocytes and peripheral T cells produce IFNγ [Figure 7E]. As this population of cells is absent from wild-type thymi, there was no corresponding effector cytokine production by wild-type $CD8^{+}$ SP thymocytes [Figure 7E]. Another functional characteristic of $CD8^{+}$ memory T cells is their ability to upregulate Bcl-xL in response to IL-15 (154). To assess this response, we incubated thymocytes from wild-type, $Itk^{-/-}$, and $Itk^{-/-}Rlk^{-/-}$ mice in media containing IL-15 for 36 h. As shown in Figure 7F, the $CD8^{+}$ SP thymocytes from $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice exhibit a dramatic upregulation of Bcl-xL in response to IL-15 compared to that seen with wild type $CD8^{+}$ SP thymocytes. Based on these findings, we conclude that the $CD8^{+}$ $CD44^{hi}$ cells in the thymus of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice are functionally, as well as phenotypically, similar to previously-activated/memory peripheral $CD8^{+}$ T cells.

The $CD8^{+}$ $CD44^{hi}$ T cell population is first detectable in day 4 post-natal thymus of $Itk^{-/-}$ mice

The finding that $CD8^{+}$ SP thymocytes in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice resemble previously-activated/memory peripheral $CD8^{+}$ T cells raised the question of the developmental origin of these cells. For instance, does this population of cells arise first in the thymus and then migrate to the periphery, or are these cells actually peripheral activated/memory $CD8^{+}$ T cells that have recirculated back

into the thymus? As a first step in examining this issue, we assessed the emergence of the $CD8^+$ $CD44^{hi}$ cell phenotype in $Itk^{-/-}$ mice by performing a longitudinal study of $CD8^+$ SP thymocyte and peripheral T cell development from birth through the first seven weeks of life. As shown in Figure 8A, an increased proportion of $CD8^+$ $CD44^{hi}$ thymocytes first appears at four days after birth in $Itk^{-/-}$ mice compared to wild type controls. Furthermore, from the age of two weeks onward, total $CD8^+$ SP thymocytes accumulate, both in percentage and absolute numbers, in $Itk^{-/-}$ mice as compared to controls [Figure 8B]. The increased numbers of $CD8^+$ SP thymocytes in $Itk^{-/-}$ mice is accompanied by a transient deficit in the numbers of peripheral $CD8^+$ T cells in these mice relative to wild type controls. This deficit persists until the mice are ~7-8 weeks of age [Figure 8B]. These kinetic data suggest that $CD8^+$ $CD44^{hi}$ T cells first arise in the thymus of $Itk^{-/-}$ mice.

Interestingly, the $CD8^+$ cells emerging into the periphery of both wild-type and $Itk^{-/-}$ mice at early time points [1-2 weeks after birth] have distinctly similar $CD44^{hi}$ profiles [Figure 8C]. This is likely due to the fact that the first migrants into the periphery of young mice undergo lymphopenia-induced proliferation in response to the “space” in the peripheral lymphoid organs, a phenomenon that is accompanied by CD44 up-regulation (155). These $CD44^{hi}$ cells remain to constitute the fraction of previously-activated cells typical of peripheral lymphoid organs (156). However, the frequency of these cells gradually diminishes in wild-type spleens, as cells continually migrate from the thymus into the periphery,

Figure 8: The altered phenotype of Itk$^{-/-}$ CD8^{+} thymocytes is detectable by day 4 post-gestation

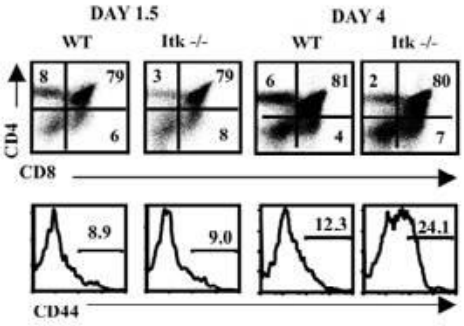
[A] Wild-type and Itk$^{-/-}$ neonatal day 1.5 [left two panels] and day 4 [right two panels] thymocytes were stained with antibodies to CD4 and CD8. The numbers in each quadrant indicate the percentage of cells in each subpopulation. Histograms of CD44 expression on gated CD8^{+} SP thymocytes are depicted below. Numbers indicate the percentage of CD8^{+} SP thymocytes expressing high levels of CD44.

[B] The percentages and total numbers of CD8^{+} SP thymocytes and peripheral T cells developing in wild-type, Itk$^{-/-}$, and Itk$^{-/-}$Rlk$^{-/-}$ mice were assessed at the indicated time points from birth to adulthood. Data were generated in a blind manner, and genotypes were determined afterwards. Data are compiled from a minimum of two experiments per time point with a minimum of two mice of each genotype per time point.

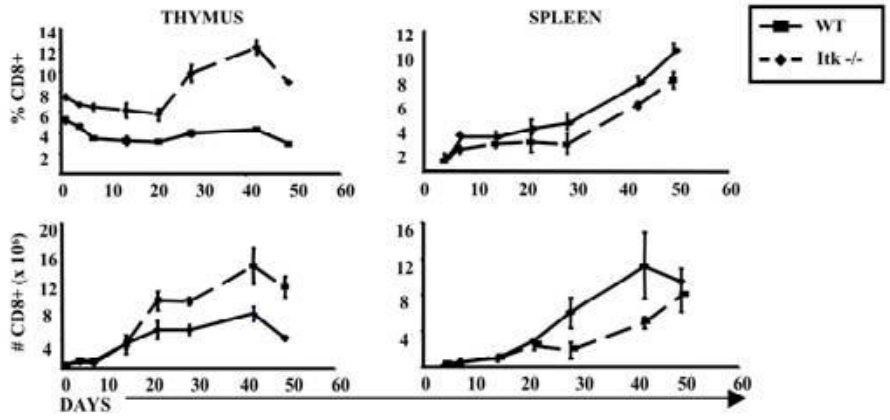
[C] CD44 expression on gated CD8^{+} thymocytes and splenocytes from 1-week, 2-week. 3-week, and 6-week old mice. Numbers indicate the percentage of CD44^{hi} cells among the gated population. Data are representative of a minimum of two experiments, each performed with a minimum of 3 mice per group.

Figure 8

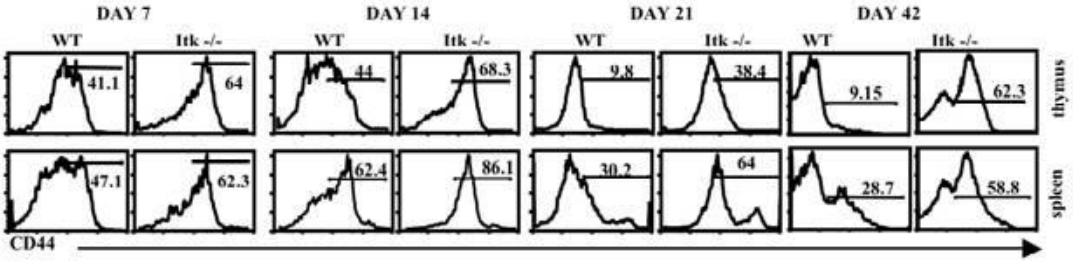
A.


DAY 1.5
DAY 4
WT
Itk -/-
WT
Itk -/-
8
79
6
3
79
8
6
81
4
2
80
7
CD4
CD8
8.9
9.0
12.3
24.1
CD44

B.


THYMUS
SPLEEN
WT
Itk -/-
% CD8+
# CD8+ (x 10⁶)
DAYS

C.


DAY 7
DAY 14
DAY 21
DAY 42
WT
Itk -/-
41.1
64
44
68.3
9.8
38.4
9.15
62.3
thymus
47.1
62.3
62.4
86.1
30.2
64
28.7
58.8
spleen
CD44

filling up space and preventing further proliferation of the newly emigrated cells from the thymus [Figure 8C]. The preferential accumulation of $CD44^{hi}$ $CD8^{+}$ SP thymocytes in the thymi of $Itk^{-/-}$ mice as compared to wild-type mice, as well as the persistence of this population over time in the periphery of $Itk^{-/-}$ mice, strongly suggests that the activated/memory $CD8^{+}$ phenotype typical of $Itk^{-/-}$ mice develops first in the thymus.

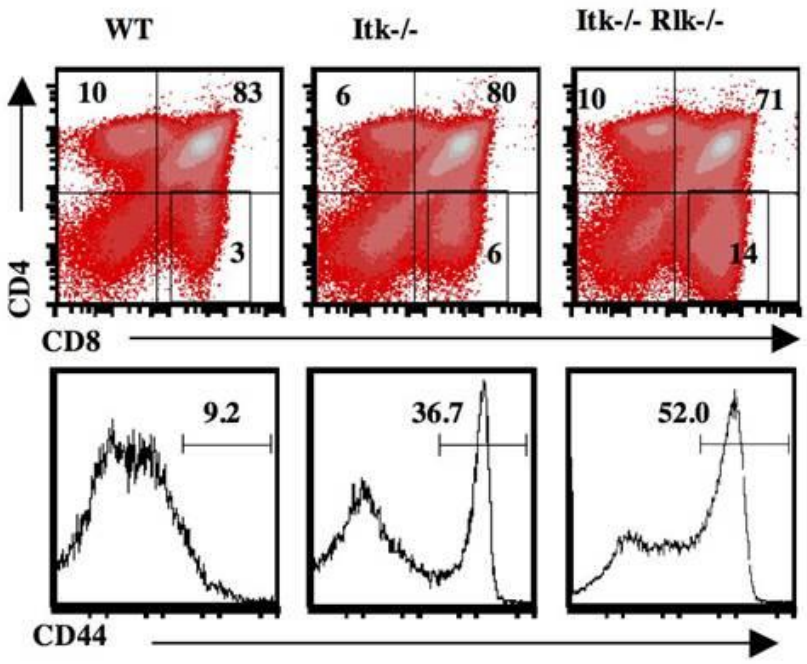
Cell-instrinsic defects lead to the altered phenotype of $CD8^{+}$ thymocytes in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice

In addition to homeostatic proliferation, $CD8^{+}$ T cells have been shown to upregulate CD44 expression and to become phenotypically and functionally mature in response to cytokines. In particular, the $CD8^{+}$ T cell populations found in both IL-7 (157) and IL-15 (158) transgenic mice that overexpress these cytokines are strikingly similar to the $CD8^{+}$ T cells present in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice. Therefore, we chose to examine whether the altered development of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ $CD8^{+}$ T cells was due to defects intrinsic to the developing T cells, or alternatively, due to an altered cytokine environment in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice. To address this question, we generated bone marrow chimeric mice in which wild-type, $Itk^{-/-}$, or $Itk^{-/-}Rlk^{-/-}$ bone marrow was injected into lethally-irradiated wild-type congenic mice. As shown in Figure 9, following reconstitution, the emergence of $CD44^{hi}$ cells in the $CD8^{+}$ SP compartment of the thymus is readily

Figure 9: Altered $CD8^+$ T cell differentiation in the absence of Tec family kinases is intrinsic to bone marrow-derived cells

Wild-type, $Itk^{-/-}$, and $Itk^{-/-}Rlk^{-/-}$ bone marrow chimeric mice were analyzed 12 weeks following reconstitution. Dot plots show CD4 versus CD8 staining on donor-derived cells [$CD45.2^+$]. The numbers in each quadrant indicate the percentage of cells in each subpopulation. The histograms below show CD44 expression on $CD45.2^+$ $CD8^+$ SP thymocytes. Numbers indicate the percentage of $CD44^{hi}$ cells among the gated population. Data are representative of four wild-type, four $Itk^{-/-}$, and eight $Itk^{-/-}Rlk^{-/-}$ chimeras analyzed.

Figure 9



apparent in mice reconstituted with Itk$^{-/-}$ or Itk$^{-/-}$Rlk$^{-/-}$ bone marrow, but not in thymi of mice reconstituted with wild-type bone marrow. These findings indicate that the altered CD8^{+} T cell development in the absence of Itk, or Itk and Rlk, is not due to abnormalities of Itk$^{-/-}$ or Itk$^{-/-}$Rlk$^{-/-}$ non-hematopoietic stromal cells.

Itk$^{-/-}$ CD44^{hi} CD8^{+} T cells are not actively proliferating and do not preferentially home to the thymus

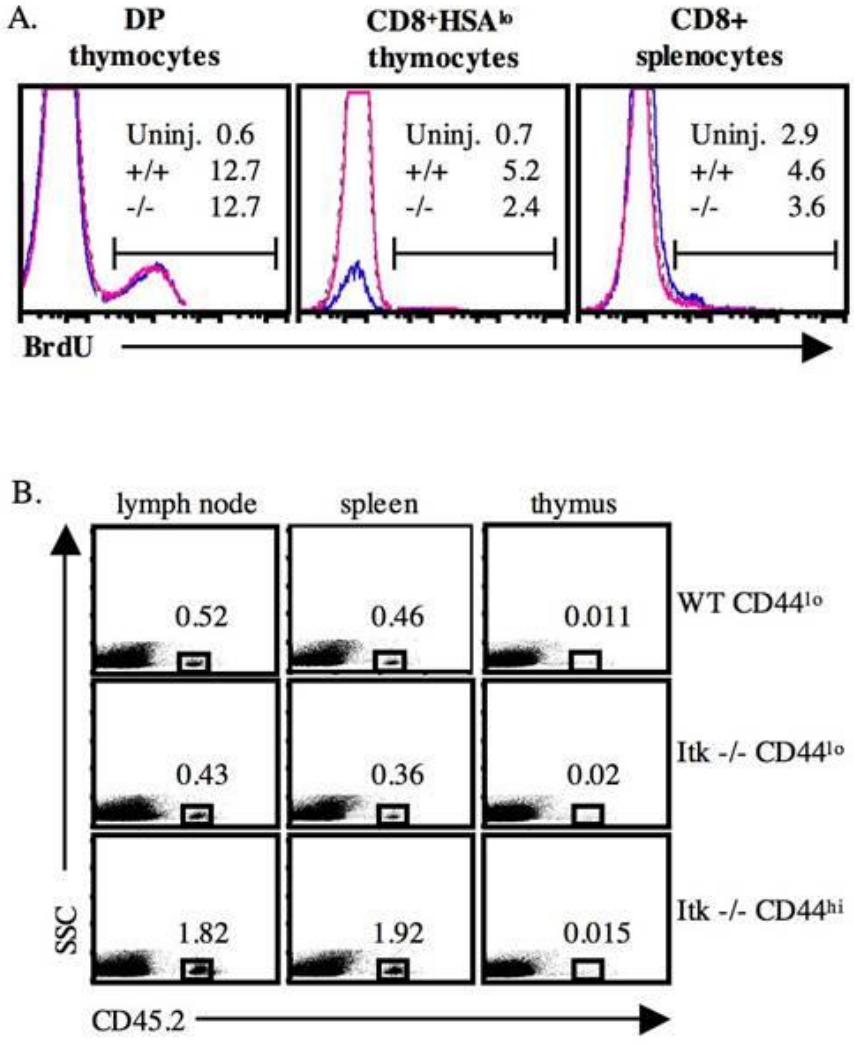
CD44 expression on peripheral CD8^{+} T cells is upregulated following antigen-specific activation and expansion, or alternatively, after cells have undergone lymphopenia-induced proliferation (159, 160). To determine whether Itk$^{-/-}$ CD8^{+} SP thymocytes and peripheral T cells were actively proliferating, we analyzed BrdU incorporation of CD8^{+} CD44^{hi} cells in the thymus and spleen of Itk$^{-/-}$ mice. Mice were injected with BrdU and analyzed 12 h later. As the majority of thymocyte proliferation occurs following pre-TCR stimulation, BrdU is primarily incorporated by CD3^{-}4^{-}8^{-} thymocytes and cells in transition to the CD4^{+}8^{+} [double positive; DP] stage; following this transition, the majority of DP thymocytes are not in cycle (161). It has also been reported that a small proportion of SP thymocytes proliferate following positive selection (162). Similarly, in the periphery of unimmunized non-lymphopenic mice, there is normally very little proliferation of naïve T cells. Following BrdU injection, a clear population of BrdU^{+} DP thymocytes is apparent in both wild-type and Itk$^{-/-}$ mice, demonstrating

Figure 10: Itk$^{-/-}$ CD8^{+} cells are not actively proliferating and do not preferentially migrate to the thymus

[A] Mice were injected with BrdU and incorporation was assessed 12 h later to determine the percentage of cells actively proliferating in wild-type [solid blue line] and Itk$^{-/-}$ [solid pink line] mice. The dashed line shows cells from uninjected mice stained with the anti-BrdU antibody. Numbers indicate the percentage of cells of each genotype in the positive gate.

[B] Wild-type CD8^{+} CD44^{lo}, Itk$^{-/-}$ CD8^{+} CD44^{lo}, and Itk$^{-/-}$ CD8^{+} CD44^{hi} cells [all CD45.2^{+}] were adoptively transferred into CD45.1^{+} wild-type congenic mice and the presence of the transferred cells in various organs was analyzed 7 days later. Numbers represent the percentage of CD45.2^{+} cells in each organ at the time of analysis. Results are representative of one of two experiments with three mice per condition.

Figure 10


A.
DP thymocytes
CD8+HSAlo thymocytes
CD8+ splenocytes
Uninj. 0.6
+/+ 12.7
-/- 12.7
Uninj. 0.7
+/+ 5.2
-/- 2.4
Uninj. 2.9
+/+ 4.6
-/- 3.6
BrdU
B.
lymph node
spleen
thymus
0.52
0.46
0.011
WT CD44lo
0.43
0.36
0.02
Itk -/- CD44lo
1.82
1.92
0.015
Itk -/- CD44hi
SSC
CD45.2

that comparable levels of BrdU were present in both mice [Figure 10A]. When $CD8^+$ SP thymocytes were examined, we detected little proliferation in the mature HSA^{lo} $CD8^+$ SP fraction of either thymus, and interestingly, the fraction of $BrdU^+$ HSA^{lo} $CD8^+$ SP cells in the $Itk^{-/-}$ thymus was even smaller than in the wild-type thymus. Analysis of the peripheral splenic $CD8^+$ T cells also revealed no increased proliferation of the $Itk^{-/-}$ cells compared to the wild-type controls [Figure 10A]. These data indicate that $Itk^{-/-}$ $CD8^+$ thymocytes and peripheral T cells are not accumulating due to increased proliferation either in the thymus or the spleen.

Although conventional T cells do not typically acquire a $CD44^{hi}$ phenotype in the thymus, they can gain access to the thymus as a result of recirculation from the periphery (163, 164). To examine the possibility that peripheral $Itk^{-/-}$ $CD8^+$ $CD44^{hi}$ cells were preferentially homing to thymus from the periphery, we assessed the trafficking patterns of $Itk^{-/-}$ cells following adoptive transfer into wild-type congenic recipients. For these experiments, purified wild type or $Itk^{-/-}$ $CD8^+$ thymocytes [$CD45.2^+$] were injected into $CD45.1^+$ congenic wild-type mice, and the ability of these transferred cells to migrate back to the thymus of the recipients was assessed. As shown in Figure 10B, even though the transferred $Itk^{-/-}$ $CD8^+$ $CD44^{hi}$ cells were easily detectable in the spleen and lymph nodes of the recipient mice, no transferred cells could be found in the thymus of the host mice. This was also the case for $CD8^+$ $CD44^{lo}$ thymocytes purified from either $Itk^{-/-}$ or wild-type mice. These data indicate that the accumulation of $CD8^+$ $CD44^{hi}$

cells in the thymi of Itk$^{-/-}$ mice is not due to preferential migration of peripheral Itk$^{-/-}$ CD8^{+} CD44^{hi} cells to the thymus.

Normal CD8+ T cell differentiation is restored in Itk$^{-/-}$ OT-1 TCR transgenic mice

Altogether these data suggest that the unusual CD8^{+} phenotype we observe in Itk$^{-/-}$ mice is likely due to altered development of CD8^{+} T cells in the thymus. Previously we have shown that the avidity of the TCR on developing CD4^{+} thymocytes influences the outcome of positive selection in Itk-deficient mice. For instance, development of mature CD4^{+} T cells is dramatically impaired when the cells express a low avidity TCR, whereas the number of mature CD4^{+} T cells that develop when the cells express a high avidity TCR is comparable to that observed in Itk-sufficient mice (150). This is in agreement with studies of TCR signaling in Itk$^{-/-}$ peripheral T cells, which have shown that the absence of Itk causes more substantial defects when the stimulus is sub-optimal (102, 138, 165). Thus, we were interested in determining if the defects in CD8^{+} T cell development observed in Itk$^{-/-}$ mice arise as a result of impaired TCR signaling during positive selection. If this were the case, one would predict that the accumulation of CD44^{hi} CD8^{+} SP thymocytes would not occur in Itk$^{-/-}$ mice crossed to an MHC class I-specific TCR transgenic line, if the TCR chosen is very efficiently selected in the thymus.

To test this possibility, we crossed Itk$^{-/-}$ mice to the TCR transgenic OT-1^{+} line. Previous data have indicated that the OT-1 TCR has a relatively high avidity

for positively-selecting ligands in the thymus (166). In support of this notion, we found that the maturation of $CD8^+$ T cells with high levels of the OT-1 TCR was only slightly reduced in the absence of Itk [Figure 11]. This is in contrast to previous data showing that the $CD8^+$ T cells expressing the H-Y TCR fail to be selected in the absence of Itk (151, 153). Furthermore, as compared to non-TCR-transgenic $Itk^{-/-}$ mice, which accumulate mature HSA^{lo} cells in the thymus, OT-1 $Itk^{-/-}$ mice have $CD8^+$ SP thymocytes that are comparable to those found in both OT-1 TCR transgenic and non-transgenic Itk-sufficient mice [Figure 11 and Figure 7A]. In addition, the CD44 profiles of wild-type versus $Itk^{-/-}$ OT-1^+ $CD8^+$ SP thymocytes and peripheral $CD8^+$ T cells were identical [Figure 11 and data not shown]. Thus, when positive selection signals are largely independent of Itk, $CD8^+$ differentiation proceeds normally, and there is no accumulation of $CD44^{hi}$ $CD8^+$ SP thymocytes or peripheral T cells. These results are consistent with those obtained by Broussard et al., in which these defects in $CD8^+$ T cell development were suppressed by the expression of a hypersensitive allele of ERK2 in Itk-deficient mice (71).

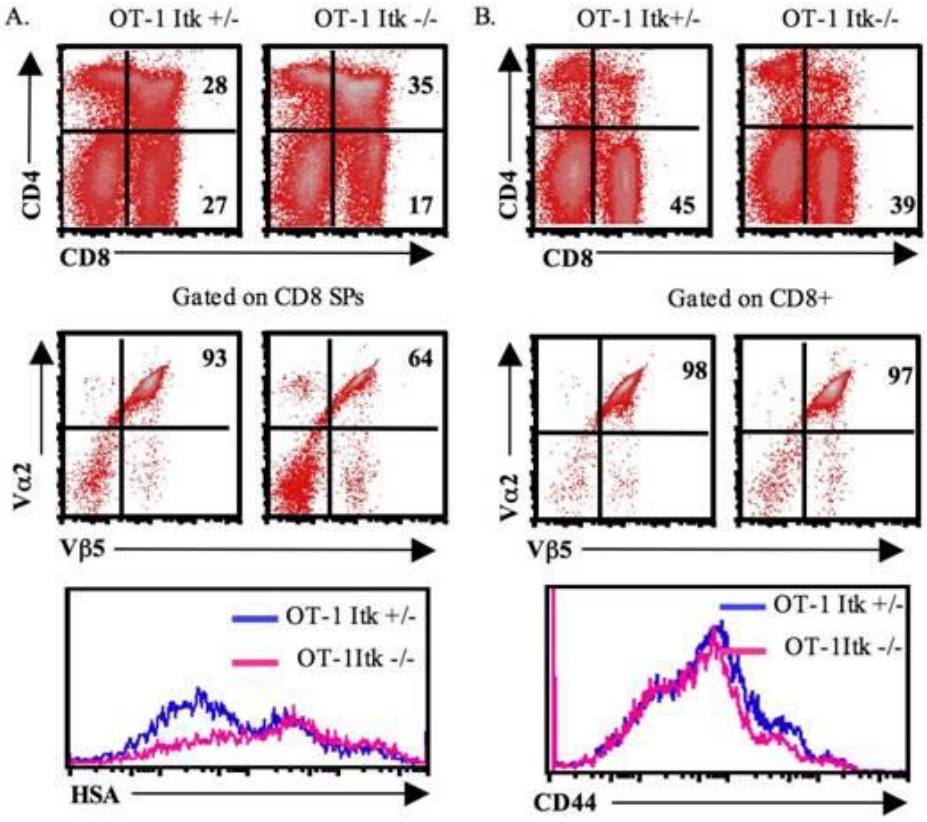
The altered $CD8^+$ T cell development in $Itk^{-/-}$ mice is dependent on IL-15

The data described above strongly suggest that $CD44^{hi}$ $CD8^+$ T cells arise first in the thymus and then accumulate in the periphery in $Itk^{-/-}$ mice, and that their appearance depends on reduced TCR signaling during selection. In addition, the data of Broussard et al. indicate that large populations of $CD8^+$

Figure 11: $CD8^+$ OT-I TCR transgenic $Itk^{-/-}$ T cells develop normally

[A] Thymocytes and [B] lymph node cells from eight-week old OT-I $Itk^{+/-}$ and OT-I $Itk^{-/-}$ mice were analyzed for expression of CD4 and CD8 [top row]. In the dot-plots below, gated $CD8^+$ populations were examined for the expression of the transgenic TCR by staining with anti-Vα2 and anti-Vβ5. The numbers in each quadrant indicate the percentage of cells in each subpopulation. Histograms [bottom row] show HSA expression on $CD8^+$ SP thymocytes and CD44 expression on the $CD8^+$ lymph nodes cells. Data are representative of four mice of each genotype analyzed.

Figure 11


A.
OT-1 Itk +/-
OT-1 Itk -/-
28
27
35
17
CD4
CD8
Gated on CD8 SPs
93
64
Vα2
Vβ5
OT-1 Itk +/-
OT-1Itk -/-
HSA
B.
OT-1 Itk+/-
OT-1 Itk-/-
45
39
CD4
CD8
Gated on CD8+
98
97
Vα2
Vβ5
OT-1 Itk +/-
OT-1Itk -/-
CD44

thymocytes still develop when Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ bone marrow are used to reconstitute irradiated β2m$^{-/-}$ hosts (71). Positive selection mediated by MHC molecules expressed on hematopoietic cells, rather than on radiation-resistant thymic stroma, is a characteristic of alternative lineages of αβ TCR^{+} T cells, such as NKT cells and H2-M3-restricted T cells, which are specific for ligands bound to nonclassical MHC class I-b molecules (13, 49, 50). In addition, where it has been examined, these T cell lineages are also exquisitely dependent on IL-15 for their maintenance in the periphery (167-169).

To test the notion that Itk$^{-/-}$ CD8^{+} CD44^{hi} T cells represent an IL-15-dependent lineage, we crossed Itk$^{-/-}$ mice to IL-15$^{-/-}$ mice. As shown in Figure 12A, both the percentage and absolute numbers of CD8^{+} SP thymocytes are reduced in Itk$^{-/-}$ IL-15$^{-/-}$ mice to a level nearing that seen in wild type mice. Moreover, the remaining CD8^{+} SP thymocytes in Itk$^{-/-}$ IL-15$^{-/-}$ mice show a normal profile of CD44 expression. Furthermore, Itk$^{-/-}$ IL-15$^{-/-}$ mice are nearly devoid of CD8^{+} T cells in the periphery [Figure 12B]. In terms of absolute cell numbers, Itk$^{-/-}$ IL-15$^{-/-}$ mice have a 23-fold reduction in peripheral CD8^{+} T cells compared to wild type mice, and a 7-fold reduction compared to IL-15-sufficient Itk$^{-/-}$ mice. This is in contrast to the ~2-fold loss in total CD8^{+} T cell numbers and ~3-fold reduction in numbers of CD8^{+} CD44^{hi} T cells in single IL-15-deficient mice compared to wild type controls. Thus, Itk$^{-/-}$ CD8^{+} CD44^{hi} T cells are far more dependent on IL-15 than their wild type counterparts, including wild type memory-phenotype CD8^{+} T cells, and in this regard, more closely resemble NKT

Figure 12: $CD8^+$ T cells are abolished in the absence of Itk and IL-15

[A] Thymocytes and [B] lymph node cells isolated from wild-type, $Itk^{-/-}$, $IL\text{-}15^{-/-}$ and $Itk^{-/-}IL\text{-}15^{-/-}$ mice were stained for expression of CD4, CD8 and CD44. Dot-plots show CD4 versus CD8 staining [top row] and CD8 versus CD44 staining [bottom row], with the numbers in each region indicating the percentage of cells in each subpopulation. The graphs below show the absolute cell numbers ± SEM for each subpopulation. Data are generated from a minimum of six mice from four independent experiments per group.

Figure 12

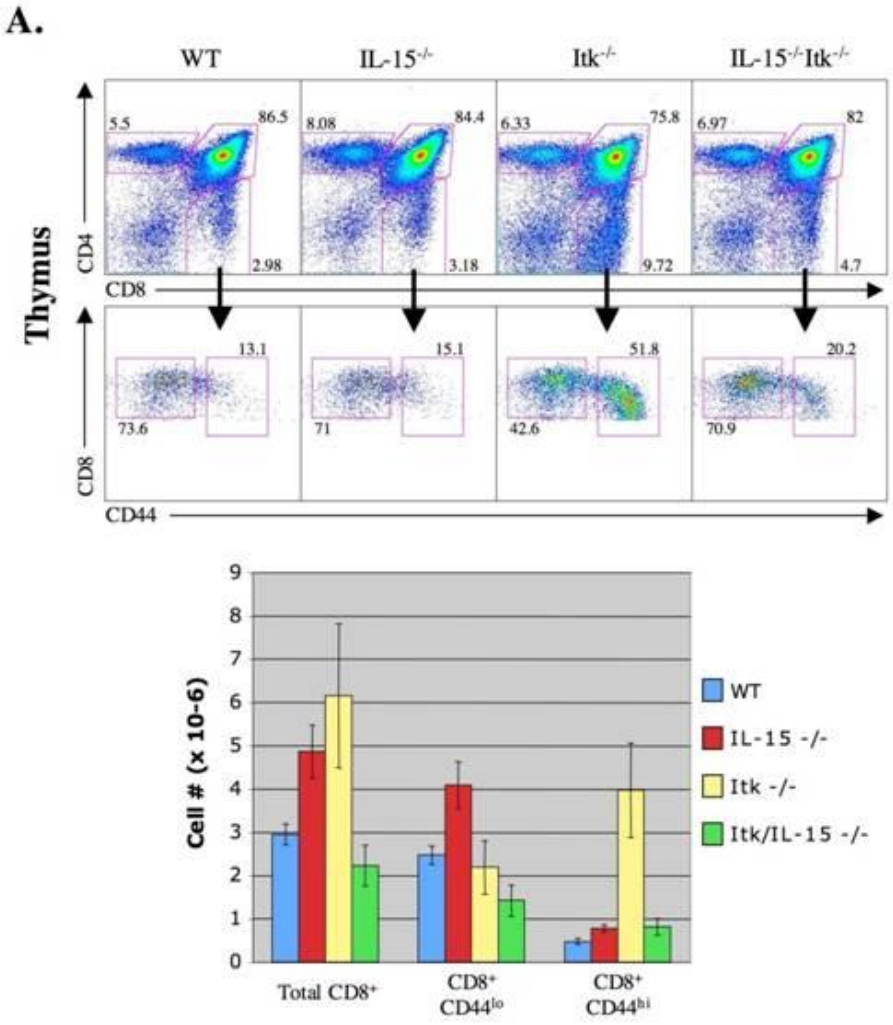
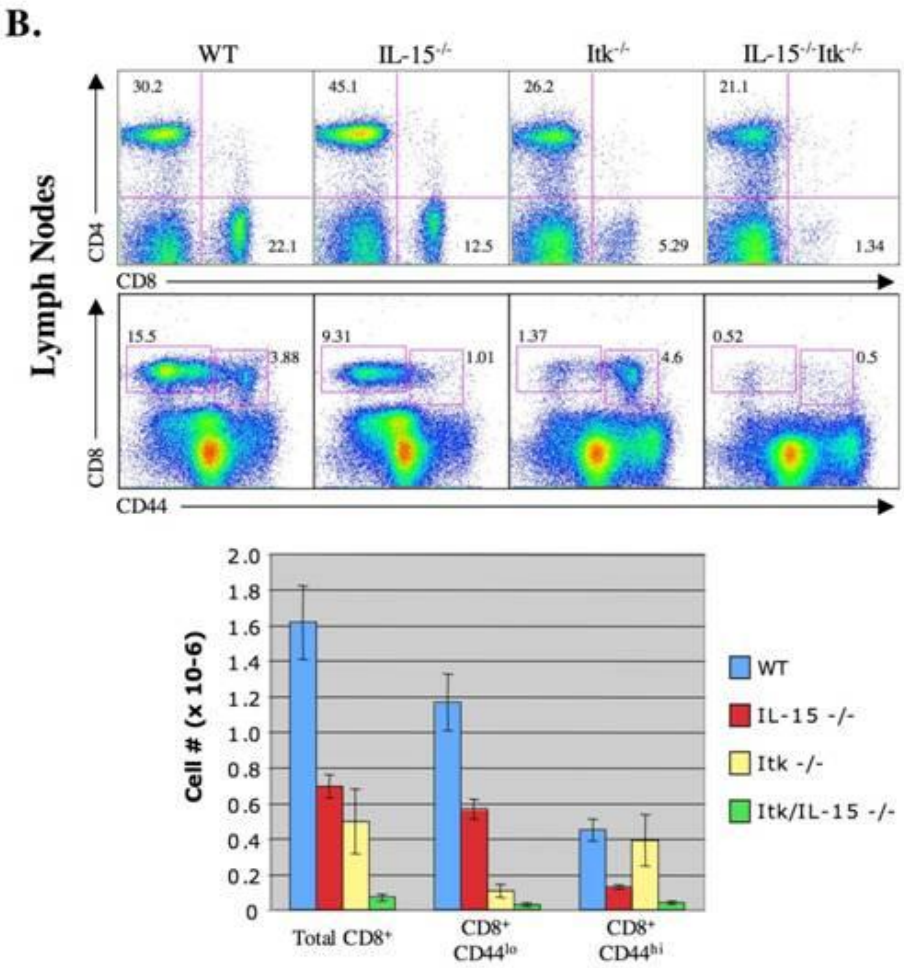

A.
WT
IL-15-/-
Itk-/-
IL-15-/-Itk-/-
Thymus
CD4
CD8
5.5
86.5
2.98
8.08
84.4
3.18
6.33
75.8
9.72
6.97
82
4.7
CD8
CD44
13.1
73.6
15.1
71
51.8
42.6
20.2
70.9
Cell # (x 10-6)
0
1
2
3
4
5
6
7
8
9
Total CD8+
CD8+ CD44lo
CD8+ CD44hi
WT
IL-15 -/-
Itk -/-
Itk/IL-15 -/-

Figure 12


B.
WT
IL-15-/-
Itk-/-
IL-15-/-Itk-/-
Lymph Nodes
CD4
CD8
30.2
22.1
45.1
12.5
26.2
5.29
21.1
1.34
CD8
CD44
15.5
3.88
9.31
1.01
1.37
4.6
0.52
0.5
Cell # (x 10-6)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Total CD8+
CD8+ CD44lo
CD8+ CD44hi
WT
IL-15 -/-
Itk -/-
Itk/IL-15 -/-

cells than they do conventional TCRαβ^{+} CD8^{+} T cells (167). Together, these data strongly suggest that the CD44^{hi} CD8^{+} SP phenotype observed in Itk$^{-/-}$ mice develops first in the thymus, and that the maintenance of these cells in peripheral lymphoid organs is stringently dependent on IL-15.

Eomesodermin is expressed at high levels in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ thymocytes undergoing positive selection

One striking aspect of the altered phenotype of Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ CD8^{+} SP thymocytes is their ability to produce IFNγ when stimulated immediately ex vivo. Since CD8^{+} T cell effector function is associated with expression of the T-box transcription factor, Eomesodermin [Tbr2] (170), we investigated whether Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ thymocytes showed altered expression of Eomesodermin. For this experiment, mRNA was prepared from sorted DP, CD4^{+} SP, and CD8^{+} SP thymocytes isolated from wild type, Itk$^{-/-}$, and Itk$^{-/-}$Rlk$^{-/-}$ mice. Eomesodermin transcript levels were then determined by real-time quantitative RT-PCR. As shown in Figure 13A, all three subsets of wild type thymocytes express low levels of Eomesodermin mRNA. In contrast, Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ CD8^{+} SP thymocytes have ~100-fold increased levels of Eomesodermin mRNA compared to wild type controls. Since IFNγ transcription in CD8^{+} T cells can also be regulated by the related transcription factor, T-bet (171), and furthermore, we have previously shown that T-bet expression is disregulated in Itk$^{-/-}$ CD4^{+} T cells (102), we also examined the levels of T-bet mRNA in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ CD8^{+} SP thymocytes.

Figure 13: $CD8^+$ thymocytes from $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice express high levels of Eomesodermin

[A] Thymocytes from wild type, $Itk^{-/-}$, and $Itk^{-/-}Rlk^{-/-}$ mice were sorted into $CD4^+CD8^+$ [DP], $CD4^-CD8^-$ SP [CD4] and $CD4^+CD8^-$ SP [CD8] cell populations. In [B], $CD4^+8^+$ DP and CD4-8+ SP cells were further sorted into $TCR\beta^{lo}$ $CD69^-$ and $TCR\beta^{hi}$ $CD69^+$ populations. In [C] cells were sorted in the same way as in [B] but animals utilized were OT-I positive and WT or OT-I positive and $Itk^{-/-}$. Total RNA was then isolated, cDNA prepared, and real-time quantitative PCR performed for Eomesodermin and β-actin. The y-axis values represent eomesodermin transcript levels normalized to the β-actin values determined for each sample. Error bars are the SD of values obtained from triplicate reactions.

Figure 13

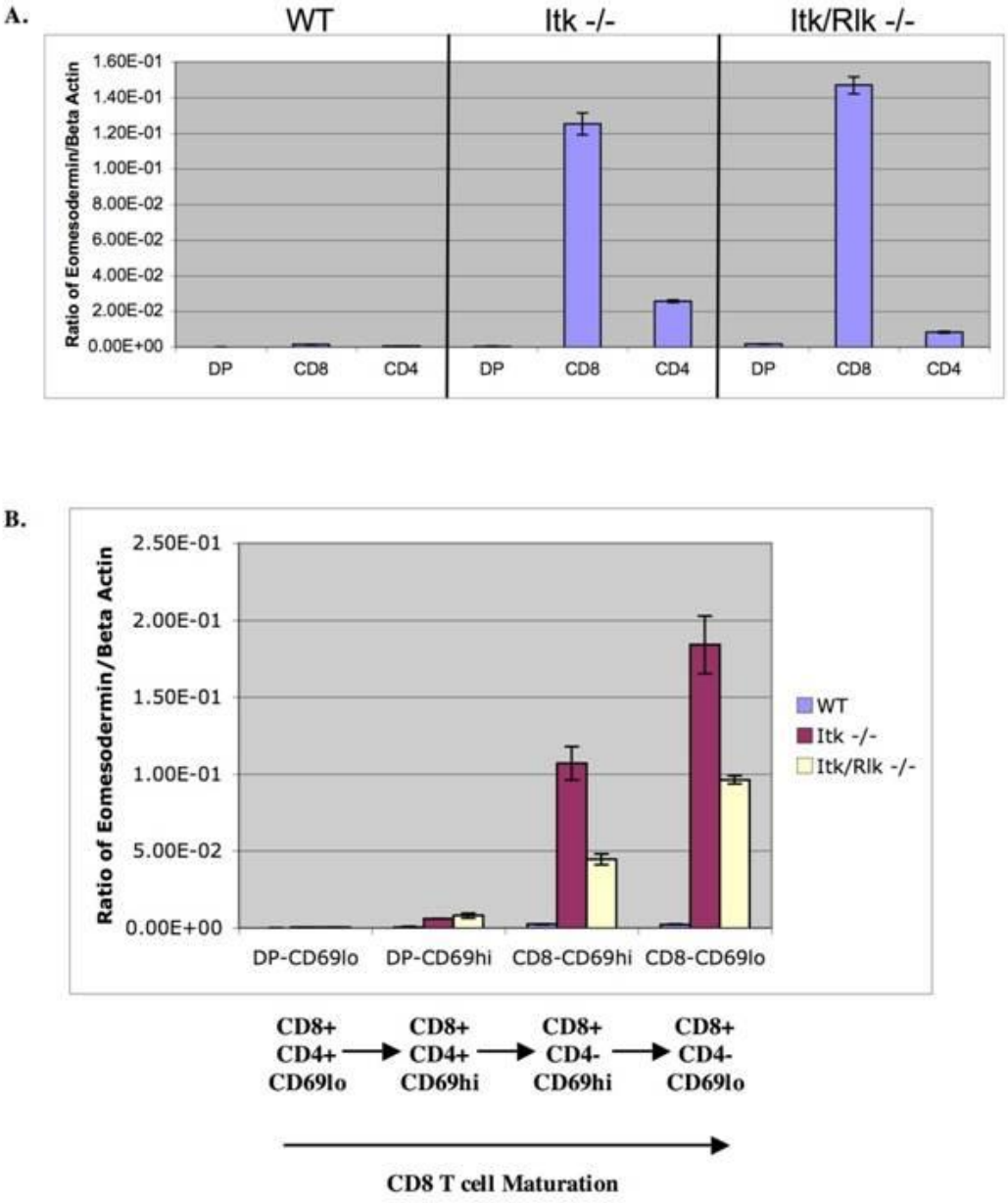
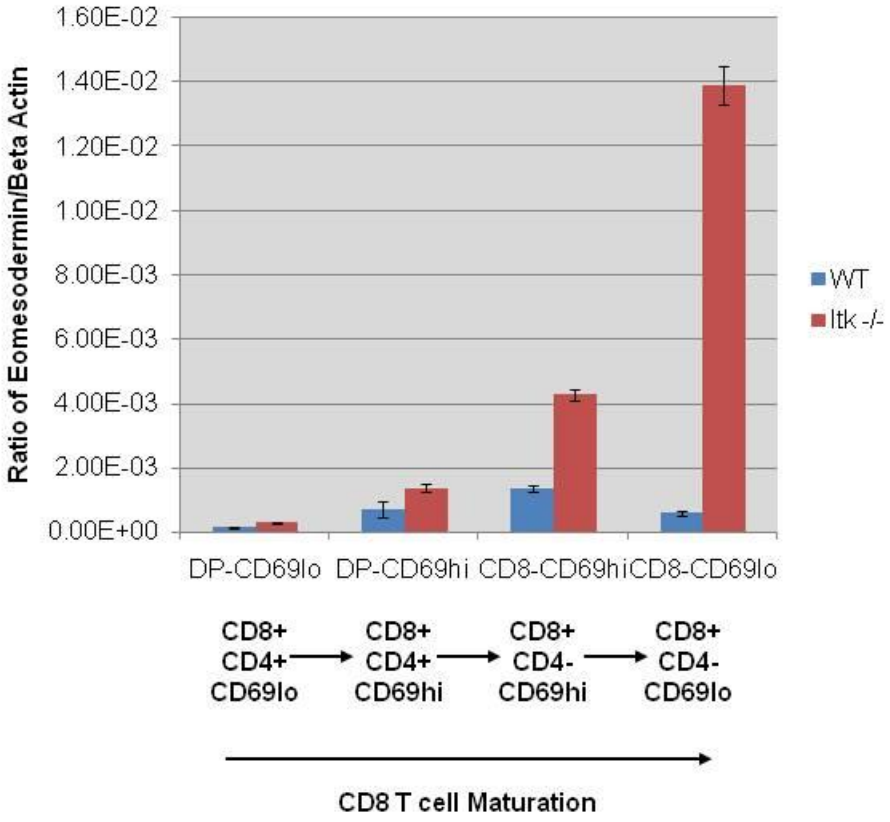

A.
WT
Itk -/-
Itk/Rlk -/-
Ratio of Eomesodermin/Beta Actin
1.60E-01
1.40E-01
1.20E-01
1.00E-01
8.00E-02
6.00E-02
4.00E-02
2.00E-02
0.00E+00
DP
CD8
CD4
B.
Ratio of Eomesodermin/Beta Actin
2.50E-01
2.00E-01
1.50E-01
1.00E-01
5.00E-02
0.00E+00
WT
Itk -/-
Itk/Rlk -/-
DP-CD69lo
DP-CD69hi
CD8-CD69hi
CD8-CD69lo
CD8+ CD4+ CD69lo
CD8+ CD4+ CD69hi
CD8+ CD4- CD69hi
CD8+ CD4- CD69lo
CD8 T cell Maturation

Figure 13

C.


1.60E-02
1.40E-02
1.20E-02
1.00E-02
8.00E-03
6.00E-03
4.00E-03
2.00E-03
0.00E+00
Ratio of Eomesodermin/Beta Actin
WT
Itk -/-
DP-CD69lo
DP-CD69hi
CD8-CD69hi
CD8-CD69lo
CD8+ CD4+ CD69lo
CD8+ CD4+ CD69hi
CD8+ CD4- CD69hi
CD8+ CD4- CD69lo
CD8 T cell Maturation

In contrast to the findings with Eomesodermin, we found no increased expression of T-bet mRNA in both Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ CD8^{+} SP thymocytes relative to wild type control cells [data not shown].

To determine the precise stage in CD8^{+} thymocyte development at which Eomesodermin transcripts are up-regulated, we further sorted thymocytes into pre-positive selection DP [CD4^{+}CD8^{+} TCR^{lo} CD69^{lo}], DP undergoing positive selection [CD4^{+}CD8^{+} TCR^{hi} CD69^{hi}], newly-arising CD8^{+} SP [CD4^{-}CD8^{+} TCR^{hi} CD69^{hi}] and more mature CD8^{+} SP [CD4^{-}CD8^{+} TCR^{hi} CD69^{lo}] subsets. As can be seen in Figure 13B, Eomesodermin mRNA is first up-regulated in Itk$^{-/-}$ and Itk$^{-/-}$ Rlk$^{-/-}$ DPs undergoing positive selection compared to wild type DPs at this stage of maturation [12-16-fold increase in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ TCR^{hi} CD69^{hi} DPs compared to wild type]. As DP cells transition into the CD8^{+} SP lineage, the increased expression of Eomesodermin becomes even more striking, resulting in 20-50-fold increases in Eomesodermin mRNA in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ TCR^{hi} CD69^{hi} CD8^{+} SPs compared to wild type control cells. These data demonstrate that the maturation of CD8^{+} SP thymocytes in the absence of Itk, or Itk and Rlk, is accompanied by a dramatic up-regulation of Eomesodermin transcripts, thereby accounting for the activated/memory cell phenotype and functional behavior of these cells.

Crossing the Itk$^{-/-}$ to OT-1 transgenics, which as shown on Figure 11 abolishes the memory [CD44^{hi}] phenotype, does not seem to completely abolish the increased expression of Eomesodermin in the later stages of CD8 T cell

maturation [Figure 13C, DP-CD69^{hi} on]. It should be noted that the changes seen in the Itk$^{-/-}$ versus the WT are now much milder: in the DP-CD69^{hi} about 2-fold increase in the OT-1^{+} versus 12-fold increase in the OT-1^{-}; in the CD8^{+}CD69^{hi} about 3-fold increase in the OT-1^{+} versus 20-fold increase in the OT-1^{-}; and in the mature CD8^{+}CD69^{lo} about 20-fold increase in the OT-1^{+} versus about 100-fold increase in the OT-1^{-}. Although it is somewhat perplexing that increasing the TCR signal with the OT-1 receptor does not completely abolish the change in Eomesodermin expression in the Itk$^{-/-}$ CD8^{+} T cells, it is somewhat reassuring that the changes seen are much lower, indicating that signal strength is a large component of the phenotype.

Discussion

Given the data presented in this chapter and the evidence indicating that the CD8^{+} T cells in the Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ cells are selected by a hematopoietic cells and a SAP dependent signaling pathway, it seems clear that the conventional CD8^{+} T cell lineage choice is extremely reliant on signaling through Tec family kinases Itk and Rlk (71, 73). Here we show that the majority of CD8^{+} SP thymocytes and peripheral T cells that arise in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice are distinct from conventional TCRαβ^{+} CD8^{+} T cells. As opposed to normal naive CD8^{+} T cells, the Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ CD8^{+} T cells resemble previously-activated/memory CD8^{+} T cells, which in addition to being CD44^{hi} and CD62L^{hi},

also express increased levels of the activation/memory markers, CD122 and NK1.1. Further supporting their status as effector or memory cells, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ $CD8^{+}$ thymocytes and peripheral T cells produce IFNγ in response to simulation immediately ex vivo. In addition, the maturation of these cells and their maintenance in the periphery are totally dependent on IL-15. What distinguishes these cells from conventional, peripheral memory $CD8^{+}$ T cells is that these features are not acquired by the cells as a result of peripheral activation signals, but instead arise during a process of intrathymic development which is dependent on SAP for selection [on hematopoietic cells] and CD28 for acquisition of the innate-like phenotype (71, 73).

In all of these aspects, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ $CD8^{+}$ thymocytes and peripheral T cells bear a striking similarity to nonconventional T cell lineages, such as cells specific for nonclassical MHC class I-b molecules. One subset of such cells is NKT cells that are specific for glycolipids bound to the CD1d molecule. The $CD8^{+}$ T cells in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice share many common features with NKT cells, including the expression of many maturation and activation markers [HSA^{lo}, $CD44^{hi}$, $CD122^{hi}$, $NK1.1^{+}$] (172). In addition, the $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ $CD8^{+}$ SP thymocytes are abundantly selected in bone marrow chimeric mice in which only the hematopoietic donor cells, but not the host thymic stromal cells, express MHC class I molecules. This positive selection behavior is a characteristic of NKT cells, and distinctly different from that of conventional $CD8^{+}$ T cells, particularly the dependence on SAP (20-22, 49, 73). Finally, we show that the

peripheral maintenance of Itk$^{-/-}$ CD8^{+} T cells is stringently dependent on IL-15, another characteristic feature of NKT cells (167).

In spite of these strong correlations, it seems unlikely that Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ CD8^{+} T cells are bona fide NKT cells. NKT cells that are specific for ligands bound to the CD1d molecule are found in the CD4^{+} and CD4^{-}8^{-} subsets, but not among the CD8^{+} T cell population (172). In addition, CD8^{+} SP thymocytes in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice fail to stain with CD1d/α-gal-cer tetramers (1). Another possibility is that Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ CD8^{+} T cells might be specific for the MHC class I-b molecule, H2-M3. H2-M3-specific T cells share many of the features of CD1d-specific NKT cells, including an activated/memory phenotype, the ability to make cytokines directly ex vivo, and the ability to be selected on MHC class I molecules expressed on hematopoietic cells in the thymus; however H2-M3-specific T cells are CD8^{+} (13, 15, 16). Based on these similarities, it is possible that Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ CD8^{+} T cells consist of H2-M3-specific cells, although this hypothesis seems unlikely due to the large numbers of CD8^{+} T cells in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice.

We propose that Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ CD8+ SP thymocytes develop as a result of altered TCR signals that resemble, or mimic, those that normally select MHC class I-b-specific cells. Consistent with this notion, our analysis of OT-1 Itk$^{-/-}$ mice, as well as the analysis of ERK^{SEM} Itk$^{-/-}$ mice by Broussard et al, indicate that the development of CD8^{+} SP thymocytes with this unusual phenotype is resolved when sustained TCR signaling during thymic development is less

dependent on Itk. Furthermore, selection of the innate-like cells in the $Itk^{-/-}$ mice is dependent on selection on hematopoietic cells, as forcing selection on MHC expressed by the thymic stroma prevents the innate phenotype (73). Finally, although selection of the innate-like $CD8^+$ T cells is not dependent on CD28 signaling, generation of the innate phenotype requires its expression. Therefore, we hypothesize that $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ $CD8^+$ SP thymocytes undergo altered lineage differentiation in response to reduced TCR-mediated signals.

One insight into the mechanism leading to altered lineage differentiation is the profound up-regulation of Eomesodermin expression in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ $CD8^+$ SP thymocytes. This transcription factor is known to regulate $CD8^+$ T cell effector functions, such as the ability to produce IFNγ upon TCR stimulation. Our data indicate that increased expression of Eomesodermin is readily detectable in the subset of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ DP thymocytes that are in the process of receiving positive selection signals. Boosting the signaling through expression of a stronger TCR [OT-1] severely reduced the upregulation of eomesodermin, but not to WT levels. These findings strongly support the conclusion that Itk, and potentially Rlk, play an important role during $CD8^+$ T cell differentiation, and that altered thymic development is responsible for the memory cell phenotype of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ $CD8^+$ SP thymocytes and T cells. These results also raise the interesting possibility that increased expression of Eomesodermin may be responsible, at least in part, for this phenotype.

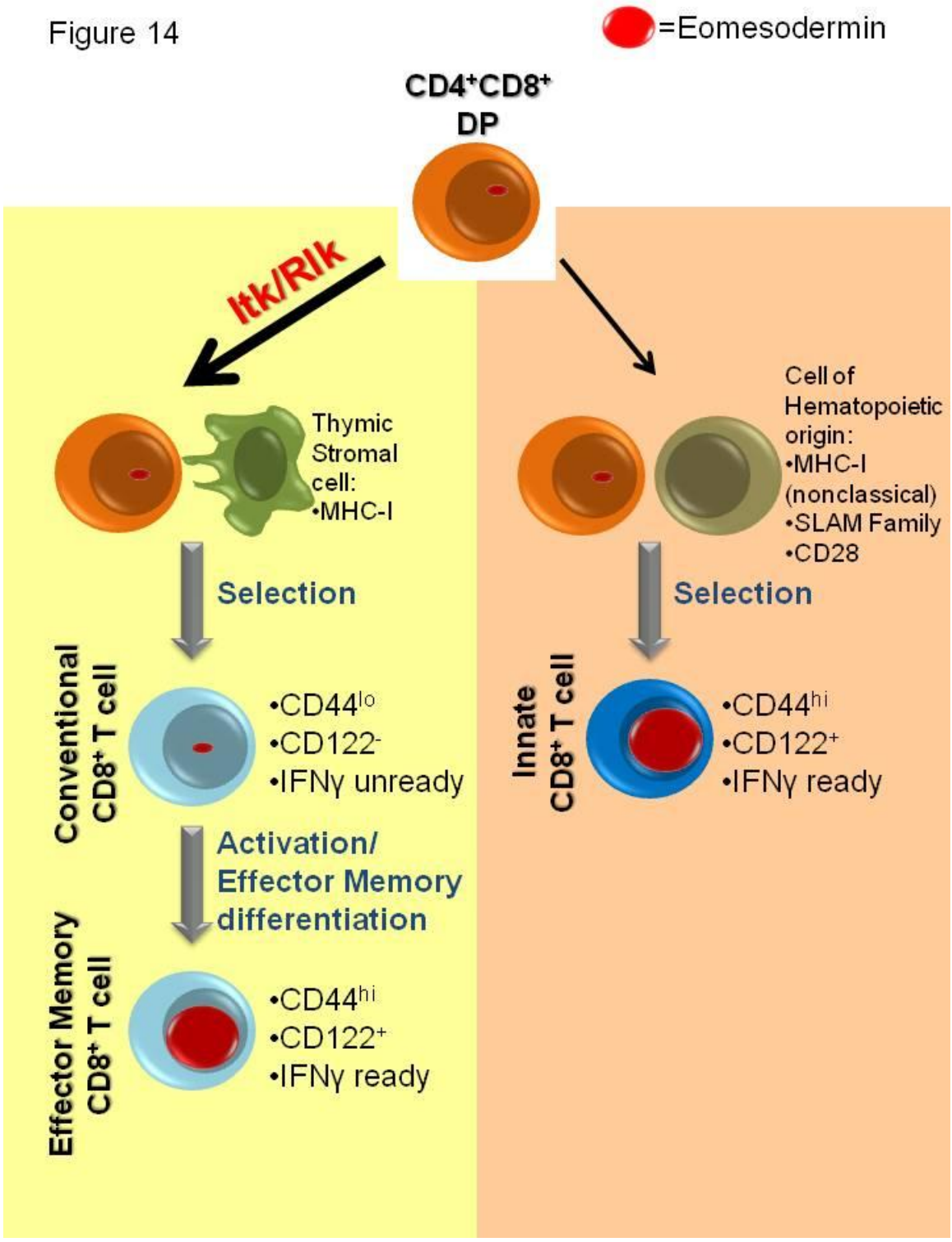
Taken together, these data suggest the following model [Figure 14]. Weaker TCR signaling during positive selection of MHC class I-specific thymocytes in the absence of Itk or Itk and Rlk promotes selection mediated by hematopoietic cells, through a SAP dependent mechanism, and the up-regulation of Eomesodermin. Eomesodermin then directly induces CD122 expression (173), thereby enhancing the responsiveness of these cells to cytokines, such as IL-15. In response to IL-15, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ $CD8^{+}$ SP thymocytes up-regulate Bcl-2 (150, 158) increasing their survival and promoting their differentiation into $CD8^{+}$ effector cells. High levels of Eomesodermin further contribute to the differentiation of $CD8^{+}$ T cells with immediate effector function capabilities, such as the ability to produce IFNγ. In addition, these findings suggest the intriguing possibility that a similar series of events regulates the maturation of MHC class I-b-specific T cells in response to weaker positive selection signals on the poorly-expressed MHC class I-b molecules. A role for Eomesodermin in the development and/or function of H2-M3-specific $CD8^{+}$ T cells may therefore be a fruitful avenue of investigation for future studies.

Figure 14: Conventional versus innate $CD8^+$ T cell differentiation model

The presence of Tec family kinases Itk and Rlk in DP thymocytes helps boost signals through the TCR, allowing for selection in thymic stromal cells mediated by classical MHC-I. This leads to selection of naïve conventional $CD8^+$ T cells [$CD44^{lo}$, $CD122^-$, and uncapable of quick IFNγ secretion] which haven't significantly upregulated eomesodermin. The generation of effector memory [through TCR mediated activation] on these cells leads to upregulation of eomesodermin, CD44, CD122, and the ability to quickly secrete IFNγ.

Absence of Tec family kinases Itk and Rlk, on the other hand, leads to a weaker signal and preferred selection on cells of hematopoietic origin. This selection is dependent on MHC-I and signaling through SLAM family members [mediated by SAP]. Selection is not dependent on CD28, but CD28 is needed for the innate phenotype of the cells. Early during the selection process eomesodermin is quickly upregulated. Selection leads to formation of innate $CD8^+$ T cells with high expression of CD44, CD122, and the ability to quickly secrete IFNγ without any prior stimulations.

Figure 14


=Eomesodermin
CD4+CD8+
DP
Itk/Rlk
Thymic
Stromal
cell:
•MHC-I
Selection
Conventional
CD8+ T cell
•CD44lo
•CD122-
•IFNγ unready
Activation/
Effector Memory
differentiation
Effector Memory
CD8+ T cell
•CD44hi
•CD122+
•IFNγ ready
Cell of
Hematopoietic
origin:
•MHC-I
(nonclassical)
•SLAM Family
•CD28
Selection
Innate
CD8+ T cell
•CD44hi
•CD122+
•IFNγ ready

Chapter III: Itk and Rlk regulate NKT cell development and function

Chapter III Attributions and Copyright information

The material in this chapter was written by me and is derived from the JI publication (174).

Felices, M., and L. J. Berg. 2008. The Tec kinases Itk and Rlk regulate NKT cell maturation, cytokine production, and survival. *J Immunol* 180:3007-3018.

Intro

NKT cells are an innate subset of αβTCR$^+$ T cells that express surface markers characteristic of both T cells and NK cells, and can rapidly produce large amounts of cytokines such as INFγ, IL-4 and IL-10 (26, 175). The great majority of murine NKT cells express an invariant TCR Vα and Jα combination [Vα14-Jα18] that is preferentially paired with a restricted TCR beta chain [mostly Vβ8.2 or Vβ7], and recognize lipid antigens in the context of CD1d (18, 19). These cells develop in the thymus from CD4$^+$8$^+$ [DP] thymocyte progenitors, and are positively selected by interactions with other CD1d$^+$/SLAM$^+$ DP cortical thymocytes (20, 22). Upon positive selection, NKT cells acquire markers characteristic of memory T cells, such as CD44 (21).

Previous studies have identified three differentiation stages of NKT cells in the thymus subsequent to their expression of a mature TCR that binds to CD1d-tetramer: stage 1, in which they have low expression of CD44 and NK1.1; stage 2, in which they acquire high expression of CD44; and stage 3, in which they acquire expression of NK1.1 as well as several other NK markers (21). Accompanying these changes in surface markers, NKT cells undergo a proliferative expansion between stages one and two, during which they already posses the ability to produce IL-4. At stage three, the cells are also competent to produce IFNγ (23-25). Interestingly, the NK1.1$^-$ NKT cells leave the thymus to

populate the periphery, where they can further mature into NK1.1^{+} NKT cells, while the more mature NK1.1^{+} NKT cells generated in the thymus remain in the thymus to become a long-lived non-dividing resident population (23, 24, 176). Furthermore, upregulation of NK1.1 on peripheral NKT cells, which denotes their terminal differentiation, is CD1d dependent (177), whereas the survival and homeostasis of these cells depends on IL-15 (168, 178).

Several cytokines, transcription factors, and signaling molecules have been implicated in the development and survival of NKT cells. As mentioned above, IL-15 is involved in NKT survival and proliferation in the periphery, while Csf-2 [granulocyte-macrophage colony-stimulating factor] is required for NKT cell differentiation and cytokine secretion (179). Members of the NF-κB transcription factor family have also been implicated in proliferation and survival of NKT cells (62). During development in the thymus, the SLAM [signaling lymphocytic activating molecule] family of receptors, the adaptor protein SAP [SLAM-associated protein], and the SRC-family tyrosine kinase Fyn have all been implicated in a signaling axis required for selection of NKT cells (20, 53-56). In addition, PKCθ [protein kinase θ], VAV1, IRF1 [interferon-regulatory factor 1], ETS1, ELF4 [E74-like factor 4 or MEF], RUNX1 [runt-related transcription factor 1] and RORγt [retinoic-acid-receptor-related orphan receptor-γt] have been shown to have significant roles in NKT cell development, function and survival (57-61, 63, 180).

Of interest, Th1 and Th2 transcriptional regulators, T-bet and GATA-3, respectively, have also been shown to have prominent roles in NKT development and homeostasis. For instance, In T-bet-deficient mice, the development of NKT cells in the thymus is blocked prior to upregulation of NK1.1 [i.e., prior to stage 3], leading to decreased numbers of NKT cells in the thymus and periphery (68). These mice also have defects in NKT cell migration, survival and effector functions, findings that are consistent with the prominent role of T-bet in CD122 expression and IFNγ transcription in T cells (181). Mice deficient in GATA-3 exhibit decreases in peripheral NKT cell numbers due to increased cell death, as well as alterations in NKT cell surface marker expression both in the thymus and the periphery; GATA-3-deficient NKT cells also show decreased cytokine secretion (67). It has recently been shown that a member of the NFAT family, NFAT2, has a more prominent role in IL-4 cytokine secretion by NKT cells than GATA-3 (182). Interestingly, Tec family kinases have been shown to modulate NFAT activation (137).

As mentioned in the general intro the Tec family of non-receptor tyrosine kinases has been shown to play a significant role in signaling downstream of the T cell receptor (1, 98). Three members of this family are expressed in conventional αβTCR+ T cells: Itk, Rlk and Tec. T cells from animals deficient in Itk have defects in TCR-induced PLC-γ phosphorylation, calcium flux generation, MAP kinase activation, and NFAT and AP-1 activation. These defects are exacerbated in T cells deficient for both Itk and Rlk, indicating possible

redundancy amongst the Tec family kinases. Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice also have impaired development of conventional CD4^{+} and CD8^{+} T cells in the thymus (70, 183).

Although Itk and Rlk are dispensable for the development of CD8^{+} "innate" T cells (69, 71-73), as discussed in the previous chapter, the role of Tec family kinases has not been thoroughly studied in other subsets of "innate" T cells, such as NKT cells. One previous study demonstrated a role for Itk in NKT cell development, indicating impaired progression of Itk$^{-/-}$ NKT cells to stage three and a resulting decrease in NKT cell numbers (184). Recently, another group has shown impaired function of Itk$^{-/-}$ NKT cells (185). In this chapter we confirm the developmental defect in NKT cells in Itk$^{-/-}$ mice, and show that this defect is exacerbated in the absence of both Itk and Rlk. We also confirm the functional defect in Itk$^{-/-}$ NKT cells and demonstrate that NKT cell function is further impaired in Itk$^{-/-}$Rlk$^{-/-}$ NKT cells, which produce virtually no effector cytokines in response to activation either *in vitro* or *in vivo*. Interestingly, expression of the T box transcription factor T-bet is reduced in NKT cells from Itk$^{-/-}$ mice, and even further reduced in Itk$^{-/-}$Rlk$^{-/-}$ NKT cells. CD122 expression is also impaired on Tec kinase-deficient NKT cells, leading to reduced NKT cell survival in the periphery. These data implicate a critical role for Tec family kinases in NKT cell development, function, and survival.

Results

Itk, Rlk and Tec are expressed in NKT cells

Three Tec family kinases are expressed in conventional αβ TCR^+ T cells, Itk, Rlk, and Tec. The hierarchy of expression of these kinases correlates with their known importance in T cell signaling, with Itk mRNA being expressed at the highest levels, followed by Rlk, and then Tec [Figure 15A] (1). To assess relative Tec kinase expression levels in NKT cells, thymic NKT cells were purified from wild type mice based on CD1d/αGAL tetramer [hereafter referred to as CD1d-tetramer]-binding, and RNA was isolated and used for quantitative real-time RT-PCR to measure the levels of Itk, Rlk, and Tec mRNA. As shown in Figure 15A, NKT cells show a similar hierarchy of expression of Tec family kinases compared to conventional αβ T cells, with Itk mRNA being expressed at the highest levels [similar to levels found in peripheral T cells], followed by Rlk and then Tec.

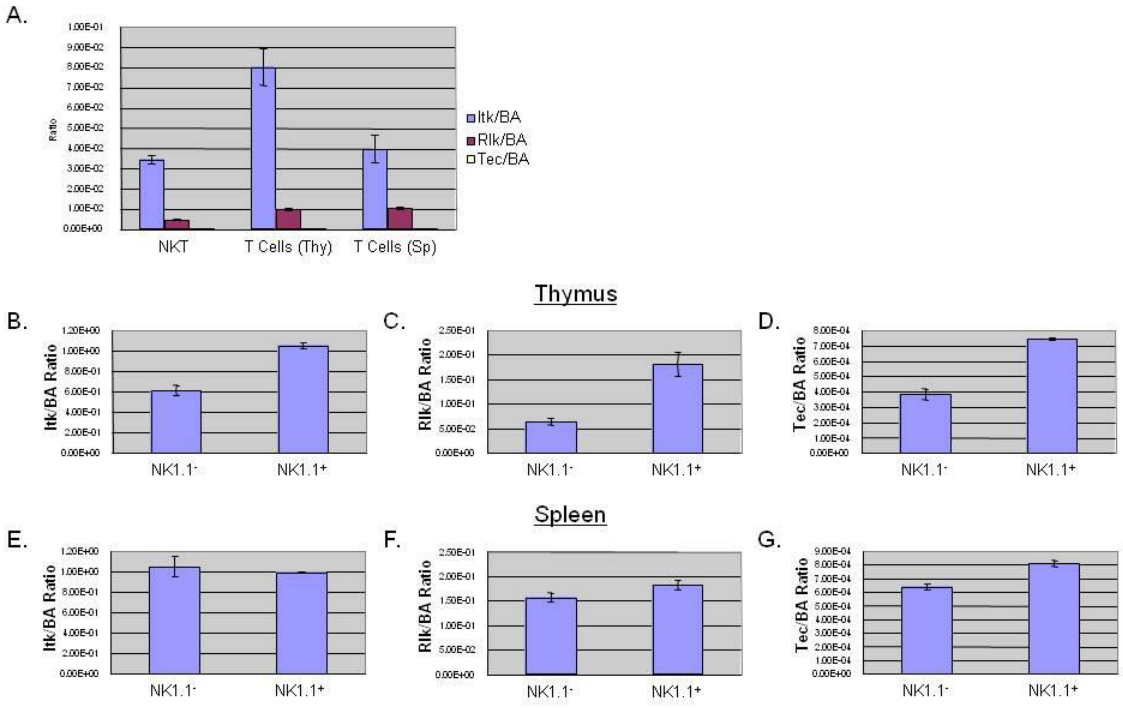
Tec family kinases are known to have altered patterns of expression during differentiation of $CD4^+$ T cells. For instance, Rlk is down-regulated following TCR stimulation, and is re-expressed in Th1 cells, but not Th2 cells. In contrast, Itk is up-regulated in Th2 cells relative to Th1 cells (183). Terminal differentiation of NKT cells is characterized by the up-regulation of NK1.1 (21). To determine if Tec family kinases are differentially expressed following terminal differentiation of NKT cells, CD1d-tetramer-binding T cells were isolated from the

Figure 15: NKT cells express Tec family kinases Itk, Rlk, and Tec

[A] NKT cells were sorted from the thymus of wild type mice by gating on CD1d-tetramer^{+} HSA^{lo} cells while CD8^{+}CD4^{-} T cells were sorted from both the thymus [Thy] and spleen [Sp] of mice for comparison. The levels of Itk [blue], Rlk [red] and Tec [yellow] mRNA were determined by real-time quantitative PCR.

[B-G] Thymocytes and splenocytes were harvested from wild type mice, enriched, gated on CD1d-tetramer^{+} HSA^{lo} cells and further sorted based on expression of NK1.1 and analyzed for Itk mRNA [B, E], Rlk mRNA [C, F], and Tec mRNA [D, G]. Data were normalized to the expression of β-actin mRNA in each sample and are representative of two [B-G] or three [A] experiments.

Figure 15


A.
Ratio
Itk/BA
Rlk/BA
Tec/BA
NKT
T Cells (Thy)
T Cells (Sp)
Thymus
B.
Itk/BA Ratio
C.
Rlk/BA Ratio
D.
Tec/BA Ratio
NK1.1-
NK1.1+
Spleen
E.
F.
G.

thymus and spleen, and separated into NK1.1$^-$ and NK1.1$^+$ populations. Itk [Figure 15B], Rlk [Figure 15C] and Tec [Figure 15D] mRNA were all up-regulated in the NK1.1$^+$ compared to the NK1.1$^-$ population in the thymus. Surprisingly, both subsets of peripheral NKT cells [NK1.1$^+$ and NK1.1$^-$] express similar levels of each Tec kinase mRNA, comparable to the levels seen in thymic NK1.1$^+$ cells [Figure 15E, F, and G]. As the only NKT cells known to emigrate from the thymus are the NK1.1$^-$ cells (23, 24, 176), this latter finding suggests that thymic emigration, in addition to terminal maturation, may induce Tec kinase up-regulation.

Altered distribution of NKT cell subsets in Tec family kinase-deficient mice

In a previous study utilizing Itk$^{-/-}$ mice, a deficiency in NKT cell numbers was seen as early as 6-8 weeks in the spleen and after 20 weeks of age in both the thymus and the spleen (184). To further address the role of Tec kinases in NKT cells, we examined whether a similar deficiency occurs in the absence of Rlk, and if the decrease in NKT cell numbers observed in the absence of Itk is exacerbated in Itk$^{-/-}$Rlk$^{-/-}$ mice. We found that in our Itk$^{-/-}$ mice, the decrease in NKT cell numbers could be seen both in the thymus and the spleen as early as 6 weeks of age [Figure 16A-F and data not shown]. While this is a more profound difference than that observed in the previous study (184), it is important to note that our Itk$^{-/-}$ mice were analyzed after 13 generations of backcrossing to

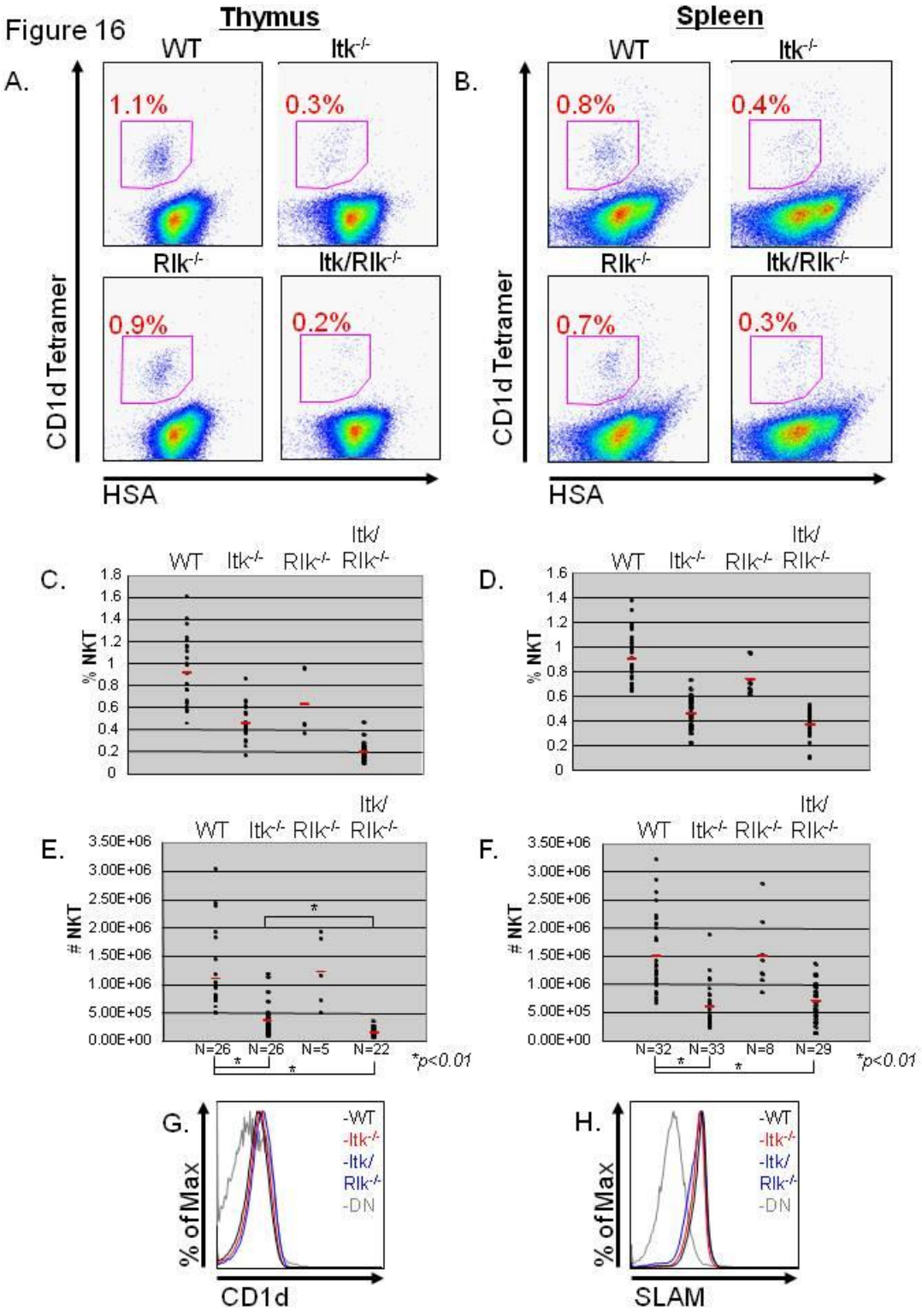
Figure 16: Tec kinase-deficient mice have reduced numbers of NKT cells in the thymus and spleen

[A, B] Cells were prepared from thymi [A] and spleens [B] of wild type, $Itk^{-/-}$, $Rlk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice and stained for CD1d-tetramer and anti-HSA. Regions in each dot-plot indicate the percentage of CD1d-tetramer^{+} HSA^{lo} cells.

[C-F] Percentages and absolute numbers of NKT cells, based on CD1d-tetramer^{+} HSA^{lo} staining, were compiled for the thymus [C, E] and spleen [D, F]. Each data point represents a different animal and red bars represent the mean. Statistical significance between two populations demarked by an asterisk. Numbers of animals analyzed are indicated at the bottom of [E] and [F].

[G, H] Thymocytes from wild type [black], $Itk^{-/-}$ [red], and $Itk^{-/-}Rlk^{-/-}$ [blue] mice were stained for CD4 and CD8, and gated $CD4^{+}CD8^{+}$ cells were analyzed for CD1d [G] or SLAM [H] expression. Data are representative of six mice analyzed per genotype in two independent experiments.

Figure 16


Thymus
Spleen
A.
B.
WT
Itk-/-
Rlk-/-
Itk/Rlk-/-
1.1%
0.3%
0.9%
0.2%
0.8%
0.4%
0.7%
0.3%
CD1d Tetramer
HSA
C.
D.
E.
F.
% NKT
# NKT
N=26 N=26 N=5 N=22
N=32 N=33 N=8 N=29
*p<0.01
G.
H.
% of Max
CD1d
SLAM
-WT
-Itk-/-
-Itk/Rlk-/-
-DN

C57Bl/10, whereas the Itk$^{-/-}$ mice used previously were backcrossed for 5 generations. We then analyzed Rlk$^{-/-}$ mice, and found a slight decrease in the percentage of NKT cells compared to wild type mice in both the thymus and periphery [Figure 16A-D]; however, this decrease in percentage did not translate into a decrease in NKT cell numbers in the absence of Rlk [Figure 16E-F]. In contrast, Itk/Rlk double-deficient mice had reduced percentages and numbers of NKT cells in the thymus when compared to wild type [7-fold reduced in number], as well as to Itk$^{-/-}$ mice [3-fold reduced in number], indicating potential redundancy between Itk and Rlk in NKT cells [Figure 16A, C, E]. Itk$^{-/-}$Rlk$^{-/-}$ mice also had reduced percentages and numbers of splenic NKT cells compared to wild type [Figure 16B, D, F]. Despite a slight but consistent decrease in NKT cell percentages in the spleen between the Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice, there was no significant difference in absolute numbers in this comparison [Figures 16B, D, F], perhaps due to the increased overall splenic and thymic cellularity seen in Itk$^{-/-}$ Rlk$^{-/-}$ when compared to the Itk$^{-/-}$ mice.

Since NKT cell progenitors in the thymus cannot be identified prior to the up-regulation of their invariant TCR, it is difficult to investigate the positive selection of this subset directly. Therefore, we examined whether or not molecules known to be involved in the positive selection of NKT cells are expressed at normal levels in the Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice. To this end, we stained DP cortical thymocytes, the cells that provide positive selection interactions for NKT cells, with antibodies to CD1d and SLAM [CD150] (20, 22).

Both CD1d [Figure 16G] and SLAM [Figure 16H] are expressed at comparable levels on DP thymocytes from wild type, Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice, indicating that altered expression of these molecules is not responsible for the decreased number of NKT cells developing in the absence of Itk, or Itk and Rlk.

Although it is not certain that CD150 is the SLAM family member involved in NKT cell selection in the thymus, it is thus far the only one reported to be expressed on the surface of cortical thymocytes (186). Nonetheless, there is indirect evidence indicating that SLAM [CD150] might be dispensable for NKT cell development, as *slam*$^{-/-}$ mice can produce IL-4 quickly post anti-CD3 activation, a response typically attributed to NKT cells (182). Alternatively, genetic evidence obtained in nonobese diabetic [NOD] mice, which are deficient in NKT cells, indicates that both SLAM and NTB-A may provide instructive signals for development of NKT cells (187). Other studies have shown that Ly9 [CD229], another SLAM family member, is not required for NKT cell development (188). Thus, while our data indicate that alterations in SLAM [CD150] expression in the absence of Itk, or Itk and Rlk, are not responsible for the observed deficiency in NKT cell selection [Figure 16H], we cannot exclude the possibility that other SLAM family members are involved in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice.

Tec family kinase-deficient NKT cells exhibit an immature phenotype

Cell surface markers distinguishing the stages of NKT cell development are well characterized (21, 184). Immature NKT cells, prior to the up-regulation of NK1.1 [fraction 1 and fraction 2] have high expression of the CD4 co-receptor. Following terminal maturation, CD4 is down-regulated, and NKT cells are either $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{-}$. CD44 up-regulation occurs in fraction 2, prior to the up-regulation of NK1.1. The final stage of maturation in NKT cells [stage 3] is accompanied by the up-regulation of NK1.1, CD69, and several additional NK cell receptors. $CD4^{+}$ or $CD4^{-}$, $CD44^{hi}$, $NK1.1^{-}$ NKT cells are exported from the thymus to the periphery, where they become $NK1.1^{+}$ following additional stimulation requiring CD1d (21).

Thymocytes and splenocytes were isolated from wild type, $Itk^{-/-}$ and $Itk^{-/-}$ $Rlk^{-/-}$ mice, gated on HSA^{lo} CD1d-tetramer-positive cells and analyzed for several developmental markers. When compared to wild type thymic NKT cells, CD4 expression on $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells remained high, similar to that seen on immature wild type NKT cells, and few to no $CD4^{-}CD8^{-}$ NKT cells were present in the thymus of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice [Figure 17A]. CD44 expression on all of the thymic NKT cells was comparable; however, expression of terminal maturation markers NK1.1 and CD69 was reduced on $Itk^{-/-}$ thymic NKT cells, and even more severely impaired on $Itk^{-/-}Rlk^{-/-}$ NKT cells [Figure 17A]. This pattern of differences between wild type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells was also observed for

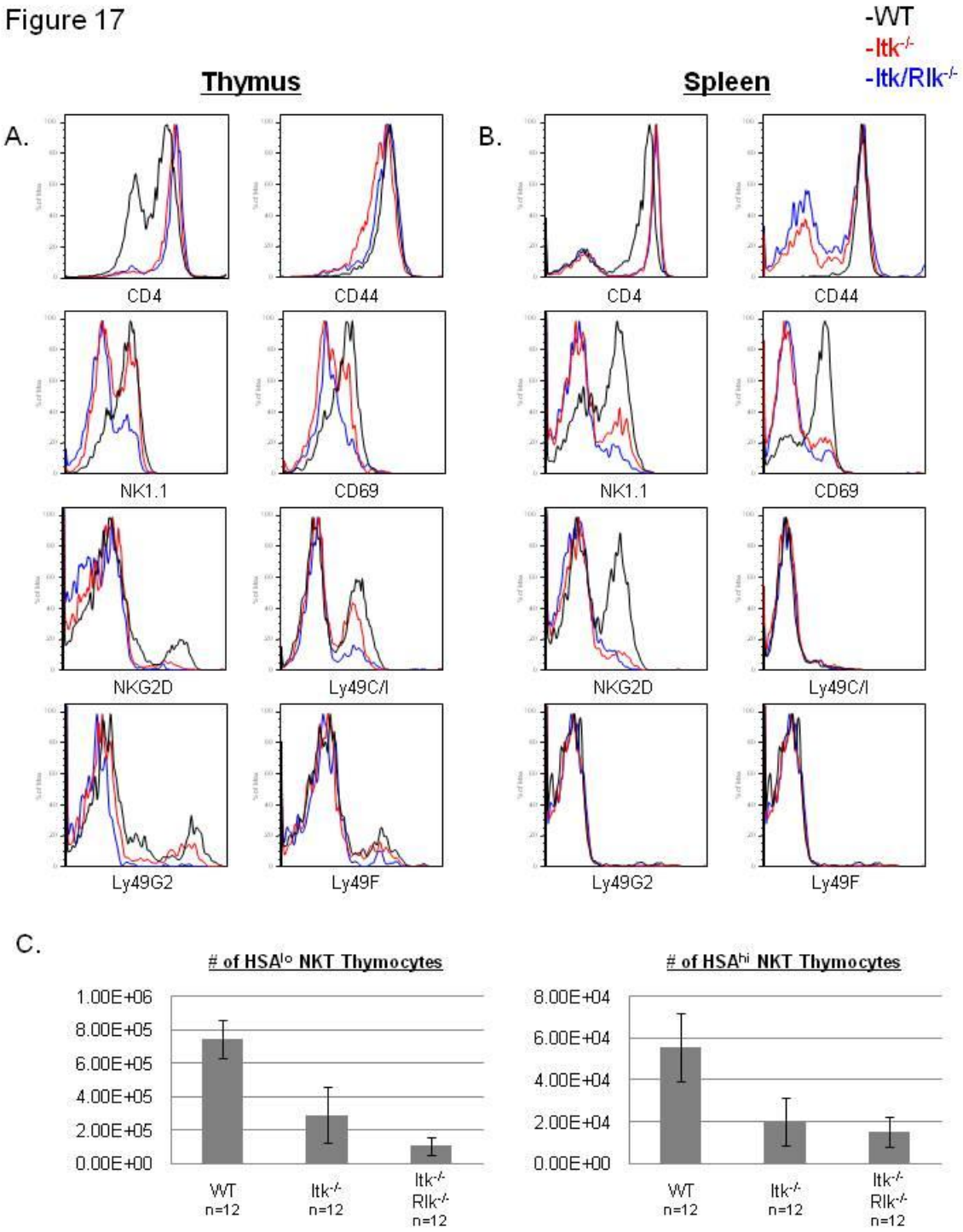
the surface markers NKG2D, Ly49C/I, Ly49G2 and Ly49F expression [Figure 17A]. Recently an earlier stage of NKT cell development has been described in which cells are either $HSA^{hi}DP^{lo}$ or $HSA^{hi}CD4^{+/hi}$ (189). As this stage precedes early developmental expansion, we considered the possibility that the substantial decrease in NKT cell numbers seen in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice was due to a block at this stage. If this were the case, we would expect to find increased numbers of HSA^{hi} NKT cells in the thymus of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice compared to wild-type mice. To assess this issue, we identified NKT cells based on CD1d-tetramer staining, and compared numbers of HSA^{hi} and HSA^{lo} thymocytes in wild type, $Itk^{-/-}$, and $Itk^{-/-}Rlk^{-/-}$ mice. As shown in Figure 17C, we did not find increased numbers of HSA^{hi} thymic NKT cells in the $Itk^{-/-}$ or $Itk^{-/-}Rlk^{-/-}$ mice relative to wild-type, arguing against a block at this stage. Furthermore, similar decreases in the $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ HSA^{lo} and HSA^{hi} NKT cell populations are seen when compared to wild-type, suggesting that the decrease in overall NKT cell numbers is likely due to a block preceding the HSA^{hi} stage. While the data shown in Figure 17C derives from mice analyzed at 6-8 weeks of age, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice analyzed at 3 weeks of age also show no apparent block at the HSA^{hi} stage of NKT cell development.

Figure 17: Tec kinase-deficient NKT cells exhibit impaired maturation

[A, B] Thymocytes [A] and splenocytes [B] were harvested from wild type [black], $Itk^{-/-}$ [red] and $Itk^{-/-}Rlk^{-/-}$ [blue] mice, stained, gated on CD1d-tetramer^{+} HSA^{lo} cells, and analyzed for expression of CD4, CD44, NK1.1, CD69, NKG2D, Ly49C/I, Ly49G2, and Ly49F. Data are representative of nine mice analyzed per genotype in three independent experiments.

[C] Thymocytes were harvested from 6-8 week old mice, were gated on CD1d-tetramer^{+}, and analyzed for expression of HSA. Total numbers of HSA^{lo} [left panel] and HSA^{hi} [right panel] NKT cells are displayed [mean±S.D.]. Data are pooled from 12 mice per group.

Figure 17


-WT
-Itk-/-
-Itk/Rlk-/-
Thymus
Spleen
A.
B.
CD4
CD44
NK1.1
CD69
NKG2D
Ly49C/I
Ly49G2
Ly49F
C.
# of HSAlo NKT Thymocytes
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
0.00E+00
WT n=12
Itk-/- n=12
Itk-/- Rlk-/- n=12
# of HSAhi NKT Thymocytes
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
WT n=12
Itk-/- n=12
Itk-/- Rlk-/- n=12

Splenic NKT cells in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice also showed a less mature phenotype than that of wild type NKT cells. CD4 expression on splenic NKT cells from Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ remained high, indicating reduced maturation; however all mice showed a comparable proportion of CD4^{-}CD8^{-} splenic NKT cells [Figure 17B]. Of interest, CD44 expression, which did not differ between the three genotypes of thymic NKT cells, is abnormal on the splenic NKT cells, with the Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice showing a substantial proportion of CD44^{lo} NKT cells. Accompanying this reduced proportion of CD44^{hi} NKT cells, Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice cells also have significant numbers of splenic NKT cells with low levels of NK1.1, CD69, and NKG2D [Figure 17B). In general the Itk$^{-/-}$Rlk$^{-/-}$ mice exhibit a more severe defect than the Itk$^{-/-}$ mice. Finally, no differences were seen for the other Ly49 markers, most likely due to lower expression of these on NKT cells in the spleen [Figure 17B] (190). Overall these data show that Itk$^{-/-}$ NKT cells fail to progress efficiently to the most mature stage of NKT cell development, and that this defect is exacerbated in the absence of Itk and Rlk, further supporting the notion of redundancy between Itk and Rlk in the maturation of NKT cells.

Tec family kinase-deficient NKT cells are functionally impaired

NKT cells are well known for their ability to rapidly produce effector cytokines following initial stimulation (26). Furthermore, the pattern of cytokines produced by NKT cells alters as the cells mature. Prior to NK1.1 up-regulation,

NKT cells can produce IL-4, but little or no IFNγ. Following up-regulation of NK1.1, NKT cell cytokine production shifts to favor production of IFNγ over IL-4 (184). Given the less mature phenotype of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells, we reasoned that these cells might produce IL-4, but little IFNγ. To examine this issue, we used a previously characterized assay (191). Briefly, wild type, $Itk^{-/-}$ or $Itk^{-/-}Rlk^{-/-}$ mice were injected i.v. with anti-CD3 antibody, and 90 minutes later splenocytes were harvested, cultured for 2 hours, and supernatants examined for cytokines by ELISA. Previous studies have shown that, in this short-term assay, the only cells capable of producing IFNγ and IL-4 are NKT cells (191). As shown in Figure 18, IFNγ [Figure 18A] and IL-4 [Figure 18B] are secreted by wild-type NKT cells. Secretion of IFNγ by $Itk^{-/-}$ splenocytes was substantially reduced, and was undetectable by $Itk^{-/-}Rlk^{-/-}$ splenocytes [Figure 18A]. Similarly, IL-4 secretion by $Itk^{-/-}$ splenocytes was decreased, whereas no IL-4 was detected in the cultures of $Itk^{-/-}Rlk^{-/-}$ splenocytes [Figure 18B]. It should be noted that decreased numbers of splenic NKT cells in the $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice may account for a portion of the decreased cytokine production observed; however, the magnitude of the reduction in IFNγ and IL-4 observed far outweighs the 50-60% decrease in proportions of NKT cells seen in the spleens of these mice.

To confirm these results with a second assay system that is more targeted to activation of NKT cells, we injected wild type, $Itk^{-/-}$ or $Itk^{-/-}Rlk^{-/-}$ mice i.v. with αGAL or PBS as a control, and examined cytokine production by intracellular cytokine staining 2hrs and 3hrs following injection (192). Splenocytes from the

injected mice were stained with CD1d-tetramer and anti-HSA antibodies, or with anti-NK1.1 and anti-TCRβ antibodies, permeabilized and stained with antibodies to IL-4 and IFNγ. Using this staining scheme, NKT cell [CD1d-tetramer^{+}, HSA^{lo}] and NK cell [TCRβ^{neg}, NK1.1^{+}] populations can each be identified, allowing us to assess NKT cell activation as well as priming of NK cells by the INFγ produced by the NKT cells [Figure 18C]. After 2 hrs of αGAL stimulation *in vivo*, a significant proportion of the wild type NKT cells are producing both IL-4 and IFNγ [16%] or IFNγ alone [17%]. At 3 hrs, the wild type NKT cells are predominantly producing IFNγ [24%], rather than both IFNγ plus IL-4 [10%]. Cytokine-producing NKT cells are decreased in Itk$^{-/-}$ mice at both 2 hrs [IL-4/IFNγ=1% and IFNγ=2%] and 3 hrs [IL-4/IFNγ=2% and IFNγ=2%] post-αGAL injection. In the Itk$^{-/-}$Rlk$^{-/-}$ mice, the defect in cytokine production by NKT cells is further exacerbated. It is worth noting that we did observe small decreases in TCR expression on the activated NKT cells, particularly in the wild type mice [data not shown]; however, this alteration did not impair our ability to identify the activated NKT cells for purposes of assessing cytokine production. Finally, as expected due to the lack of IFNγ production by Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NKT cells, priming of the NK cells in these mice was also impaired, with 48% of the wild type NK cells producing IFNγ, while only 2% of Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NK cells produced IFNγ at the 3hr time point [Figure 8C]. In addition to the reduced IFNγ production by Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NKT cells, it is likely that the reduced numbers of NKT cells in these mice are contributing to the impaired priming of NK cells.

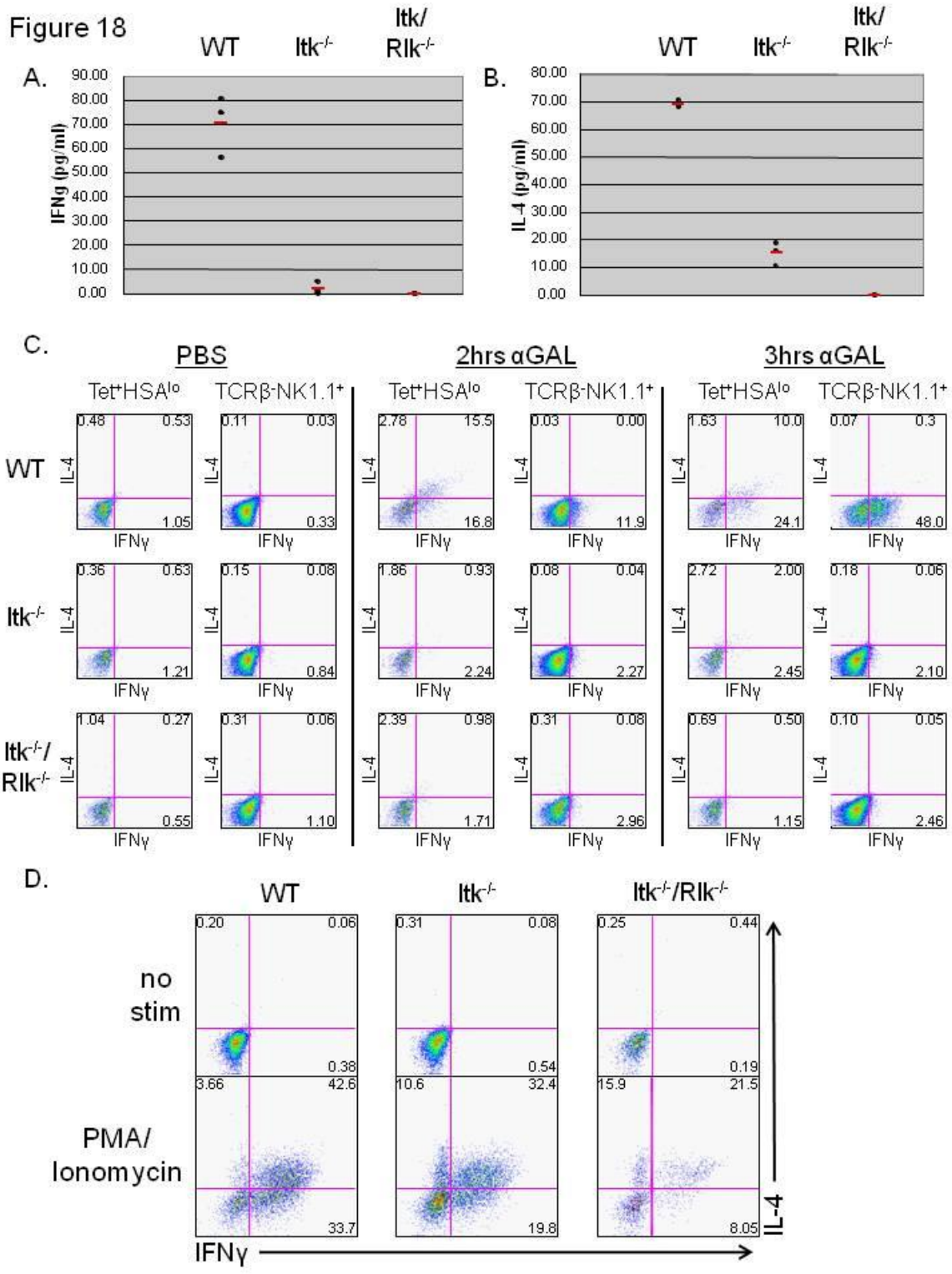
Figure 18: Tec kinase-deficient NKT cells are functionally impaired following *in vivo* activation

[A, B] Wild type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice were injected i.v. with 1 µg anti-CD3 antibody. Splenocytes were harvested after 90 minutes, incubated in vitro for 2 hours, and supernatants were analyzed for an IFNγ [A] or IL-4 [B] by ELISA. Each data point represents one mouse and the red bar indicates the mean. Data are representative of three experiments.

[C] Wild type, $Itk^{-/-}$ or $Itk^{-/-}Rlk^{-/-}$ mice were injected with PBS or 2 µg of α-Galactosylceramide [αGAL] and spleens were harvested after two or three hours [three hours for the PBS]. Cells were either stained with CD1d-tetramer and anti-HSA or with anti-NK1.1 and anti-TCRβ antibodies. NKT cells [CD1d-tetramer^{+} HSA^{lo}] and NK cells [$NK1.1^{+}TCR\beta^{-}$] were analyzed for intracellular IL-4 and IFNγ production. The three rows of dot-plots show gated NKT and NK cell populations from wild type [top], $Itk^{-/-}$ [middle] and $Itk^{-/-}Rlk^{-/-}$ [bottom]. Data are representative of results from two independent experiments each with two mice per time point.

[D] Thymocytes were harvested from wild type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice and were activated with PMA [10 ng/ml] and Ionomycin [2000 ng/ml] for 4 hrs; Golgi stop was added for the last 3 hrs of incubation. Cells were then surface stained for CD1d-tetramer and anti-HSA, fixed and permeabilized, and stained with antibodies to IL-4 and IFNγ. Dot-plots show gated CD1d-tetramer^{+} HSA^{lo} cells. Data are representative of results from four independent experiments.

Figure 18


A.
WT
Itk-/-
Itk/ Rlk-/-
IFNg (pg/ml)
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
B.
WT
Itk-/-
Itk/ Rlk-/-
IL-4 (pg/ml)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
C.
PBS
2hrs αGAL
3hrs αGAL
Tet+HSAlo
TCRβ-NK1.1+
WT
Itk-/-
Itk-/- / Rlk-/-
IL-4
IFNγ
0.48 0.53 1.05
0.11 0.03 0.33
2.78 15.5 16.8
0.03 0.00 11.9
1.63 10.0 24.1
0.07 0.3 48.0
0.36 0.63 1.21
0.15 0.08 0.84
1.86 0.93 2.24
0.08 0.04 2.27
2.72 2.00 2.45
0.18 0.06 2.10
1.04 0.27 0.55
0.31 0.06 1.10
2.39 0.98 1.71
0.31 0.08 2.96
0.69 0.50 1.15
0.10 0.05 2.46
D.
WT
Itk-/-
Itk-/-/Rlk-/-
no stim
PMA/ Ionomycin
IL-4
IFNγ
0.20 0.06 0.38
3.66 42.6 33.7
0.31 0.08 0.54
10.6 32.4 19.8
0.25 0.44 0.19
15.9 21.5 8.05

To further examine the functional capabilities of Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NKT cells, thymic NKT cells were activated *in vitro* with anti-CD3 antibody, and cytokine production was assessed. These studies produced similar results to the *ex vivo* analysis described above, indicating impaired cytokine production by Itk$^{-/-}$ compared to wild type NKT cells, and a more severe defect seen in Itk$^{-/-}$Rlk$^{-/-}$ versus Itk$^{-/-}$ cells [data not shown]. Bypassing any proximal defects in TCR signaling by stimulating cells with PMA and ionomycin indicated that slightly reduced proportions of Tec kinase-deficient NKT cells were capable of producing effector cytokines when compared to wild type NKT cells [Figure 18D]. This reduction is substantially less in magnitude than that observed when triggering Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NKT cells through the TCR using anti-CD3 antibody or αGAL, indicating that the majority of the defect in cytokine production is due to a defect in proximal TCR signaling. This experiment also confirmed that the less mature phenotype of Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NKT cells corresponds to a bias towards IL-4 production versus the predominant lFNγ production observed from wild type NKT cells. As can be seen in Figure 18D, only 58% of the cytokine-producing wild-type NKT cells made IL-4, whereas 68% and 82% of the cytokine-producing NKT cells in the Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$, respectively, made IL-4; these data also indicate a corresponding decrease in IFNγ production by Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NKT cells. These results correlate well to a recent report by Au-Yeung et al. in which Itk$^{-/-}$ NKT cells were activated with αGAL and then stimulated *in vitro* with ionomycin,

also showing a shift in cytokine production towards IL-4 and away from IFNγ (185).

Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NKT cells stimulated in vitro proliferate, but fail to accumulate

In an effort to determine the underlying cause of the decreased numbers of NKT cells in Itk$^{-/-}$ and the Itk$^{-/-}$Rlk$^{-/-}$ mice, we examined the proliferative responses and survival of NKT cells following *in vitro* stimulation. For these experiments, we adapted a previously-described NKT cell *in vitro* proliferation assay (193). Briefly, splenocytes were harvested from wild type, Itk$^{-/-}$ and Itk$^{-/-}$ Rlk$^{-/-}$ mice, labeled with CFSE, and incubated in vitro for 4 or 6 days with a variety of stimuli. Cultures were initiated with 2x10^{6} cells [pooled from three mice] of each genotype, and cell numbers were assessed at each time point. The number of NKT cells for each sample was then calculated based on surface staining for CD1d-tetramer and HSA [HSA^{lo}CD1d-tetramer^{+}]. Although the magnitude of the responses varied from experiment to experiment, the relative responses of wild-type versus Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NKT cells showed a consistent pattern between experiments. In all experiments we saw reductions in the percentages, as well as absolute numbers of NKT cells detected at day 4, for all three genotypes analyzed. This result reflects both a decrease in overall cell numbers at this timepoint, coupled with a downregulation of the TCR on the NKT cells, particularly those cultured with αGAL.

The majority of NKT cells that received either no stimulus [NS], or were cultured with a low dose of IL-2 [20 ng/ml] alone, succumb to cell death [Figure 19A]. Increasing the concentration of IL-2 to 2 µg/ml promotes the survival of wild-type NKT cells accompanied by proliferation, leading to a modest expansion of this population by day 6; in contrast neither $Itk^{-/-}$ nor $Itk^{-/-}Rlk^{-/-}$ NKT cell populations expand under these conditions, in spite of the fact that some proliferative response is observed [Figure 19A, B]. Stimulation with IL-7 promotes the survival of all wild-type and $Itk^{-/-}$ NKT cell populations above that seen in the absence of any stimulus [NS], but is less effective at maintaining $Itk^{-/-}Rlk^{-/-}$ NKT cells in culture. These data indicate that $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells are more responsive to IL-7 than they are to IL-2 for *in vitro* survival.

In vitro stimulation with αGAL, either alone or together with IL-2, promotes a dramatic expansion of the wild type NKT cells by day 6 of culture [~20 fold; Figure 19A]. For $Itk^{-/-}$ NKT cells αGAL alone promotes a modest expansion [~2.5 fold], which is slightly enhanced when combined with a low dose of IL-2 [~4 fold expansion]. $Itk^{-/-}Rlk^{-/-}$ NKT cells did not expand at all following *in vitro* αGAL stimulation. This lack of expansion of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells cannot be attributed to a predominant defect in proliferation, as all three genotypes of NKT cells show comparable dilution of CFSE by day 6. Instead, our findings indicate that $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells exhibit a profound deficiency in survival following *in vitro* stimulation.

Figure 19: In vitro expansion of Tec kinase-deficient NKT cells is impaired

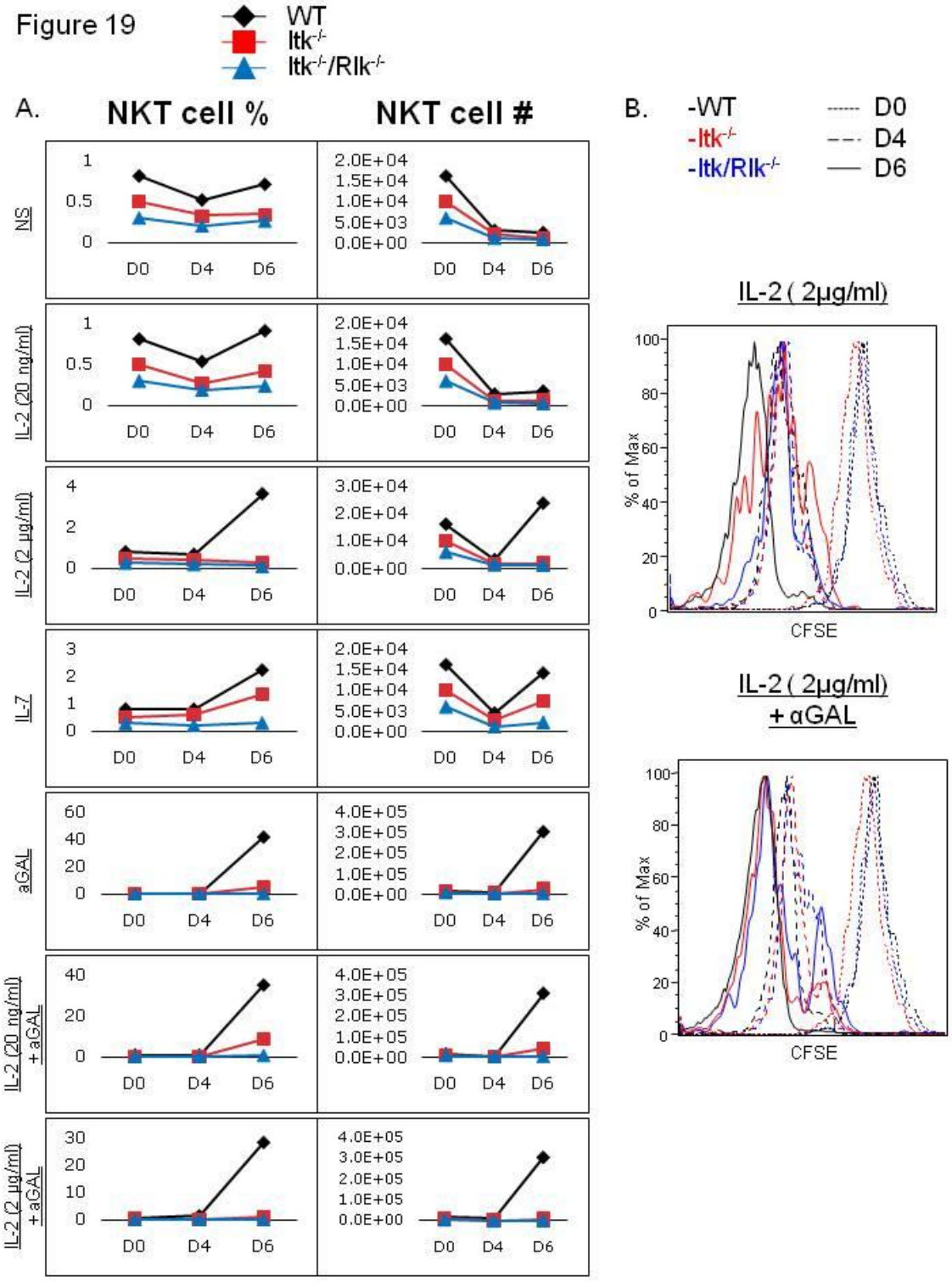
Pooled splenocytes from three wild type [black diamond], three $Itk^{-/-}$ [red square] and three $Itk^{-/-}Rlk^{-/-}$ [blue triangle] mice were stained with CFSE and incubated with the stimuli indicated for 4 or 6 days.

[A] At each time point cells were harvested, counted, stained and analyzed for NKT cells by gating on CD1d-tetramer^{+} HSA^{lo} cells. Left column shows the percentages of CD1d-tetramer^{+} HSA^{lo} cells, while the right column shows the absolute numbers of NKT cells detected at each time point.

[B] CFSE fluorescence of CD1d-tetramer^{+} HSA^{lo} cells are shown for day 0 [short dashed lines], day 4 [long dashed lines] and day 6 [solid lines].

Data shown are representative of three independent experiments.

Figure 19


WT
Itk-/-
Itk-/-/Rlk-/-
A.
NKT cell %
NKT cell #
NS
IL-2 (20 ng/ml)
IL-2 (2 µg/ml)
IL-7
aGAL
IL-2 (20 ng/ml) +aGAL
IL-2 (2 µg/ml) +aGAL
D0
D4
D6
B.
-WT
-Itk-/-
-Itk/Rlk-/-
D0
D4
D6
IL-2 (2µg/ml)
% of Max
CFSE
IL-2 (2µg/ml) + αGAL
% of Max
CFSE

Tec family kinase-deficient NKT cells show impaired survival and reduced CD122 and T-bet expression

The data shown above suggest that Tec kinase-deficient NKT cells have a survival defect. To test this notion more directly, we examined freshly-isolated *ex vivo* NKT cells for markers of apoptosis/death. Thymocytes and splenocytes from wild type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells were analyzed for co-expression of Annexin V and 7AAD. While no striking differences were seen in the percentages of apoptotic/dead NKT cells in the thymus [Figure 20A], we consistently observed an increased proportion of $AnnexinV/7AAD^{+}$ NKT cells in the spleens of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ compared to wild type mice [Figure 20B]. As IL-15 has a key role in peripheral maintenance of NKT cells (168, 178), we considered that defective survival of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells might result from impaired expression of CD122, the IL-2/IL-15 receptor β-chain. As shown in Figure 20C and D, CD122 was dramatically decreased on the $Itk^{-/-}$ and the $Itk^{-/-}Rlk^{-/-}$ NKT cells, both in the thymus and the spleen. This reduced expression of CD122 correlated well with the numbers of NKT cells in the mice, as $Itk^{-/-}Rlk^{-/-}$ NKT cells had lower expression of CD122 than NKT cells from $Itk^{-/-}$ mice. Further, this defect correlates with the peripheral survival data, and is consistent with the known role of IL-15 in the peripheral survival of all NKT cell subsets. Interestingly, this decrease in CD122 expression also correlates with the impaired up-regulation of NK1.1 in the $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells, and is consistent with the observation that IL-15-deficient mice have a marked decrease

in mature [$NK1.1^+$] NKT cells, with no effect on the immature NKT cells present in the thymus (178). Putting these data together may also account for why we fail to see an increase in apoptotic/dead NKT cells in the thymus of $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice, as these thymi contain only small numbers of mature NKT cells, with few cells reaching the stage at which they would be susceptible to an absence of IL-15.

Up-regulation of CD122 has been associated with the T-box transcription factors T-bet and Eomesodermin in conventional αβ TCR^+ T cells (173). Furthermore, T-bet-deficient mice also have severe defects in NKT cell maturation, peripheral survival, and cytokine production. Given the similarities seen between T-$bet^{-/-}$ and $Itk^{-/-}$ or $Itk^{-/-}Rlk^{-/-}$ NKT cells, we investigated T-bet expression in these cells. To this end, NKT cells were sorted from the wild type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice, RNA was harvested and the samples were analyzed for T-bet mRNA expression using quantitative real-time RT-PCR. A substantial reduction in T-bet mRNA was found in the NKT cells from the $Itk^{-/-}$ mice [3-4 fold decrease] compared to wild type NKT cells, with a more profound defect seen in NKT cells from the $Itk^{-/-}Rlk^{-/-}$ mice [7-8 fold decrease] [Figure 20E]. Since T-bet expression is up-regulated during the final stage of NKT cell development, this result is not unexpected, as most $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells do not reach this stage of maturation. However, we also found that a comparison between purified thymic $NK1.1^-$ NKT cells from the three types of mice also showed the same reduction in T-bet mRNA expression in Tec kinase-deficient cells versus wild

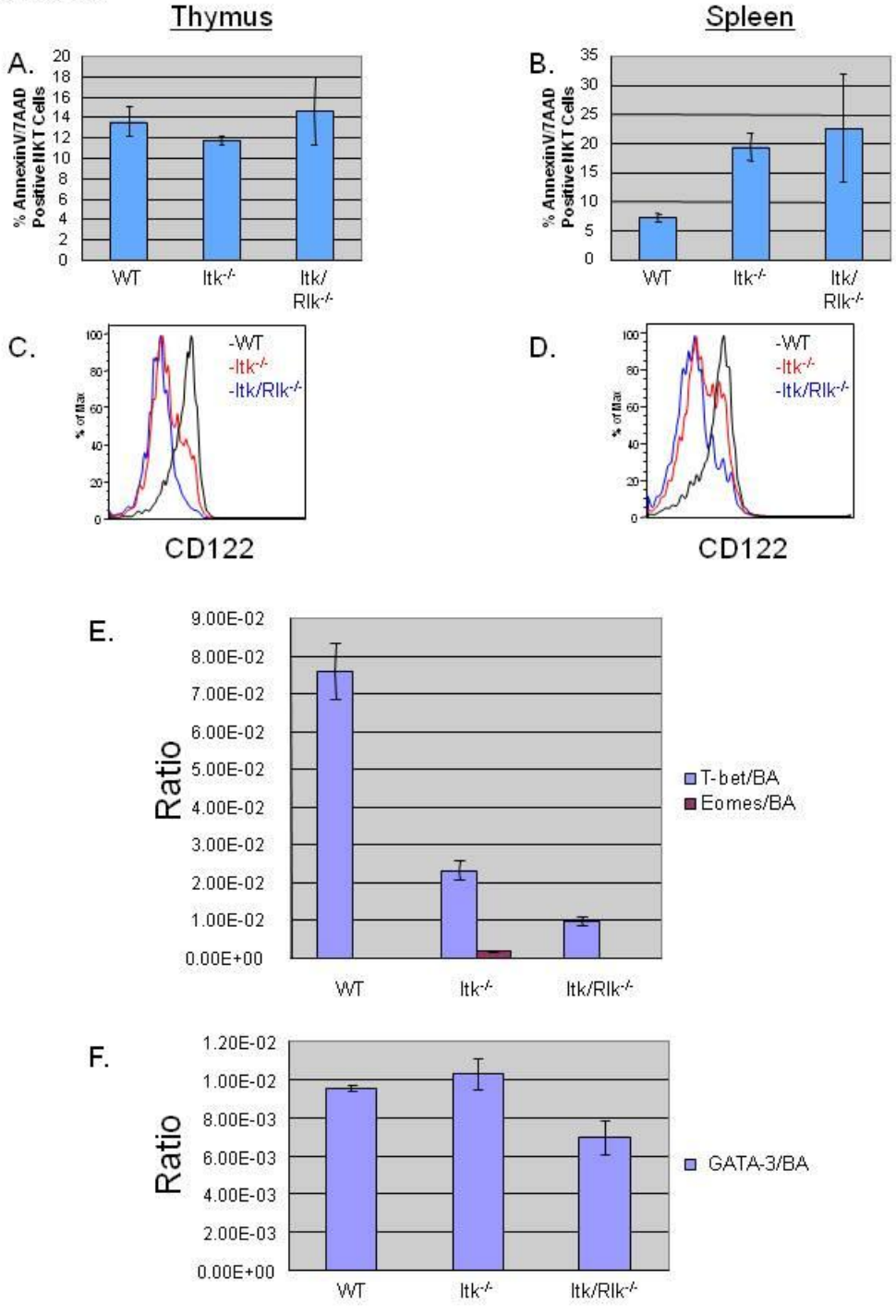
Figure 20: Impaired survival of Tec kinase-deficient NKT cells correlates with reduced CD122 and T-bet expression

[A, B] Thymocytes [A] and splenocytes [B] from three wild type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice were stained for CD1d-tetramer, HSA, Annexin V, and 7AAD. Gated CD1d-tetramer^{+} HSA^{lo} cells were analyzed for co-expression of Annexin V and 7AAD. The graph shows the mean percentage of Annexin V+ 7AAD+ NKT cells for each genotype. Data are representative of three experiments.

[C, D] Gated CD1d-tetramer^{+} HSA^{lo} thymocytes [C] or splenocytes [D] from wild type [black], $Itk^{-/-}$ [red], and $Itk/Rlk^{-/-}$ [blue] mice were analyzed for expression of CD122. Data are representative of six mice of each genotype analyzed in two independent experiments.

[E, F] cDNA was prepared from sorted NKT cells isolated from the thymus of wild type, $Itk^{-/-}$ or $Itk^{-/-}Rlk^{-/-}$ mice. Samples were then analyzed for T-bet [E, blue bars], Eomesodermin [E, red bars], and GATA-3 [F] mRNA levels. Graphs show data normalized to β-actin mRNA levels in each sample, and are representative of three independent experiments.

Figure 20:


Thymus
Spleen
A.
B.
% AnnexinV/7AAD Positive IIKT Cells
WT
Itk-/-
Itk/ Rlk-/-
C.
D.
-WT
-Itk-/-
-Itk/Rlk-/-
% of Max
CD122
E.
Ratio
T-bet/BA
Eomes/BA
F.
GATA-3/BA

type [data not shown]. Interestingly, no decrease in T-bet expression was seen in a recent study comparing Itk$^{-/-}$ to wild-type NKT cells (185). Both our study and that of Au-Yeung and colleagues used quantitative RT-PCR to assess T-bet mRNA levels in NKT cell populations; however, our experiments examined T-bet expression in thymic NKT cells, while the previous study examined splenic NKT cells. We have also analyzed splenic NKT cell populations from wild-type and Itk$^{-/-}$ mice for T-bet expression, and find results consistent with those reported by Au-Yeung et al. (185), that T-bet mRNA is, if anything, slightly increased in Itk$^{-/-}$ compared to wild-type splenic NKT cells [data not shown]. In contrast, T-bet mRNA levels in the small number of Itk$^{-/-}$Rlk$^{-/-}$ splenic NKT cells is decreased when compared to the wild type NKT cells [data not shown]. These findings suggest that peripheral NKT cell survival may require T-bet, leading to a strong selection for those cells able to up-regulate T-bet expression. This hypothesis could account for the differences seen between thymic versus splenic Itk$^{-/-}$ NKT cells regarding T-bet mRNA levels, and also, the more severe loss of Itk$^{-/-}$Rlk$^{-/-}$ NKT cells compared to those lacking Itk alone.

Eomesodermin has also been shown to regulate CD122 expression in conventional T cells, similar to T-bet (173), yet is not normally expressed in NKT cells (68). As described in the previous chapter we found aberrantly high expression of Eomesodermin in CD8^{+} thymocytes and T cells from Itk$^{-/-}$ and Itk$^{-/-}$ Rlk$^{-/-}$ mice (69). Therefore we also examined Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ NKT cells for Eomesodermin mRNA expression. While no Eomesodermin mRNA was

detected in the wild type or $Itk^{-/-}Rlk^{-/-}$ NKT cells, we consistently found low levels of Eomesodermin mRNA in the $Itk^{-/-}$ NKT cells [Figure 20E]. Additionally, GATA-3 has recently been implicated in regulating NKT peripheral survival, function and maturation, based on studies of GATA-3-deficient mice (67). Although no consistent differences were seen in expression of GATA-3 between the wild type and $Itk^{-/-}$ NKT cells, confirming results from Au-Yeung and colleagues (185), a 15-30% decrease was seen in the $Itk^{-/-}Rlk^{-/-}$ NKT cells compared to those of wild-type mice [Figure 20F]. Overall, the failure of Tec kinase-deficient NKT cells to regulate expression of these key transcription factors is likely to impact NKT cell survival, due to impaired CD122 expression, NKT cell effector functions, specifically IFNγ secretion, and NKT cell terminal maturation.

Discussion

The role of Tec family kinases in conventional αβ T cell development, differentiation and function has been well documented (1, 98, 183). Of the three family members expressed in T cells, Itk, Rlk and Tec, Itk has the predominant role in TCR-dependent thymic selection events, as well as in TCR-dependent activation of mature T cells. In contrast, Tec appears to have no important role during T cell development or in the primary activation of naïve T cells (194). While the function of Rlk is still uncertain, a combined deficiency in Itk and Rlk

leads to more severely impaired signaling than a deficiency in Itk alone, indicating some redundancy between Itk and Rlk in conventional αβ T cells (139). Unlike the well-characterized role of Itk and Rlk in conventional T cells, little is currently known about the function of these kinases in non-conventional "innate" T cells, such as γδTCR^{+} T cells, NKT cells, and other subsets of CD8^{+} and CD4^{-} 8^{-} T cells with specificity for nonclassical MHC class I molecules. One previous report showed decreased numbers of NKT cells in the thymus and periphery of Itk-deficient mice, and showed that Itk$^{-/-}$ NKT cells had reduced expression of NK1.1 (184), while a more recent study focused on cytokine secretion defects of Itk$^{-/-}$ NKT cells (185). Based on the initial study indicating a possible role for Tec family kinases in NKT cells, we set out to determine whether Rlk could compensate for Itk in NKT cell development, and also whether the Tec kinases Itk and Rlk are required for other aspects of NKT cell biology, including effector function and peripheral homeostasis.

Overall, our data indicate that both Itk and Rlk together are critical for the terminal maturation, cytokine production, and peripheral survival of NKT cells. Interestingly, the phenotype we observe for NKT cells in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice shares strong similarities with defects observed in the absence of the cytokine IL-15 (168, 178), and more strikingly, in the absence of the T-box transcription factor, T-bet (68); although the overall decrease in cell numbers is indicative of an early developmental block similar to that in the SAP and Fyn deficient mice [described later on]. As T-bet is known to regulate transcription of CD122, the IL-

15 receptor β chain (173), the ability of NKT cells to respond to IL-15 would likely depend on prior T-bet expression. Furthermore, a study aimed at identifying other genes regulated by T-bet in NKT cells identified a wide array of T cell effector molecules, including cytokines, chemokines, and cytolytic proteins, as well as survival factors (181). Thus, T-bet together with IL-15 are essential for terminal maturation, development of effector function, and long-term survival of NKT cells.

One important function of T-bet is regulation of IFNγ transcription (195). This correlates well with the fact that NKT cell maturation to the $NK1.1^{+}$ stage, which coincides with T-bet up-regulation, is accompanied by a switch in NKT cell effector cytokine production from IL-4 to IFNγ (184). Thus, it is not surprising that $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells exhibit a severe defect in IFNγ production, as few of these cells progress to the $NK1.1^{+}$ stage of NKT cell maturation. While at first glance these findings appear inconsistent with a recent study showing modest reductions in IL-4 and IFNγ transcript levels in $Itk^{-/-}$ NKT cells (185), this earlier study examined only constitutive levels of IL-4 and IFNγ transcripts, and did not examine cytokine mRNA levels following NKT cell activation. In fact, both studies agree that $Itk^{-/-}$ NKT cells are dramatically impaired in cytokine protein production in response to TCR stimulation, suggesting a defect in TCR-induced transcription at these cytokine loci.

As mentioned above, we also find a substantial defect in IL-4 production by Tec kinase-deficient NKT cells. This latter defect is unlikely to be due to altered NKT cell maturation, but instead, is likely explained by impaired TCR signaling in the absence of Itk and Rlk. In fact PMA/ionomycin activation, which bypasses proximal TCR signaling and the requirement for Tec kinases, largely restores IL-4 production to $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells. Of interest, we also see low, but detectable expression of Eomesodermin mRNA in $Itk^{-/-}$, but not $Itk^{-/-}Rlk^{-/-}$, NKT cells. This finding may in part account for the increased ability of $Itk^{-/-}$ over $Itk^{-/-}Rlk^{-/-}$ NKT cells to respond to *in vivo* stimulation by producing some IFNγ, and may also contribute to the IFNγ transcripts seen by Au-Yeung and colleagues (185) in freshly-isolated splenic $Itk^{-/-}$ NKT cells.

Based on our findings in comparison to published data, we propose that Itk and Rlk are required for the up-regulation of T-bet mRNA during the final stage of NKT cell maturation [Figure 21]. Should this be the case, it implicates TCR signaling in regulating T-bet expression, as Tec kinases are predominantly activated in T cells downstream of the TCR. This notion is at odds with current evidence regarding the regulation of T-bet transcription, which in conventional $CD4^+$ T cells has been shown to be dependent solely on IFNγ receptor signaling, and independent of TCR signaling (196). Thus, an alternative possibility is that TCR signaling leading to activation of Itk and Rlk is required for NKT cell maturation, but not directly responsible for regulation of T-bet transcription. As additional signaling proteins in the TCR pathway have been shown to be

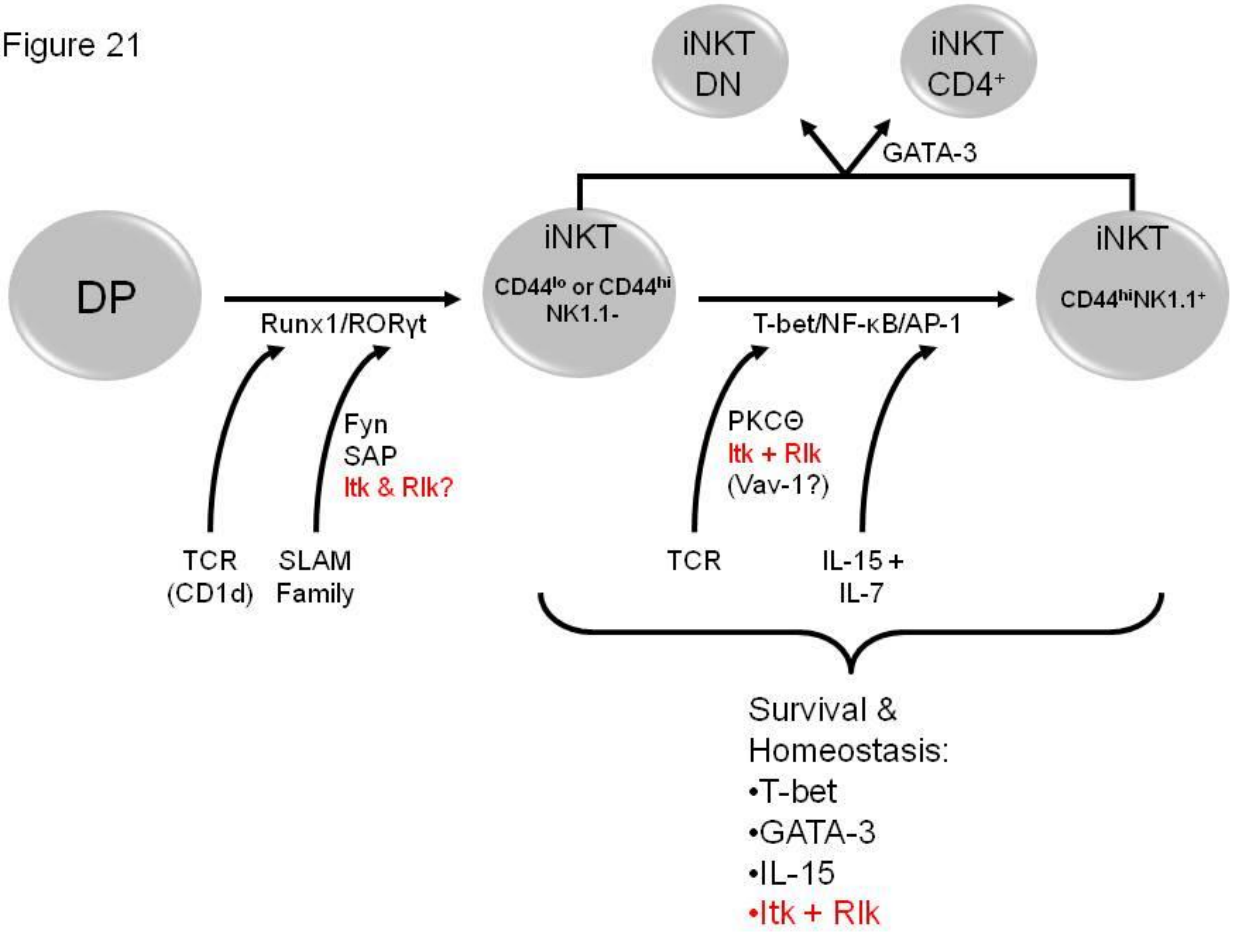
important for NKT cell development, including PKCθ and Vav-1 (59-61), there is strong evidence supporting an ongoing role for TCR signaling in NKT cell maturation. However, the precise mechanism of T-bet regulation in developing NKT cells remains to be determined.

In contrast to the defects in NKT cells observed in the absence of Itk and Rlk, a deficiency in the Src kinase, Fyn, leads to a more severe and earlier block in NKT cell ontogeny (52, 53). Recently these data have been extended to include the adapter protein SAP, which activates and recruits Fyn to SLAM-family receptors (20, 54-56). Interestingly Tec family kinases, specifically Btk and Itk, have been shown to bind to the Fyn SH3 domain through an amino terminal region that is conserved between these kinases (197, 198). While these findings suggest a connection between Tec family kinases and the SLAM-SAP-Fyn pathway, this possibility seems unlikely. Specifically, the defect in NKT cell numbers is much more severe in the Fyn-deficient mice, and occurs at an earlier stage of NKT cell maturation. Similarities between the Fyn- and SAP-deficient mice, and mice lacking CD1d (175) or the transcription factors Runx1 and RORγt(57, 58), suggest instead that TCR plus SLAM-family receptor signaling are required for early steps in NKT cell positive selection, and that this developmental step is Tec kinase-independent [Figure 7]. However there is an early block in the Itk deficient and [especially] the Itk and Rlk doubly deficient

Figure 21: A model of pathways important for NKT cell development

Invariant NKT cells [iNKT] are derived from DP [$CD4^+CD8^+$] thymocyte precursors. These cells are selected by interactions with other DP thymocytes involving TCR binding to CD1d plus activation of a SLAM family receptor. SLAM-family receptor signals are transduced by SAP and Fyn. Together, these two pathways induce transcription factors Runx1 and RORγt. It still remains elusive whether or not there is a role for Tec family kinases at this early stage. Once selected, immature NKT cells [$CD44^{lo}$ or $CD44^{hi}$, $NK1.1^-$] require further signals through the TCR plus cytokine signals [IL-15 and IL-7] to upregulate NK1.1 and achieve terminal maturation [$CD44^{hi}$, $NK1.1^+$]. For this transition, TCR signals require PKCθ, Tec kinases Itk and Rlk, and perhaps Vav-1. The transcription factors T-bet, NF-κB, and AP-1 all have important roles in regulating this terminal maturation. The transcription factor GATA-3 is important in generating $CD4^+$ NKT cells at both the $NK1.1^-$ and $NK1.1^+$ stages. Homeostasis and survival of NKT cells is intrinsically dependent on the cytokine IL-15, whose receptor expression is tightly regulated by the transcription factor T-bet. GATA-3, as well as Itk and Rlk, are also important for NKT cell survival and peripheral homeostasis. DN, $CD4^-CD8^-$.

Figure 21


iNKT
DN
iNKT
CD4+
GATA-3
DP
Runx1/RORγt
iNKT
CD44lo or CD44hi
NK1.1-
T-bet/NF-κB/AP-1
iNKT
CD44hiNK1.1+
Fyn
SAP
Itk & Rlk?
PKCΘ
Itk + Rlk
(Vav-1?)
TCR
(CD1d)
SLAM
Family
TCR
IL-15 +
IL-7
Survival &
Homeostasis:
•T-bet
•GATA-3
•IL-15
•Itk + Rlk

mice which precede the earliest identifiable NKT developmental stage [HSA^{hi}]. This would attribute another defect, besides the terminal maturation defect, to the absence of Tec family kinases. Perhaps if all three Tec family kinases, Itk, Rlk and Tec, were missing in the NKT cells the defect seen would mimic that of the Fyn or SAP deficient mice. Unfortunately a triple deficient mouse is not available yet and identification of early defects prior to upregulation of the TCR in NKT cells is quite difficult.

A final clue on the role of Tec kinases in NKT cell development comes from studies of the transcription factor, BATF [B cell-activating transcription factor] (65, 66). BATF is an inhibitory component of the AP-1 family of transcription factors, which reduces AP-1 binding activity when expressed in T cells in a transgenic mouse model. In these transgenic mice, conventional T cell development is normal; however, NKT cell numbers are substantially reduced. Similar to the phenotype of NKT cells in Tec kinase-deficient mice, the BATF-transgenic NKT cells fail to undergo the final stage of maturation as measured by up-regulation of NK1.1. This finding indicates that AP-1 activation plays a crucial role in the terminal maturation of NKT cells. As Itk and Rlk are known to be essential for optimal AP-1 activation in conventional T cells (140), these data suggest that a similar pathway operates in NKT cells, and is required for the final step of NKT cell development.

The role of Tec family kinases in innate immune cell subsets is an interesting and complex issue. In one subset of T cells, αβ $CD8^+$ T cells, Tec family kinases are required to promote development into the conventional T cell lineage; in the absence of Itk, or Itk and Rlk, $CD8^+$ T cell development proceeds into an innate-like lineage (69-73). In this chapter we show that in another subset of innate T cells, αβ NKT cells, Tec family kinases have a critical positive function in the development, maturation, function and survival of these cells. Together, these findings indicate that Itk and Rlk have a distinct function in each lineage of innate T cells. In this context, the Tec family kinases may be required for optimal TCR signaling, and defects in this pathway may lead to alterations in lineage development in the thymus. Alternatively, it remains possible that Itk and Rlk may be important for a distinct signaling pathway, such as that mediated by a costimulatory receptor, that differentially impacts NKT cell, conventional, and innate $CD8^+$ T cell maturation.

Chapter IV: Itk is important in γδ T cell development and is needed to maintain the Th1 profile of cytokine secretion in these cells

Chapter IV Attributions and Copyright information

The material in this chapter was written by me and consists of a collaboration between me and Catherine Yin [CY]. Specific contributions for each figure are as follows: figure 22 [CY], figure 23 [MF and CY], figure 24 [MF], figure 25 [MF and CY], figure 26 [MF], and figure 27 [MF].

Intro

Itk has been shown to play a principal role, within the Tec family of non-receptor tyrosine kinases, in signaling downstream of the T cell receptor(1, 98). Particularly, Itk deficient animals have defects in PLC-γ phosphorylation, calcium flux generation, MAP kinase activation, and AP-1 and NFAT activation. Itk has also been linked with conventional αβ T cell development, selection, and function. Of particular importance, Itk is involved in $CD4^+$ T helper cell differentiation (183). Assays, both *in vitro* and *in vivo*, looking at T-helper cell differentiation show impaired Th2 responses in the absence of Itk (102, 137, 139, 140, 199). Itk is also required for clearance of virus by $CD8^+$ T cells independently of the role Itk has on $CD4^+$ T cell help (200).

As described in chapters II and III, several studies have described a role for Itk in innate versus conventional αβ T cell development. In the absence of Itk $CD8^+$ T cells develop with an innate phenotype; specifically they are highly dependent on IL-15, express NK1.1, secrete cytokines directly *ex vivo* and are selected on hematopoietic cells (69-72). A later study found that generation of these “innate-like” $CD8^+$ T cells is dependent on the kinase domain of Itk (74). SAP is required for the selection of these “innate-like” $CD8^+$ T cells in the Itk deficient mice while CD28 is required for acquisition of the innate characteristics found on these cells (73). While absence of Itk seems to promote generation of

"innate-like" $CD8^+$ T cells, it is detrimental to the generation of another innate subset, NKT cells. Three studies have shown that Itk is involved in NKT cell selection, maturation, survival and cytokine secretion (174, 184, 185). However, to date no work has been done on the role of Itk in a third subset of innate T cells, γδ T cells.

γδ T cells are a well conserved subset of T cells which constitutes 1-5% of the lymphocytes in the blood and peripheral organs, but can account for up to 50% of the lymphocytes in the epithelial rich tissues. They can develop, in the absence of MHC restriction, from double negative thymocytes and can recognize soluble protein and non-protein antigens of endogenous origin. γδ T cells are capable of secreting Th1 and Th2 cytokines (27). Amongst the many functions attributed to γδ T cells, immune modulation of adaptive immunity, specifically humoral immunity, is of particular interest (45).

Initial studies utilizing $TCR\alpha^{-/-}$ mice showed B cell expansion, differentiation, and secretion of IgE and IgG_1, which are both "T dependent isotypes", indicating that another subset of cells could be providing B cell help (201). $TCR\beta^{-/-}$ deficient mice challenged repeatedly with parasitic infections could induce germinal center formation and increased antibody production, although not against the pathogen (202). Utilizing a model of pulmonary allergic inflammation, decreased IgE and IgG_1 were seen in mice that are deficient in γδ T cells when compared to wild type mice (203). Upon immunization with a well-

defined Ag in the absence of αβ T cells, γδ T cells can induce germinal center formation and Ig hypermutation, albeit the hypermutation occurs in a different set of B cells as usually seen when αβ T cells are present (204). Even though they account for a very small number of γδ T cells, the $CD4^+$ cells seem to be the subset of γδ T cells involved in GC formation, at least in *Mycobacterium* induced models of GC formation (205).

Human γδ T cells can migrate into germinal centers and upregulate B-cell costimulatory molecules, including CD40L, OX40, CD70 and ICOS post TCR stimulation (5). Furthermore, upon stimulation a subset of human γδ T cells can also express MHC-II, CD80/CD86 and CD40, as well as several adhesion molecules found normally in mature monocyte derived DCs, indicating that they could participate in humoral immunity by mimicking a professional APC (45, 206). Another way for γδ T cells to modulate humoral immunity is by regulation of maturation of DCs (207, 208). Of interest a recent article has shown that human γδ T cells moving into GCs respond to T_{FH}-derived IL-21 by producing CXCL10 and CXCL13 aiding in the recruitment of more B cells, T_{FH} cells, and monocytes to the GCs (209). Taken together, all these studies indicate that γδ T cells can participate, directly or indirectly, in humoral immunity.

Conventional αβ $CD4^+$ T cells from Itk deficient mice can't mount proper Th2 responses *in vitro* and *in vivo* (183). As mentioned previously NKT cells in the Itk deficient mice also have a severe defect in cytokine production (174, 185).

Despite these results, the Itk deficient mice have a systemic phenotype demarking elevated Th2 responses, illustrated by the spontaneously high levels of IgE found in these mice (140). IL-4 induced TCR mediated secretion, required for IgE class switching, can be found in three separate subsets of T cells: conventional αβ $CD4^+$ T cells, NKT cells, and γδ T cells (210-212).

In this chapter we show increased numbers of γδ T cells in the thymi and spleens of Itk deficient mice. The increase in numbers is accompanied by an increase in the proportion of $CD4^+$ γδ T cells [both thymus and spleen] and $NK1.1^+$ γδ T cells [thymus only]. We also report a shift towards expression of the Th2 transcription factor GATA3 in the Itk deficient γδ T cells, which fits the strong increase in IL-4 secretion seen in the γδ T cells from these mice upon TCR engagement. Congruent with the Th2 cytokine secretion seen on the Itk deficient γδ T cells, we also see elevated expression of markers usually associated with B cell help, such as ICOS and CD40L, on these cells. Finally we show that absence of γδ T cells in the Itk deficient mice causes a strong decrease in the elevated levels of IgE (140) and spontaneously enriched germinal centers usually found on these mice. Taken together this data shows that proper expression of Itk might have an important role in controlling γδ T cell mediated dysregulation of B cell help.

Results

Itk deficient mice have increases in γδ T cells

Work on Itk deficient animals has demonstrated that absence of Itk leads impaired selection and decreased numbers of conventional αβ $CD4^+$ and $CD8^+$ T cells as well as NKT cells (1, 54, 174, 184). On the other hand absence of Itk seems to favor selection of "innate-like" $CD8^+$ T cells denoted by the large increase in numbers of these cells seen in the Itk deficient mice (69, 71). Much like these "innate-like" $CD8^+$ T cells, selection of γδ T cells seems to be favored in the Itk deficient mice.

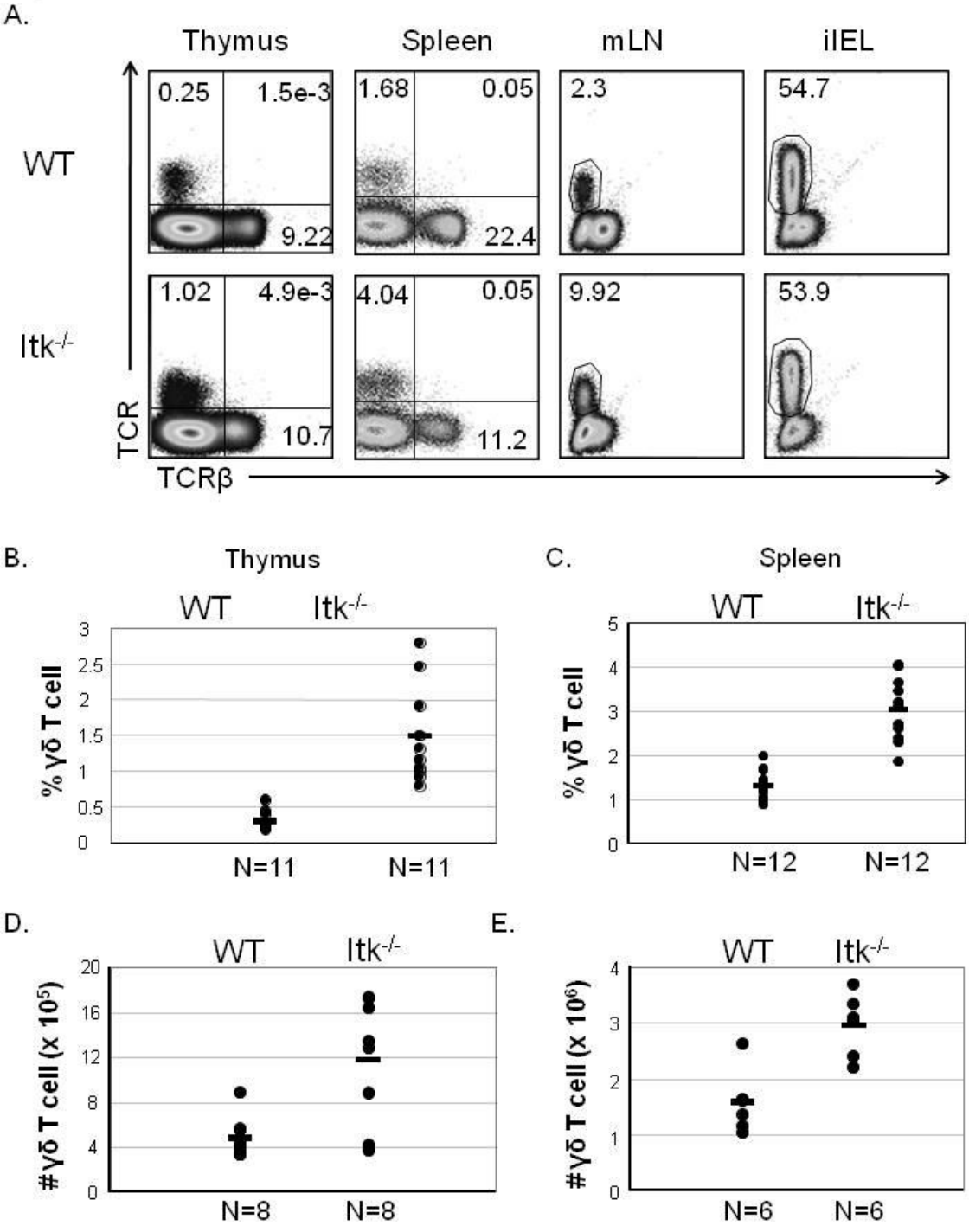
As seen in Figure 22[A], a comparison between the distribution of wild type [top row] versus Itk deficient [bottom row] γδ T cells shows an increase in the proportion of γδ T cells found in the Itk deficient animals. Specifically, there is a fourfold increase in the proportion of thymic Itk deficient γδ T cells [first column], and greater than a two fold increase in the proportion of splenic γδ T cells. These values are statistically significant [Figure 22B and C] and they correspond to increases in the absolute numbers of γδ T cells found in these compartments [Figure 22D and E]. Of interest we also see an important increase in the proportion of mesenteric lymph node [mLN] γδ T cells in the Itk deficient mice, probably due to the stark decrease in αβ T cells found in this compartment. We see no differences in the proportions of γδ T cells from the intestinal intraepithelial lymphocytes [iIELs].

Figure 22: Itk deficient mice have increased proportions of γδ T cells in the thymus and spleen

[A] Cells were prepared from thymus, spleen, mLN and iIELs of wild type, and $Itk^{-/-}$ mice, and stained with anti-TCRγ and anti-TCRβ Abs. Data are representative of 4 independent experiments with 2-3 mice per group for spleen and thymus and 2 independent experiments with 2-3 mice per group for mLN and iIELs.

[B-E] Percentages and absolute numbers of $TCR\gamma^{+}$ cells, based on $TCR\gamma^{+}TCR\beta^{-}$ staining, were compiled for the thymus [B and D] and spleen [C and E]. Statistical significance for thymus [B] is $p<0.0001$, [D] is $p=0.0207$; and for splenocytes [C] is $p<0.0001$, [E] is $p=0.0087$. Each data point represents a different animal, and the bars represent the mean. Numbers of animals analyzed are indicated as N values at the bottom of B-E. ***, extremely significant; **, very significant; *, significant.

Figure 22


A.
Thymus
Spleen
mLN
iIEL
WT
Itk-/-
0.25
1.5e-3
9.22
1.68
0.05
22.4
2.3
54.7
1.02
4.9e-3
10.7
4.04
0.05
11.2
9.92
53.9
TCR
TCRβ
B.
Thymus
WT
Itk-/-
% γδ T cell
N=11
N=11
C.
Spleen
WT
Itk-/-
% γδ T cell
N=12
N=12
D.
WT
Itk-/-
#γδ T cell (x 10^5)
N=8
N=8
E.
WT
Itk-/-
#γδ T cell (x 10^6)
N=6
N=6

Itk deficient mice contain increased proportions of $CD4^+$ and $NK1.1^+$ γδ T cells

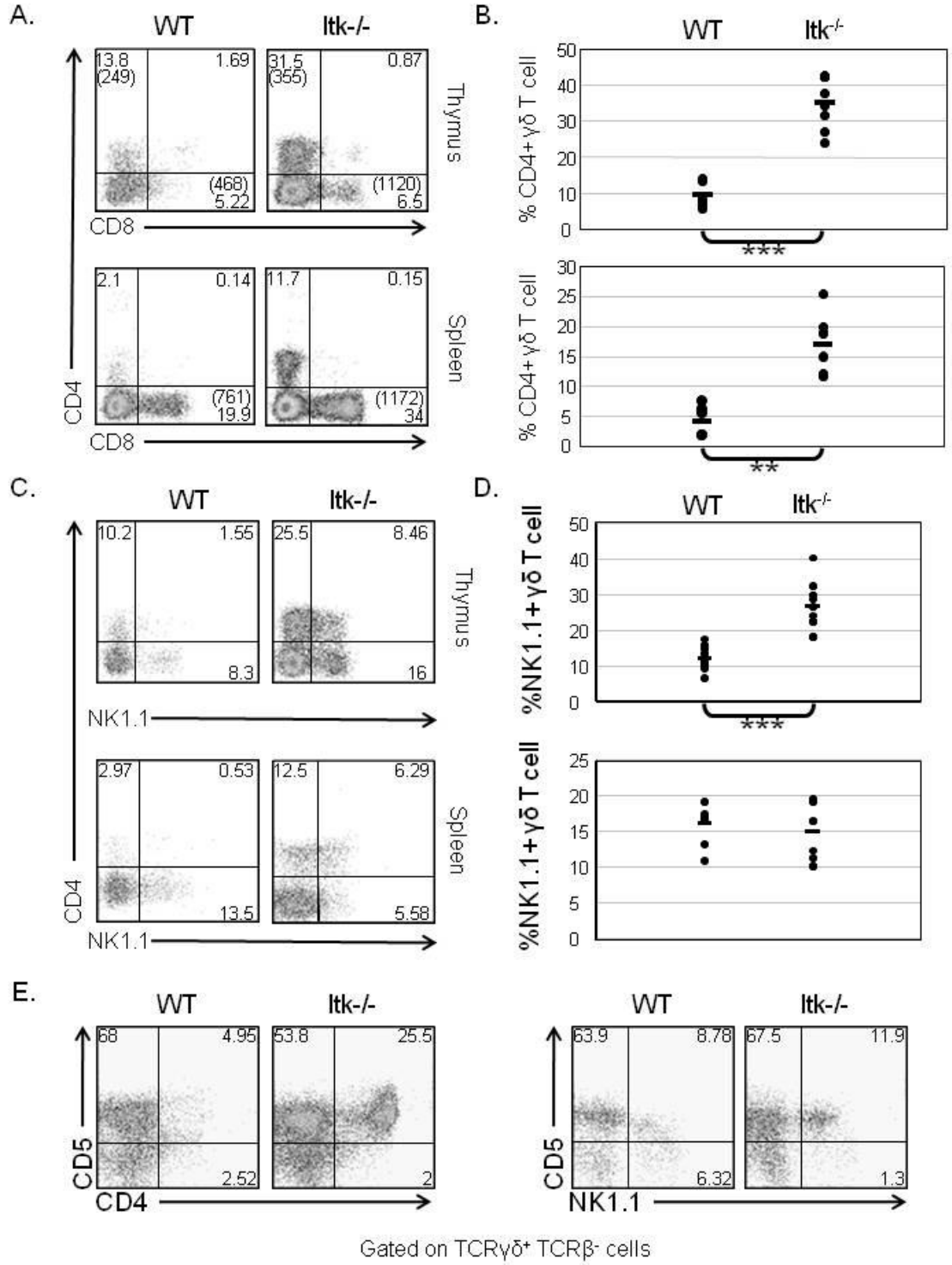
Given the increase in proportion and numbers of γδ T cells in the Itk deficient animals we next set out to identify if this increase was due to a global increase of γδ T cell subsets, or if it was due to an enhancement of a specific γδ T cell subset. We investigated proportions of Vγ2, Vγ1.1, and Vγ3 γδ T cells in the thymic and splenic compartments and found no discernable differences between the WT and Itk deficient animals [data not shown].

Previous work on Itk deficient animals has identified important increases in the proportion of $CD8^+CD4^-$ "innate-like" αβ T cells (69, 71). These cells also had increased expression of NK1.1. Paradoxically, another subset of innate αβ T cells in the Itk deficient animals, NKT cells, actually have decreases in NK1.1 expression (174, 184, 185). Due to this information we decided to study whether γδ T cells have normal proportions of $CD8^+$ and $NK1.1^+$ cells. Although we found an increase in the proportion of Itk deficient $CD8^+CD4^-$ γδ T cells in the thymus [Figure 23A, top row], particularly if MFI is taken into account, and spleen [Figure 23A, bottom row], we found a surprisingly large increase in the proportion of $CD4^+CD8^-$ γδ T cells both in the thymus [figure 23A and B, top row] and the spleen [Figure 23A and B, bottom row] of the Itk deficient mice. The proportion of $NK1.1^+$ γδ T cells in the Itk deficient mice was also increased in the thymus [figure 23C and D, top row] but not the spleen [Figure 23C and D, bottom row]. However, if one takes into account the overall increase in Itk deficient γδ T cells

Figure 23: Itk deficient mice have altered γδ T cell subsets in the spleen and thymus

Cells were prepared from thymi and/or spleens of wild type and $Itk^{-/-}$ mice, stained and gated on $TCR\gamma^{+}TCR\beta^{-}$ cells. [A] $TCR\gamma^{+}TCR\beta^{-}$ cells were analyzed for CD4 and CD8 expression. [B] Shows the percentage of $CD4^{+}TCR\gamma^{+}$ cells [n=8 in thymus and n=6 in spleen]. Each data point represents a different animal, and the bars represent the mean. Statistical significance for $CD4^{+}TCR\gamma^{+}$ cells for thymus is p=0.0002 and for spleen is p=0.0022. Data are representative of 2 independent experiments for the spleen and 3 independent experiments for the thymus with 2-3 mice per group. [C] $TCR\gamma^{+}TCR\beta^{-}$ cells were analyzed for CD4 and NK1.1 expression. [D] Shows the percentages of $NK1.1^{+}TCR\gamma^{+}$ cells [n=11 in thymus and n=9 in spleen]. Each data point represents a different animal, and the bars represent the mean. Statistical significance for $NK1.1^{+}TCR\gamma^{+}$ cells for thymus is $p<0.0001$ and for spleen is p=0.5363. Experiments are representative of 4 independent experiments for the thymus and 3 independent experiments for the spleen with 2-3 mice per group. [E] Splenic $TCR\gamma^{+}TCR\beta^{-}$ cells were analyzed for CD5 and CD4 expression [left panels] and CD5 and NK1.1 expression [right panels]. Representative of 2 independent experiments with 3 mice per group. ***, extremely significant; **, very significant; *, significant.

Figure 23



Gated on TCRγδ+ TCRβ- cells

seen in this compartment [figure 22E], it is clear that the total number of NK1.1$^+$ γδ T cells is increased. Interestingly, even though there are few to none NK1.1$^+$CD4$^+$ γδ T cells in the thymi and spleens of WT mice, a large proportion of the Itk deficient NK1.1$^+$ γδ T cells, over half in the spleen, are CD4$^+$. These increases in the CD4$^+$, NK1.1$^+$, and CD4$^+$NK1.1$^+$ populations might account for the increases in γδ T cells seen in the Itk deficient mice.

Increased proportions of CD4$^+$ γδ T cells have also been demonstrated by abolishing expression of both TCRβ and CD5 at the same time (205). This is not entirely unheard of as CD5 has been shown to suppress αβ CD4$^+$ differentiation (213). Due to these findings we decided to check if CD5 expression was decreased in the Itk deficient animals in order to allow for expansion of CD4$^+$ γδ T cells. Opposite to the results in the previous study, not only did the Itk deficient mice have a larger proportion of splenic γδ T cells expressing CD5, but nearly all the CD4$^+$ γδ T cells Itk deficient mice expressed CD5 [Figure 23E, left panels]. Similarly, most of the NK1.1$^+$ splenic γδ T cells in the Itk deficient animal express CD5 whereas little over half of the WT NK1.1$^+$ splenic γδ T cells express CD5 [Figure 23E, right panels]. These discrepancies between the two studies might be accounted by the different mouse model systems used.

Tec family kinase expression is decreased in NK1.1$^+$ γδ T cells

Due to the discrepancy in proportion and numbers of γδ T cells found in the Itk deficient mice, we decided to evaluate expression of Tec family kinases in γδ T cells. To this end we sorted TCRγδ^{+}TCRβ^{-} and TCRβ^{+}TCRγδ^{-} and evaluated Itk, Rlk, and Tec mRNA levels [Figure 24A, B, and C, outer bars respectively]. Albeit decreased when compared to αβ T cells, the pattern of Tec family kinase expression in the γδ T cells remained the same, with Itk having the highest levels of expression followed by Rlk and then Tec. Due to the increases in NK1.1^{+} γδ T cell numbers seen in Itk deficient mice, we also decided to evaluate Tec family kinase expression in NK1.1^{+} versus NK1.1^{-} WT γδ T cells [Figure 24A, B, and C, center bars respectively]. Interestingly we saw a decrease in expression of all Tec family kinases in the NK1.1^{+} γδ T cells versus the NK1.1^{-} γδ T cells.

Basal levels of the GATA-3 are increased in Itk deficient γδ T cells

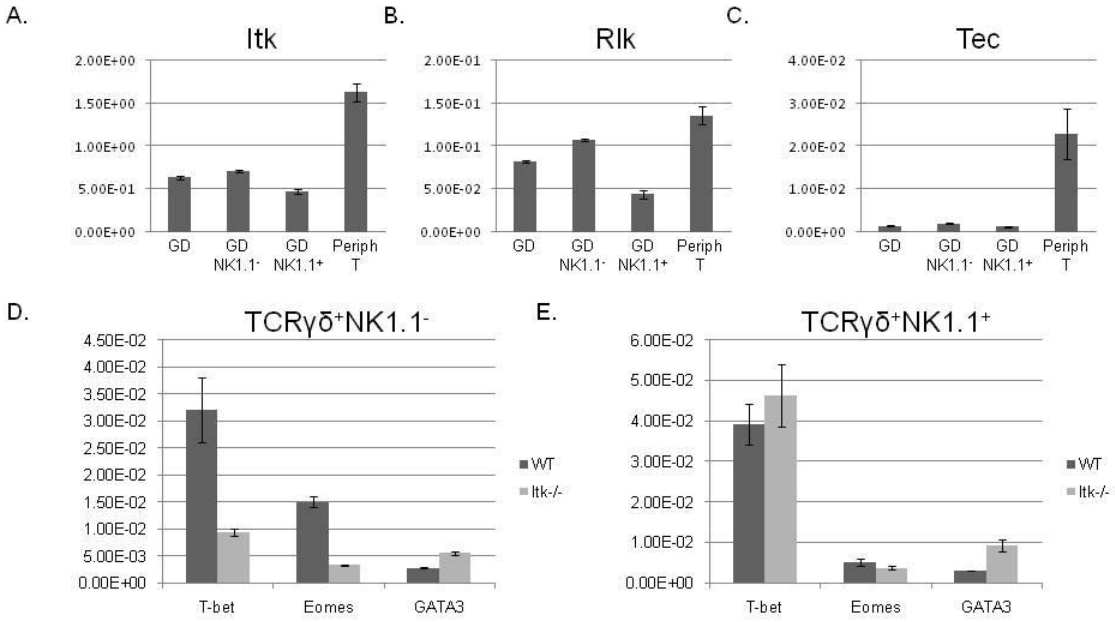
Previous work on Itk deficient animals has demonstrated altered expression of transcription factors involved in Th1 and Th2 cytokine secretion. In the case of conventional αβ T cells, Itk deficient CD4^{+} cells show aberrant regulation of T-bet post stimulation yielding high levels of the transcription factor (102). As discussed in chapter II, we found dysregulation of a T-box family member closely related to T-bet, Eomesodermin [also involved in Th1 cytokine secretion], leading to its high expression in the “innate-like” CD8^{+} T cells found in

Figure 24: γδ T cells have decreased Tec family kinase expression in NK1.1^{+} subsets and there is an increase in GATA-3 levels in Itk$^{-/-}$ γδ T cells

[A-C] Pooled splenocytes and LNs were isolated from WT mice and sorted into γδ T cells, NK1.1^{-} and NK1.1^{+} γδ T cells, and peripheral αβ T cells. Levels of Itk [A], Rlk [B], and Tec [C] mRNA were determined by real-time quantitative PCR.

[D-E] cDNA from NK1.1^{-} [D] and NK1.1^{+} [E] γδ T cells were further analyzed for T-bet, eomesodermin, and GATA-3 mRNA levels. Graphs show data normalized to β-actin mRNA levels in each sample, and are representative of two independent experiments.

Figure 24


A.
Itk
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
GD
GD NK1.1-
GD NK1.1+
Periph T
B.
Rlk
2.00E-01
1.50E-01
1.00E-01
5.00E-02
0.00E+00
GD
GD NK1.1-
GD NK1.1+
Periph T
C.
Tec
4.00E-02
3.00E-02
2.00E-02
1.00E-02
0.00E+00
GD
GD NK1.1-
GD NK1.1+
Periph T
D.
TCRγδ+NK1.1-
4.50E-02
4.00E-02
3.50E-02
3.00E-02
2.50E-02
2.00E-02
1.50E-02
1.00E-02
5.00E-03
0.00E+00
T-bet
Eomes
GATA3
WT
Itk-/-
E.
TCRγδ+NK1.1+
6.00E-02
5.00E-02
4.00E-02
3.00E-02
2.00E-02
1.00E-02
0.00E+00
T-bet
Eomes
GATA3
WT
Itk-/-

the Itk deficient animals (69). Finally, chapter III presented data showing decreased expression of T-bet in thymic NKT cells (174). Based on this information and given the opposing role that T-bet has on GATA-3 we decided to evaluate expression of these transcription factors in the γδ T cells from the Itk deficient versus the WT mice (116, 214).

In order to asses expression of these transcription factors we sorted WT and Itk deficient γδ T cells into NK1.1 negative and positive fractions, and utilizing quantitative real time PCR we looked at the basal [non-activated] levels of expression of each transcription factor. In the Itk deficient $NK1.1^-$ γδ T cell fraction [Figure 24D] we found decreases in the expression of T-box family members [3.5 fold for T-bet and 4.5 fold for Eomes], which are involved in Th1 immunity, when compared to the WT. On the other hand, we found a consistent increase in GATA-3 [2 fold], which is involved in Th2 immunity, when compared to the WT. An increase in GATA-3 [3 fold] could also be seen in the Itk deficient $NK1.1^+$ γδ T cell fraction [Figure 24E], but no important differences were seen for the T-box transcription factors in this fraction.

Itk deficient γδ T cells are prone to Th2, but not Th1, cytokine secretion

The increases seen in Th2 versus Th1 transcription factors in the Itk deficient γδ T cells propelled us to investigate if there were also differences in the cytokine secretion profiles of these cells. To ascertain this we first investigated

the IL-4 [Figure 25A] and IFNγ [Figure 25B] mRNA levels found in WT versus Itk deficient γδ T cells post TCR mediated activation. Although basal levels of IFNγ mRNA were similar in the WT and Itk deficient γδ T cells, basal IL-4 mRNA was increased in the Itk deficient γδ T cells. Consistent with the transcription factor data, we saw increases in IL-4 mRNA [over 10 fold] in the Itk deficient γδ T cells accompanied by decreases in IFNγ mRNA [over 3.5 fold] when comparing to the WT cells at 10 and 20 hours post activation.

Next we set out to study protein levels of cytokine secretion by γδ T cells in the WT versus Itk deficient cells. It has previously been shown that $NK1.1^+$ γδ T cells and $CD4^+$ γδ T cells are responsible for the majority of cytokine secretion when compared to the negative subsets in γδ T cells (215-219). In accordance to this information we decided to split up the WT and Itk deficient cells into NK1.1 negative and positive [Figures 25C-F] and CD4 negative and positive [Figures 25G-J]. We then activated these cells through the TCR and after three days checked for secretion of Th2 and Th1 cytokines. We could readily see strong induction of Th2 cytokines IL-4, IL-10, and IL-13 in the Itk deficient $NK1.1^+$ cells when compared to the WT cells. Likewise, we saw a reverse effect involving higher induction of the Th1 cytokine IFNγ in the WT γδ T cells, both in the NK1.1 positive and NK1.1 negative fractions now, when compared to those from the Itk deficient animal. When looking at the CD4 positive subset, IL-4 induction was still higher and IFNγ secretion was impaired in the Itk deficient γδ T cells, when compared to those from the WT. However, there seemed to be no differences

Figure 25: Itk$^{-/-}$ γδ T cells are functionally impaired following ex vivo activation

Pooled LNs and spleens from wild type and Itk$^{-/-}$ mice were harvested and sorted for TCRγδ^{+} cells [A and B] or NK1.1^{+}TCRγδ^{+} and NK1.1^{-}TCRγδ^{+} cells [C-F].

[A-B] 2 x 10^5 cells were stimulated with 10 μg/ml of anti-TCRγ for 0, 10 and 20 hours. IL-4 [A] and IFNγ [B] mRNA expression levels were determined by real-time quantitative PCR. Data shown are normalized to β-actin mRNA levels in each sample. Data is representative of at least 2 individual experiments.

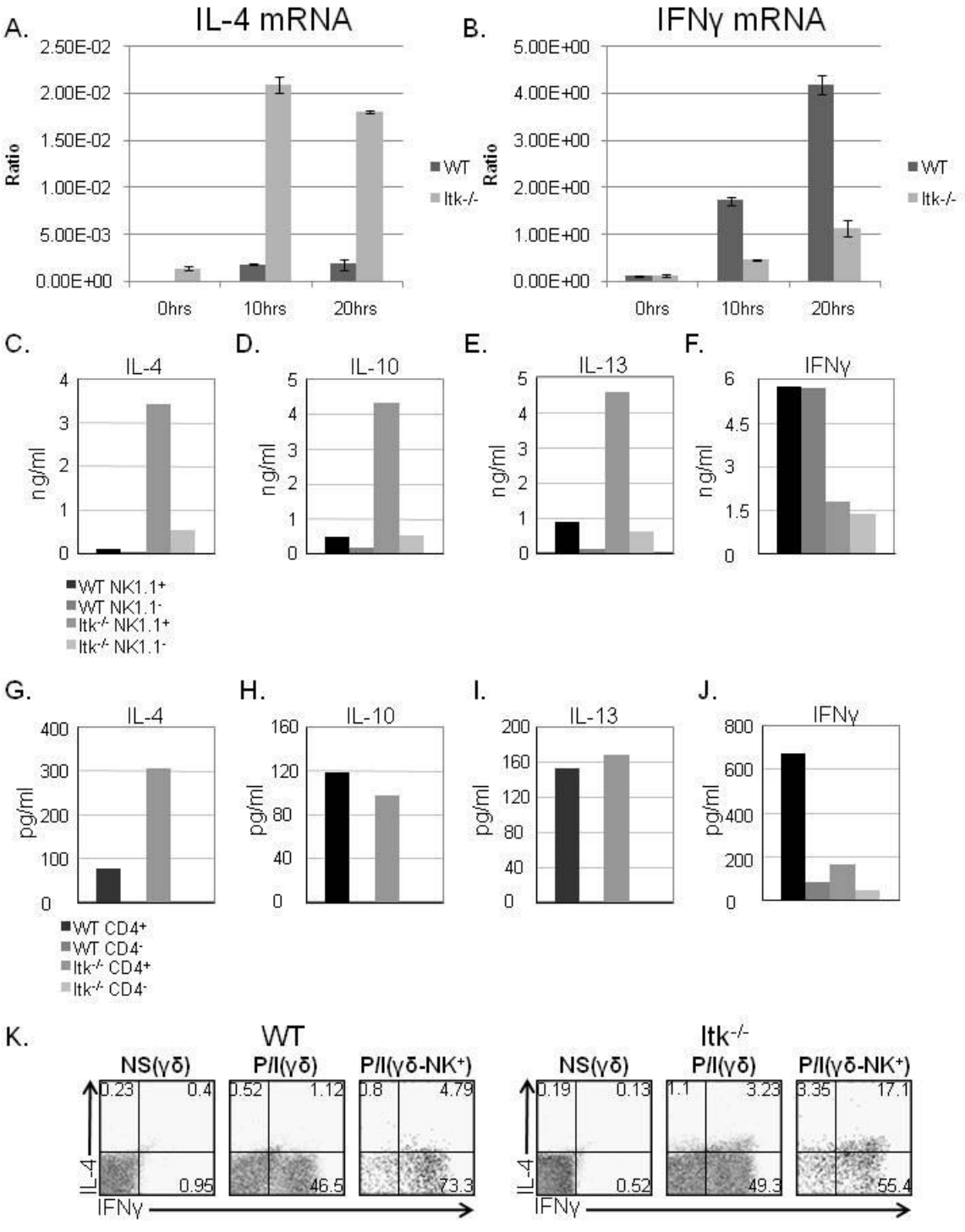
[C-F] 5 x 10^4 cells were stimulated with 10 μg/ml of anti-TCRγ for 72 hours and supernatants were analyzed for the presence of IL-4 [C], IL-10 [D], IL-13 [E], and IFNγ [F] by CBA [Cytometric Bead Array]. Data is representative of 3 independent experiments.

[G-J] Pooled LNs and spleens from wild type and Itk$^{-/-}$ mice were harvested and sorted for CD4^{+}TCRγδ^{+} and CD4^{-}TCRγδ^{+} cells. 3 x 10^4 cells were stimulated as in C-F. Supernatants were analyzed for the presence of IL-4 [G], IL-10 [H], IL-13 [I], and IFNγ [J] by CBA. Data is representative of 1 independent experiment.

[K] Unstimulated [NS] and stimulated [P/I] wild type [left] and Itk$^{-/-}$ [right] γδ T cells were analyzed for intracellular IL-4 and IFNγ production. Stimulated wildtype and Itk$^{-/-}$ NK1.1^{+}γδ T cells were also stimulated and analyzed for intracellular IL-4 and IFNγ production. Cells were stimulated with 10 ng/ml PMA and 2 μg/ml

Ionomycin [P/I] for 4 hours. Data are representative of two independent experiments, each with three mice per group.

Figure 25


A.
IL-4 mRNA
Ratio
2.50E-02
2.00E-02
1.50E-02
1.00E-02
5.00E-03
0.00E+00
0hrs
10hrs
20hrs
WT
Itk-/-
B.
IFNγ mRNA
Ratio
5.00E+00
4.00E+00
3.00E+00
2.00E+00
1.00E+00
0.00E+00
0hrs
10hrs
20hrs
WT
Itk-/-
C.
IL-4
ng/ml
D.
IL-10
ng/ml
E.
IL-13
ng/ml
F.
IFNγ
ng/ml
WT NK1.1+
WT NK1.1-
Itk-/- NK1.1+
Itk-/- NK1.1-
G.
IL-4
pg/ml
H.
IL-10
pg/ml
I.
IL-13
pg/ml
J.
IFNγ
pg/ml
WT CD4+
WT CD4-
Itk-/- CD4+
Itk-/- CD4-
K.
WT
Itk-/-
NS(γδ)
P/I(γδ)
P/I(γδ-NK+)
0.23
0.4
0.95
0.52
1.12
46.5
0.8
4.79
73.3
0.19
0.13
0.52
1.1
3.23
49.3
3.35
17.1
55.4
IL-4
IFNγ

amongst the other Th2 cytokines [IL-10 and IL-13]. Cytokine secretion was also assessed for the $CD8^+$ subset of γδ T cells, but this subset was found to produce less cytokines and the differences between the WT and Itk deficient cells were minimal [data not shown].

Given that Itk functions proximal to the T cell receptor upon TCR engagement in conventional αβ T cells and the defects in signaling through these cells can be fixed by bypassing proximal TCR signaling through PMA and Ionomycin, cytokine secretion post PMA and Ionomycin activation was studied in γδ T cells from WT and Itk deficient cells [Figure 25K]. This experiment helps predict whether the differences seen in the Itk deficient γδ T cells are due to just defects in signaling upon TCR stimulation, in which case WT and Itk deficient secretion profiles should be the same, or due to altered development and cytokine secretion profiles in the Itk deficient animals, in which case the differences in cytokine secretion can still be seen by bypassing the TCR. As it can be seen, PMA and Ionomycin activation in the whole γδ T cell population [center square] yields similar amounts of IFNγ in the WT and Itk deficient cells, indicating a rescue of IFNγ secretion. However, a slight increase in IL-4 positive cells can be detected in the Itk deficient cells. If one gates on the $NK1.1^+$ γδ T cells then the increase in IL-4 secretion and decrease in IFNγ in the Itk deficient cells becomes more evident. This data is seems to indicate that the increase in IL-4 secretion is due to developmental issues rather than issues with signaling upon TCR activation. It is somewhat perplexing that the Itk deficient cells are not

producing as much IL-4 as observed in the TCR activation experiments, but this might be due to differences in length of activation, as the PMA/Ionomycin activated cells cannot be activated for prolonged periods of time due to toxicity.

Itk deficient γδ T cells have an upregulation of markers involved in B cell help

In the previous figures it has been shown that the Itk deficient γδ T cells are geared towards secretion of Th2 cytokines. Given this information we set out to investigate if these cells had other characteristics of Th2 immunity, particularly expression of markers needed for humoral immunity. As mentioned in the intro, human γδ T cells can upregulate B cell costimulatory molecules CD40L, CD70, OX40, and ICOS (5). In order to asses Itk deficient γδ T cell potential for upregulation of B cell costimulatory markers, WT and Itk deficient thymocytes and splenocytes were evaluated directly *ex vivo* or placed in culture for 24 hours in the presence and absence of TCR stimulus.

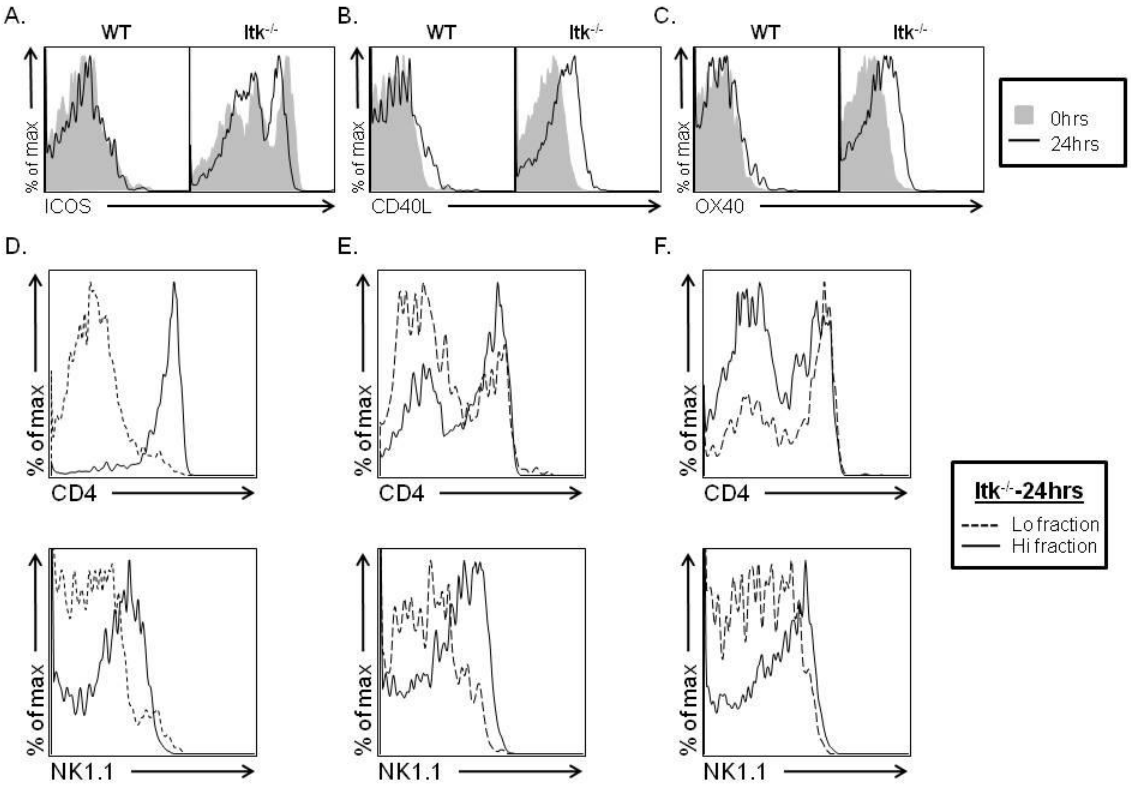
Neither the Itk deficient nor the WT γδ T splenocytes were able to upregulate B cell costimulatory markers in this assay [data not shown]. Interestingly, levels of ICOS were upregulated in a large proportion of the Itk deficient γδ T cell thymocytes directly *ex vivo* when compared to those from the WT [Figure 26A, gray fill]. 24 hour *in vitro* stimulation [Figure 26A, solid line] didn't seem to alter the high levels of ICOS in the Itk deficient γδ T cell thymocytes and didn't seem to induce expression in the WT γδ T cells. Longer

Figure 26: Itk$^{-/-}$ γδ T cells upregulate markers involved in B cell help

[A-C] TCRγδ^{+}TCRβ^{-} T cells from pooled thymocytes from wild type and Itk$^{-/-}$ mice were stimulated with 10 μg/ml α-TCRγ 0 and 24 hours. Unstimulated and stimulated TCRγδ^{+}TCRβ^{-} T cells were then analyzed for the expression of ICOS [A], CD40L [B], and OX40 [C].

[D-F] The high [solid line] and low fractions [dashed line] [from A-C [respectively under]] from Itk$^{-/-}$ TCRγδ^{+}TCRβ^{-} T cells were further analyzed for CD4 [top] and NK1.1 [bottom] expression.

Figure 26


A.
WT
Itk-/-
% of max
ICOS
B.
WT
Itk-/-
% of max
CD40L
C.
WT
Itk-/-
% of max
OX40
0hrs
24hrs
D.
% of max
CD4
E.
% of max
CD4
F.
% of max
CD4
Itk-/--24hrs
Lo fraction
Hi fraction
% of max
NK1.1
% of max
NK1.1
% of max
NK1.1

stimulations had the same pattern of expression [data not shown]. Evaluation of the ICOS^{hi} fraction in the Itk deficient γδ T cells indicated that nearly all of them were CD4^{+} and NK1.1^{+} [Figure 26D top and bottom respectively].

Little to no difference was seen in the basal levels of CD40L [Figure 26B, gray fill] and OX40 [Figure 26C, gray fill] expression when comparing the thymic Itk deficient γδ T cells to those of the WT. However 24 hour *in vitro* stimulation in α-TCRγδ coated plates led to upregulation of both CD40L and OX40 in the Itk deficient thymic γδ T cells, while this had no effect on the WT γδ T cells [Figure 26B and C respectively, solid line]. Of note, albeit the increase was less noteworthy, 24 hour *in vitro* culture in uncoated plates also led to an increase of these molecules in the thymic Itk deficient γδ T cells while having no effect on the WT cells. This seems to indicate that, at least in part, the increases in expression of CD40L and OX40 might not just be TCR mediated but rather adhesion molecule mediated or cytokine mediated [as the cultures contain IL-2]. Regardless of this, the CD40L^{hi} fraction of Itk deficient thymic γδ T cells is composed mostly of CD4^{+} and NK1.1^{+} cells [Figure 26E, top and bottom respectively]. The OX40^{hi} fraction of the Itk deficient thymic γδ T cells was composed mostly of NK1.1^{+} cells [Figure 26F, bottom], but unlike with ICOS and CD40L only half of the OX40^{hi} thymic γδ T cells were CD4^{+} [Figure 26F, top]. No upregulation of CD70 was seen in either the WT and Itk deficient thymic γδ T cells [data not shown].

γδ T cells are largely responsible for the hyper IgE and enriched GC phenotype seen in the Itk deficient mice

The Th2 cytokine secretion potential and ability to upregulate B cell costimulatory molecules exhibited by the Itk deficient γδ T cells seems to be indicative of these subset of cells having a role in humoral immunity in the Itk deficient mice. Of consequence, previous work has shown spontaneously high levels of IgE in the Itk deficient mice (140). IL-4, which as shown here is highly secreted by the Itk deficient γδ T cells, greatly enhances IgE class-switching and production (220, 221). Given this information, Itk deficient mice were crossed to TCRδ deficient mice, which have no γδ T cells, in order to see if the γδ T cells are, at least in part, responsible for the spontaneously high levels of IgE.

As shown in figure 27[A], we can recapitulate the high IgE titers described previously on the Itk deficient mice [third column compared to first column]. Strikingly, the Itk-TCRδ double deficient mice have an extremely significant reduction in serum IgE levels when compared to those of the Itk single deficient mice [fourth column compared to third column]. Individual cohorts of mice were tested at 2 months of age, 3.5 months of age, and 5 months of age all yielding the same pattern of IgE titters [data not shown].

IgE class-switching and secretion requires B cell activation and proliferation, and given that data shown here indicates that B cell costimulatory

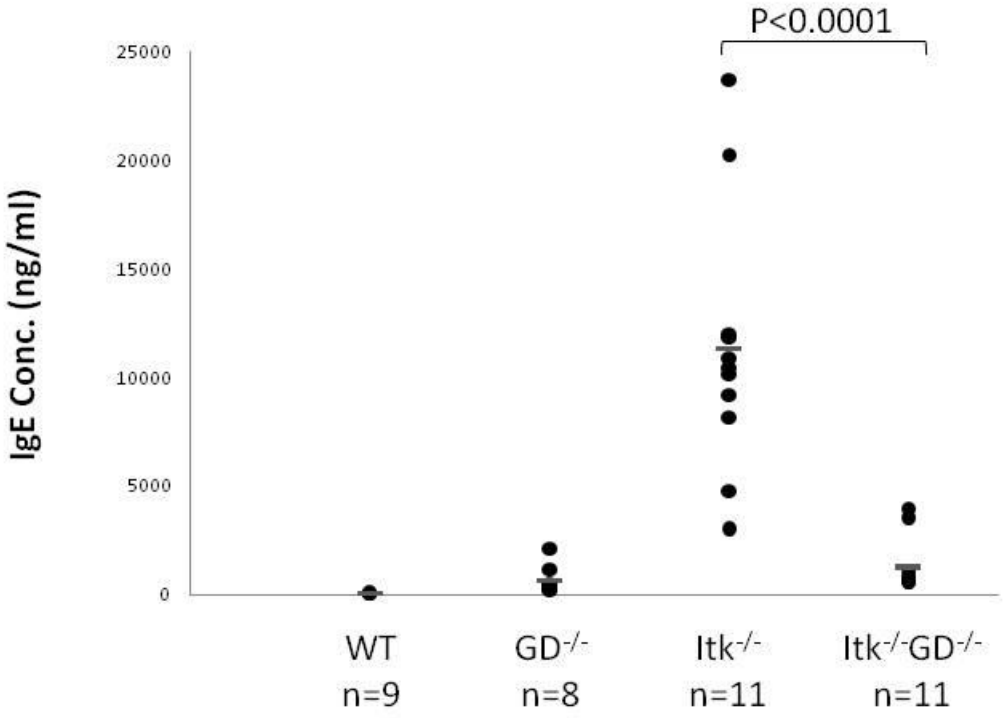
Figure 27: Hyper IgE and enriched germinal center phenotype in Itk$^{-/-}$ mice largely due to γδ T cells

[A] Serum obtained from wild type [WT], TCRδ$^{-/-}$ [labeled GD$^{-/-}$], Itk$^{-/-}$, and Itk$^{-/-}$ TCRδ$^{-/-}$ [Itk$^{-/-}$GD$^{-/-}$] mice were analyzed for the presence of IgE by ELISA.

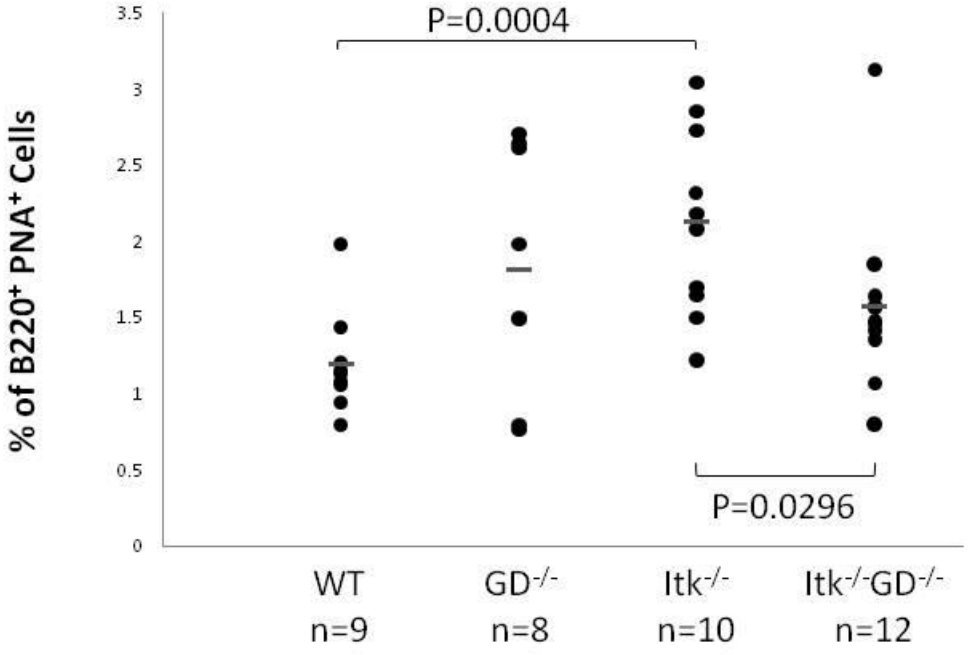
[B] Splenocytes from wild type [WT], TCRδ$^{-/-}$ [labeled GD$^{-/-}$], Itk$^{-/-}$, and Itk$^{-/-}$TCRδ$^{-/-}$ [Itk$^{-/-}$GD$^{-/-}$] mice were stained with α-B220 and α-PNA for detection of germinal center B cells. Each data point represents a different animal, and the bars represent the mean. Numbers of animals analyzed are indicated as N values at the bottom.

Figure 27

A.


P<0.0001
IgE Conc. (ng/ml)
25000
20000
15000
10000
5000
0
WT
n=9
GD-/-
n=8
Itk-/-
n=11
Itk-/-GD-/-
n=11

B.


P=0.0004
P=0.0296
% of B220+ PNA+ Cells
3.5
3
2.5
2
1.5
1
0.5
0
WT
n=9
GD-/-
n=8
Itk-/-
n=10
Itk-/-GD-/-
n=12

molecules are upregulated in the Itk deficient γδ T cells, changes in GC formation were also evaluated. As seen in figure 27[B], there is a quite significant increase in the proportion of GC B cells [$B220^{+}PNA^{+}$] seen in the Itk deficient mice when compared to the WT mice. Again, the Itk-TCRδ double deficient mice exhibited a significant decrease in the proportion of GC B cells when compared to the Itk single deficient mice [fourth column compared to third column]. All in all, this data indicates that the spontaneous B cell activation and IgE class switching is strongly subdued by the absence of γδ T cells in the Itk deficient mice.

Discussion

The previous chapters have delineated a complex role for Tec family kinases, particularly Itk, in innate immune development and function (69, 71-74, 174, 184, 185). In the case of $CD8^{+}$ αβ T cells, absence of Itk seems to promote generation of an innate subset of these cells that is dependent on hematopoietic cells and SAP signaling for selection. On the other hand absence of Itk seems to be detrimental for the selection and survival of NKT cells. In either case Itk seems to have a significant role in innate αβ T cell development and function; preventing it in the case of $CD8^{+}$ T cells and aiding it in the case of NKT cells. Given the findings of these previous studies we wanted to investigate whether Itk had a significant role in another subset of innate T cells, γδ T cells.

In this chapter we show that, much like in the CD8 studies, absence of Itk seems to promote γδ T cell development, yielding larger proportions and numbers of these cells. The increases in γδ T cell numbers could be seen both in the thymus [little less than 3-fold] and spleen [little less than 2-fold]. The increase in numbers was not caused by a particular increase in the proportion of specific γδ T cell subsets as we didn't see differences in the proportions of Vγ1.1, Vγ2, and Vγ3 cells [data not shown]. However, we saw increases in the proportion of $NK1.1^+$ [thymus], $CD8^+$ [both thymus and spleen], and $CD4^+$ [both thymus and spleen] γδ T cells. Of particular interest we see a population of $NK1.1^+CD4^+$ γδ T cells in the Itk deficient mice which is absent in the WT mice. Taking into account the overall increase in numbers, the increase in proportion of these populations of cells yields a pretty big increase in numbers in both the thymus and spleen. It seems likely though that the enhancement of the $NK1.1^+$, $CD4^+$, and $CD8^+$ subsets alone is not totally responsible for the overall increase in numbers as these can't yield a 3-fold increase in the thymus and a two-fold increase in the periphery.

One previous study utilizes a model system that also capitulates an increase in the $CD4^+$ compartment of γδ T cells (205). In this study the increase in $CD4^+$ γδ T cells is seen in TCRβ/CD5 doubly deficient mice. CD5 expression in αβ T cells has been previously reported to suppress $CD4^+$ T cell differentiation (213). Given these two pieces of information it seemed likely that CD5 expression in the Itk deficient γδ T cells might be decreased. Not only it wasn't

decreased, but rather CD5 was expressed in a much higher proportion of $CD4^+$, and for that matter $NK1.1^+$, γδ T cells than in the WT controls. Differences between the two studies might be accounted by the fact that the previous study utilized TCRβ deficient mice, and double positive cells are required for proper selection and function of γδ T cells (222). The increase in the proportion of CD5 positive γδ T cells seen in our study could be indicative of the reason as to why we see increased proportions and numbers of γδ T cells. CD5 is usually utilized as evidence of TCR signaling and signal strength during development of αβ T cells (223-225). Studies utilizing γδTCR transgenes with altered signaling potential have shown that CD5 expression also correlates with TCR signal strength in γδ T cells (81, 226). Perhaps the reason behind the increases seen in γδ T cells in the Itk deficient mice might be explained by increases in signal strength in γδ T cells in these mice. This would however go against the mantra of Itk function in αβ T cells which states that Itk is required to boost signal strength downstream of the TCR (98). An alternative, derived from completely opposite lines of thinking, could be that reduced signaling in the Itk deficient mice only allows the γδ T cells with highest affinity TCRs to develop, giving these cells a competitive advantage and explaining the higher proportions of CD5 positive γδ T cells. However, more biochemical work and γδ TCR repertoire analysis would need to be done to conclude anything on this matter.

The increase in $NK1.1^+$ γδ T cells prompted us to look at expression of Tec family kinases in these cells. In the previous chapter we described

increases [particularly in the thymus, and to a much lesser extent in the spleen] of Itk, Rlk, and Tec kinases in the NK1.1 positive versus the NK1.1 negative NKT cells (174). This is of interest because in NKT cells absence of Itk is detrimental and leads to an absence of NK1.1 positive cells. On the other hand absence of Itk in γδ T cells seems to be advantageous and leads to generation of more NK1.1 positive cells. Likewise, we saw the opposite kinase expression pattern in these cells, with the NK1.1 positive γδ T cells having less Itk, Rlk, and Tec than the NK1.1 negative γδ T cells. Increases in NK1.1 expression have also been reported in the Itk deficient $CD8^+$ "innate-like" αβ T cells (69). Given this information it is tempting to speculate on the role Itk [negative in γδ and $CD8^+$ T cells and positive in NKT cells] has in NK1.1 expression, and furthermore on selection of one innate subset versus the other. A great deal more work remains to be done in order to validate this speculation.

Having defined a defect in regulation of γδ T cells in the Itk deficient mice we next set out to evaluate function of these cells. Previous work on innate Itk deficient cells has shown defects in upregulation of T-bet in NKT cells and exacerbation of expression of another T-box family member, eomesodermin, in the "innate-like" $CD8^+$ T cells (69, 174). This drove us to investigate whether these transcription factors, and their Th2 counterpart, GATA-3, were aberrantly regulated in the γδ T cells. Both T-bet and Eomesodermin were strongly downregulated in the Itk deficient $NK1.1^-$ γδ T cells, whereas no big differences were seen in the $NK1.1^+$ cells. GATA-3, on the other hand, was upregulated in

both the NK1.1^{-} and [particularly] the NK1.1^{+} γδ T cells. GATA-3 and T-bet have been shown to cross regulate each other. While GATA-3 regulates T-bet through inhibition of Th1 cell-specific factors, T-bet regulates GATA-3 through kinase mediated interactions which prevent GATA-3 binding to the DNA (116, 227). This latter study also showed that Itk can phosphorylate T-bet and furthermore presence of Itk facilitates direct association between T-bet and GATA-3. Another study claims that the reason γδ T cells produce IFNγ by default is simply that GATA-3 is not as efficient in cross regulating T-bet in these types of cells (228).

Given the previously stated information, the question then arises of what is the cytokine secretion outcome in a γδ T cell system in which T-bet cannot cross regulate GATA-3. Clearly elevated levels of GATA-3 are seen in the Itk deficient mice, but according to the aforementioned study this alone does not preclude IFNγ dominance. From our results it can be seen that when Itk is missing, and therefore T-bet regulation of GATA-3 is lost, the default cytokine secretion seems to turn to a more impressive IL-4 secretion profile. However this matter is a bit more complicated than just Itk helping T-bet regulate GATA-3, as bypassing the TCR restores secretion of IFNγ. The IL-4 secretion profile could also be enhanced by selection of a population of γδ T cells which preferentially secrete this cytokine. CD4^{+} γδ T cells have been previously described to preferentially secrete Th2 cytokines (219). Not only do we see an enhancement in the CD4^{+} population, but the NK1.1^{+} population of γδ T cells is also secreting Th2 cytokines rather than Th1 cytokines in the Itk deficient mice. It should be pointed

out that while the NK1.1$^+$ Itk deficient γδ T cell population has increases in IL-4, IL-10, and IL-13, the CD4$^+$ population has increases in only IL-4, and they are not as impressive. Both populations have notable decreases in IFNγ production. This might indicate that the NK1.1$^+$ and the CD4$^+$ populations are two separate, both phenotypically and functionally, populations. We do see a third elevated population of NK1.1 and CD4 double expressing cells the Itk deficient animals. Further separation and activation experiments would need to be done in order to narrow down the individual functions of each population of γδ T cells in the Itk deficient mice. It should be said that the changes seen in cytokine secretion in the Itk deficient γδ T cells are not just due to lack of T-bet crossregulation of GATA-3, but also due to aberrant development in the Itk deficient mice as the NK1.1 negative γδ T cells, which usually don't secrete Th2 cytokines, also have the capability of secreting IL-4, IL-13, and IL-5. Exactly what is responsible for the new found ability of these cells to secrete cytokines remains a mystery which cannot easily be addressed by our current model [Figure 28] or any previously published data.

The high IL-4 and IL-13 produced by the Itk deficient γδ T cells explains part of the spontaneous high IgE secretion previously reported on the Itk deficient mice (140). However, this alone cannot explain the phenotype as in order to produce and switch to IgE the B cells must first receive help from certain costimulatory molecules [ICOS, CD40L, CD70, and OX40]. Ability to upregulate such molecules has previously been reported on human γδ T cells (5). When

studying thymic γδ T cells from the Itk deficient mice we found a large proportion of cells expressing ICOS. A recent study has indicated that both T-bet and GATA-3 can positively regulate ICOS expression via ICOS promoter [T-bet] and a 3'-untranslated region element [GATA-3] (229). In this study, overexpression of either T-bet or GATA-3 could enhance ICOS transcription. It is important to note that the $ICOS^{hi}$ γδ T cells in the Itk deficient mice are $NK1.1^{+}$ and $CD4^{+}$. As mentioned earlier, although T-bet expression is not affected in the $NK1.1^{+}$ cells [unlike in the negative cells], GATA-3 is specially upregulated in this group. We do not however see $ICOS^{hi}$ expressing cells in the spleens of these mice, but we speculate that perhaps high expression of ICOS is quickly downregulated in the periphery of the deficient mice, giving it a short window of time to activate the B cells. We also observed upregulation of both CD40L and OX40 in the Itk deficient γδ T cells, but this was trickier as cytokine responsiveness or aberrant adhesion molecule signaling might be responsible for part of this as we also saw some upregulation of these molecules in wells that didn't receive TCR stimulus. Regardless of the cause of this upregulation, the WT γδ T cells were not able to induce these molecules.

Taken together, only a proof of concept remained to show that the γδ T cells in the Itk deficient mice could induce aberrant B cell activation and class-switching. This was achieved by crossing the Itk deficient mice to the TCRδ deficient mice. Sure enough a remarkable decrease was seen in the serum IgE concentrations of the Itk/ TCRδ doubly deficient mice. This decrease was also

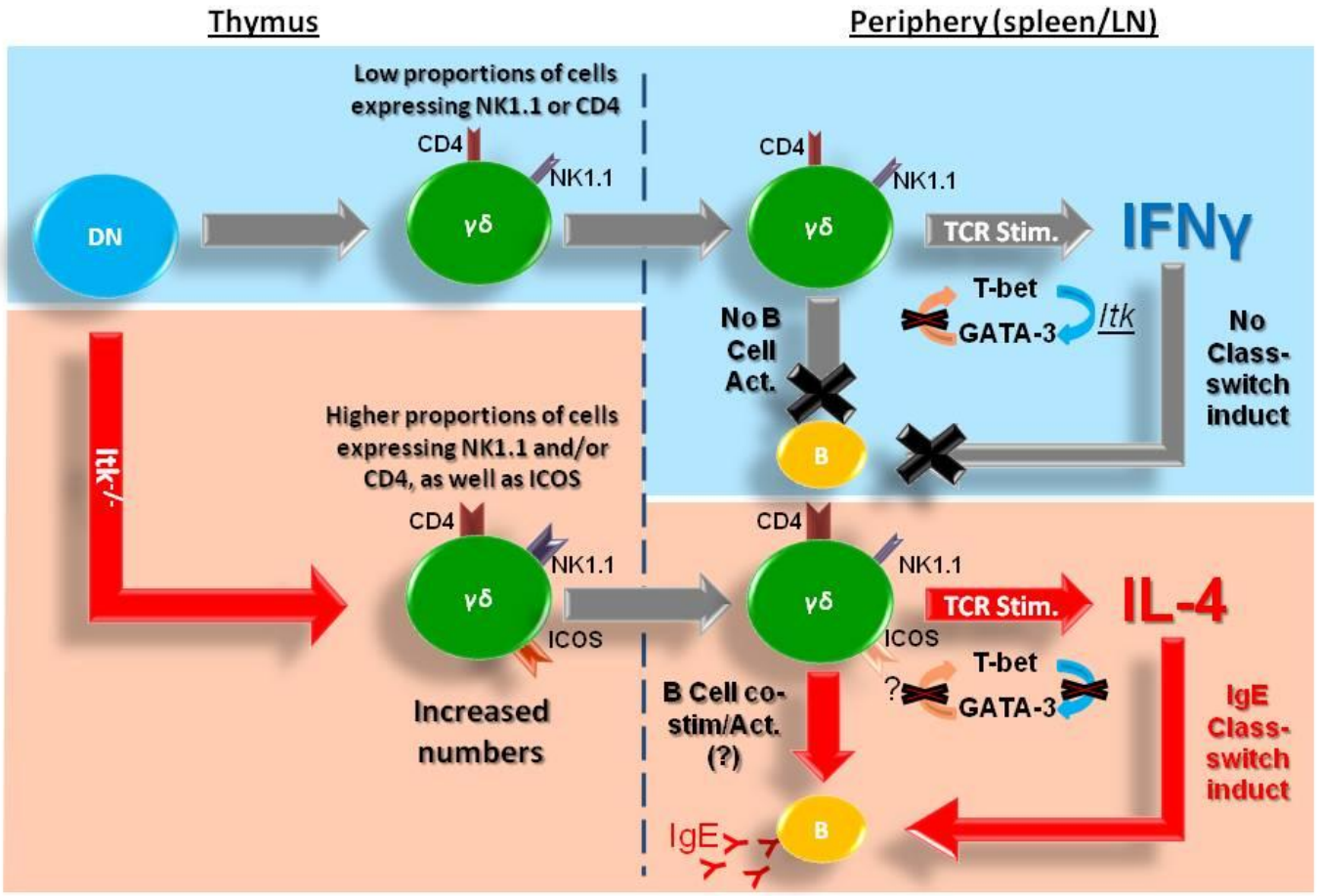
accompanied by a significant decrease in germinal center B cells. Our study illustrates how aberrant expression of Itk in γδ T cells can lead to an excess in IgE production [Figure 28]. A similar spontaneous enhancement in IgE production mediated by γδ T cells was also recently reported in Itch deficient mice and a role for γδ T cells in systemic IgE responses in allergic eosinophilic airway inflammation has been previously described (230, 231). Coupled to the detrimental role of IgE in allergic disease, and the fact that Itk inhibitors are being actively researched to fight allergy, it seems clear that a better understanding of the role of Itk in γδ T cells must be attained in order to address this pathology properly (232-237). Moreover, the results presented in this chapter might illustrate how discrepancies in γδ signaling during development might incur pathologies later on in life through altering the balance between Th1 and Th2.

Figure 28: A model for the Th2 bias in Itk deficient mice

γδ T cells develop from $CD4^-CD8^-$ thymocytes. Normally [blue background] a small proportion of $CD4^+$ or $NK1.1^+$ γδ T cells develop in the thymus. Similar small proportions of $CD4^+$ or $NK1.1^+$ γδ T cells can be found in the periphery. γδ T cells cannot normally upregulate B cell costimulatory molecules. Upon TCR mediated activation, crossregulation of GATA-3 by T-bet, mediated by Itk, yields production of IFNγ and not IL-4.

Absence of Itk in γδ T cells [red background] seems to enhance the development/selection of these cells yielding a larger number of total γδ T cells both in the thymus and periphery. An increased proportion of γδ T cells expressing, CD4, NK1.1 or both is seen in the thymus, whereas in the spleen increased only increased proportions of $CD4^+$ and $CD4^+NK1.1^+$ can be seen. ICOS is also upregulated in the thymus and other B cell costimulatory molecules [CD40L and OX40] can also be upregulated upon TCR stimulation. This upregulation seems to be transient and is probably downregulated quickly in the periphery, but not prior to the γδ T cells providing the B cells "help". TCR stimulation in the $Itk^{-/-}$ γδ T cells yields primarily IL-4, probably due to the fact that T-bet can no longer crossregulate GATA-3 in the absence of Itk. It might be possible that GATA-3 can now crossregulate T-bet, enhancing the Th2 bias. The switch to IL-4 production now induces IgE class switching in the activated B cells, leading to the eventual secretion of abnormal amounts of IgE.

Figure 28


Thymus
Periphery (spleen/LN)
Low proportions of cells expressing NK1.1 or CD4
CD4
NK1.1
DN
γδ
CD4
NK1.1
γδ
TCR Stim.
IFNγ
T-bet
GATA-3
Itk
No B Cell Act.
No Class-switch induct
B
Itk-/-
Higher proportions of cells expressing NK1.1 and/or CD4, as well as ICOS
CD4
NK1.1
γδ
ICOS
Increased numbers
CD4
NK1.1
γδ
TCR Stim.
IL-4
ICOS
T-bet
?
GATA-3
B Cell co-stim/Act. (?)
IgE Class-switch induct
IgE
B

CHAPTER V: Discussion

Chapter V Attributions and Copyright information

One of the figures [Figure 30] on this chapter was previously published in a manuscript submitted by Julie Lucas and I [the experiment was performed by me] (238).

Lucas, J. A., M. Felices, J. W. Evans, and L. J. Berg. 2007. Subtle defects in pre-TCR signaling in the absence of the Tec kinase Itk. *J Immunol* 179:7561-7567.

Synopsis of the material

The work presented in the data chapters of this thesis clearly document an important, and novel, role for Tec family kinases in innate T cell lineage decision/selection, development, and function. It is important to study the role of Tec family kinases in innate T cell subsets because it gives us an idea of how similar or different signaling pathways are downstream of the TCR in innate versus adaptive T cells. Furthermore, better understanding of these kinases can help us expand our knowledge on the signaling processes required for conventional lineage choice as opposed to adaptive lineage choice. Prior to this body of work, only one study existed documenting very briefly a possible role for Itk in an innate T cell subset, NKT cells (184). We now know that Itk is critical for the development of conventional $CD8^+$ T cells, is necessary for terminal maturation of NKT cells and proper cytokine secretion by said cells, and is necessary to maintain the Th1 cytokine secretion bias seen in γδ T cells.

Chapter II described the role of Itk and Rlk in $CD8^+$ T cells. We show that in the $Itk^{-/-}$ and the $Itk^{-/-}Rlk^{-/-}$ mice $CD8^+$ T cells develop with a memory like phenotype denoted by high expression of NK1.1, CD44, CD122, dependence on IL-15, and quick cytokine secretion. Furthermore, $CD44^{hi}$ expression arises in the thymus [not the periphery], and there is high expression of the transcription factor Eomesodermin in these cells. Another study, submitted side by side with ours, showed that the $CD8^+$ T cells arising in these mice were selected on cells

from hematopoietic origin (71). Finally this same group later on showed that the $CD8^+$ T cells in the $Itk^{-/-}$ and the $Itk^{-/-}Rlk^{-/-}$ mice are dependent on expression of SAP for their selection (73). Taken together all these data demonstrate that mostly innate-like $CD8^+$ T cells develop in the $Itk^{-/-}$ and the $Itk^{-/-}Rlk^{-/-}$ mice, indicating that Tec family kinases Itk and Rlk have a critical role in conventional $CD8^+$ T cell lineage decision.

Contrary to the detrimental role in the development of innate-like $CD8^+$ T cells, chapter III described an important role for Itk and Rlk in terminal maturation of NKT cells. The $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NKT cells had defects in: initial selection, denoted by a decrease in NKT cell numbers prior to the earliest developmental stage we could study; terminal maturation, illustrated by an inability to upregulate NK cells markers usually associated with this stage; cytokine secretion, a defect probably caused mostly by decreased signaling downstream of the TCR and not faulty development; peripheral survival, most likely caused by decreased levels of CD122, which is needed for signaling via IL-15; and the upregulation of T-bet, which probably governs, for the most part, a good amount of the terminal maturation associated defects. This work, along with another study released at the same time, showed a clear role for Tec family kinase signaling in the proper development and function of NKT cells (185). It also points to a less clear role for these kinases in initial selection of NKT cells.

Work presented on the last data chapter [IV] shows that Itk is involved in maintaining the Th1 cytokine secretion profile of γδ T cells. Whether or not this is due simply to altered development of these cells or just an inability of T-bet to crossregulate GATA-3 upon TCR mediated activation in the absence of Itk, it is not clear. It is clear however that absence of Itk gives the γδ T cells a selective advantage, denoted by increased numbers of these cells. $Itk^{-/-}$ mice also have increased numbers of $NK1.1^{+}$ and/or $CD4^{+}$ γδ T cells. The increase in IL-4 cytokine production, accompanied by the ability to upregulate B cell costimulatory molecules, in $Itk^{-/-}$ γδ T cells seems to be responsible for the spontaneously high levels of IgE seen in the $Itk^{-/-}$ mice. Therefore, data in this chapter demonstrates that Itk is needed to maintain proper γδ T cell development and function.

Each of the data sections contained a grounded discussion, based on the data obtained and the literature available, about each of the subsets mentioned. Rather than regurgitate these discussions, the sections that follow in this chapter will be of a more speculative nature, attempting at times to reconcile a common role for Tec family kinases in all of these subsets, and at times merely speculating on why they differ.

A role for signal strength in conventional and innate T cell development

If one were to take a minimalistic approach to T cell selection, in which signal strength alone governs selection of innate T cells and conventional T cells, then the data presented on this thesis could be used to gauge and model the signaling requirements for different subsets of T cells. The main assumption taken here would be that absence of Itk only causes a decrease in the overall signal strength downstream of the TCR, but changes nothing else. Another assumption of this model system would be that all the subsets of T cells are interacting with equal amounts of MHC molecules to be selected and all TCR-MHC interactions are of similar strength. Given this model system [Figure 29] one could conclude that selection of conventional $CD8^+$ T cells has the highest requirement for signal strength given the stark decrease in numbers upon decreasing signal amplitude (69, 71). NKT cell selection is also decreased quite a bit in the Itk deficient animals, also denoted by a large decrease in numbers (174). Although not as impressive as the decreases just discussed, selection of conventional $CD4^+$ T cells is impaired as well (150). γδ T cell selection is favored slightly by the decrease in signaling, indicating [in this model] that this subset of cells has lower signaling requirements for selection. Finally, given the vast increase in innate like $CD8^+$ T cells, we could infer that this subset of cells has the lowest requirement of signal strength for selection (69, 71). Of course the

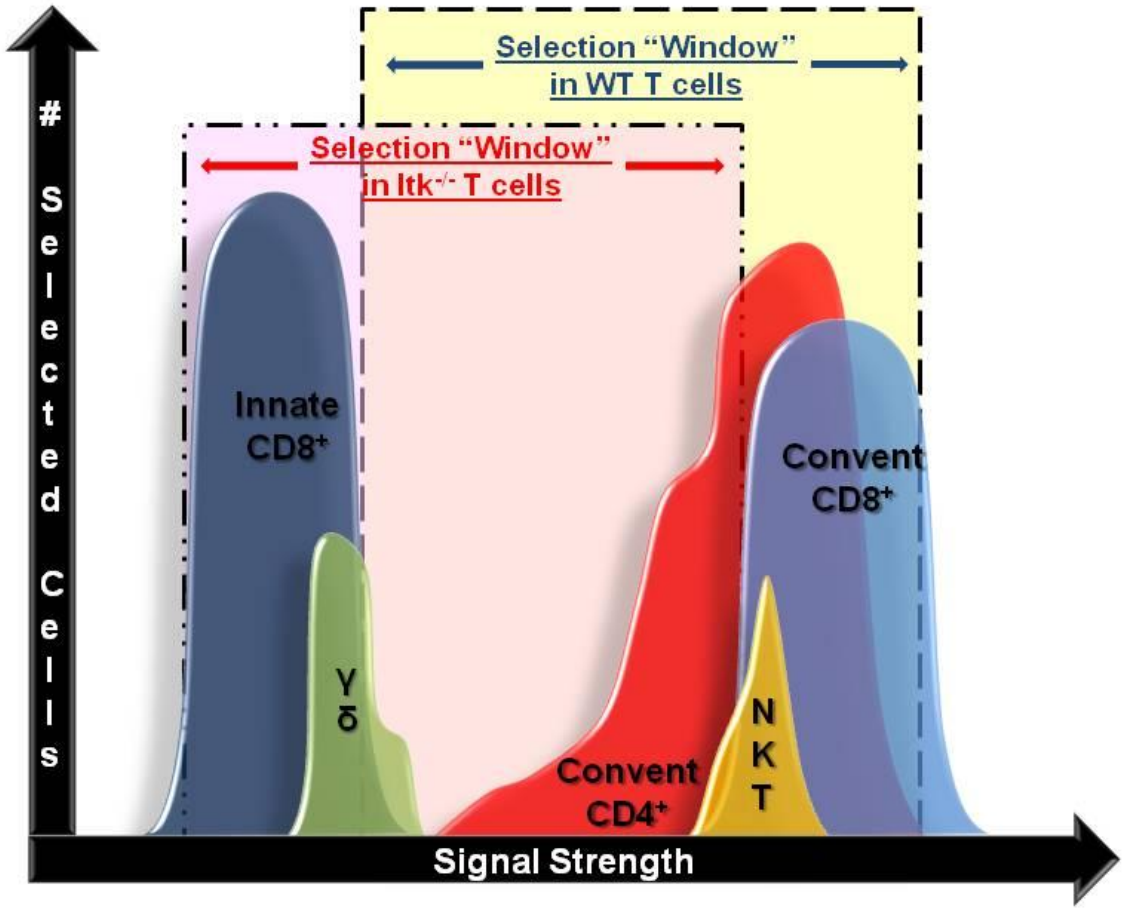
increase in the number of innate $CD8^+$ T cells and γδ T cells is most likely dependent on the space created by the decrease in other T cell types.

There are several issues with this simplified model that would need to be addressed in order to make it more realistic. For starters conventional T cells are selected on thymic stromal cells while innate T cells get selected on cells of hematopoietic origin, which have been shown to have lower levels of MHC expression on their surface (9, 75). To normalize the difference in MHC expression levels, we would have to shift up the signaling strength of innate subsets selected on hematopoietic cells because these cells are getting selected on lower amounts of MHC. One piece of evidence that demonstrates that signaling strength in innate subsets is modulated by MHC expression is based on a study which forces CD1d expression in the thymic epithelia, increasing the number of molecules NKT cells can be selected on (77). The increase in CD1d molecules causes the NKT cells to undergo negative selection, indicating that their signal strength is normally quite high [or at least close to their threshold for negative selection], and increasing the number of selecting molecules pushes the signal above the acceptable threshold (76). A separate matter, which has been studied in conventional $CD4^+$ versus $CD8^+$ T cell selection, is the importance of duration of signals for positive selection (239-243). One could hypothesize that perhaps decreased amounts of MHC in the hematopoietic cells might cause a decrease in the duration of the signals transduced in the innate cells, thereby affecting selection of these cells.

Figure 29: A simplified model for signal strength modulating T cell selection

Under circumstances where conventional amounts of signal are transduced downstream of the TCR [dashed window] large amounts of conventional [convent] $CD8^+$ and $CD4^+$ T cells are selected. Smaller amounts of NKT cells and γδ T cells are also selected. Finally, very few innate $CD8^+$ T cells get selected. Decreasing signal strength through ablation of Itk [dash, dot, dot, dash window] cause cells with high signaling requirements for selection to not be selected. This allows other cells with lower signaling requirements, such as γδ and innate $CD8^+$ T cells in this model, to fill the void.

Figure 29


Selected Cells
Selection "Window" in WT T cells
Selection "Window" in Itk-/- T cells
Innate CD8+
γδ
Convent CD4+
NKT
Convent CD8+
Signal Strength

Another issue not addressed by a simple signal strength model is the role of other receptors in boosting signals downstream of the TCR. Case in point, both CD4 and CD8 coreceptors have been shown to boost TCR signals by recruiting Lck to the TCR complex (244, 245). In fact, the CD4 receptor can recruit Lck better than the CD8 receptor, causing increased signaling in the $CD4^+$ T cells (246-249). This might explain why the conventional $CD4^+$ T cells are affected less than the $CD8^+$ T cells in the Itk deficient mice. Given that selection of some innate subsets is also dependent on SLAM family mediated signaling, then perhaps these receptors could also have a role in boosting the signals downstream of the TCR, albeit this has not been proved. It should be pointed out that SLAM family receptors, like 2B4 [CD244], have also been shown [in NK cells] to have a negative role in signaling leading to activation(250-252), indicating that perhaps SLAM family receptors are actually involved in dampening signals rather than boosting them. In this case, SLAM family signaling would theoretically lead to preventing certain T cell subsets [conventional CD8 T cells for instance], from undergoing a productive positive selection.

One final problem with the simplistic signal strength model is that it assumes that signaling in different T cell subsets utilizes the same molecules. As discussed in detail in the general intro, some innate T cell subsets, most notably NKT cells, have requirements for different signaling molecules. This makes it quite hard to compare signaling requirement s of conventional T cells

versus innate T cells. This model also assumes that absence of one set of T cells allows for expansion of another set of T cells, and does not take into account T cell lineage choice.

Lineage choice in innate versus conventional T cells

The argument that Itk and Rlk might have a role in lineage choice of innate versus conventional T cells, or lineage choice amongst different types of innate T cell subsets, is also a valid one. This idea, of course, does not exclude signal strength, as signal strength is bound to have an important role in this process. A lineage choice model would, however, include the notion of signal quality. In other words, it's not just how strong the signals are downstream of the TCR that are of consequence to the lineage outcome, but also the signaling molecules, presenting cells, and coreceptors involved that will determine the final lineage decision. To be perfectly clear, in this thesis signal strength refers to modulation of signals in a pathway where all the components are the same and are linearly ordered. Signal quality in this thesis refers to modulation of signals in pathways were the components vary.

Since lineage decision occurs early on during thymic T cell development, DN stage for γδ bifurcation and DP stage for other T cell bifurcations, it is

important to know if Tec family kinases are even expressed at these stages. Figure 30 shows that not only are Tec family kinases Itk and Rlk expressed at the DN and DP stages, but they are expressed at significant levels at the DN1 stage, preceding any lineage bifurcation. In fact, we have previously shown that absence of Itk has mild, yet noticeable, consequences on pre-TCR signaling (238). However exactly how Tec family kinases influence lineage decisions remains a mystery.

Out of all the subsets of cells discussed in this thesis, the role of Tec family kinases in $CD8^+$ T cell lineage decision seems the clearest. As shown in chapter II, Itk and Rlk are required for the selection of conventional $CD8^+$ T cells; their absence give rise to innate $CD8^+$ T cells (69). It seems apparent that $Itk^{-/-}$ $CD8^+$ T cells get selected on hematopoietic cells rather than on the thymic epithelium (71). As discussed in the previous section, this could be due to conventional $CD8^+$ T cells requiring more signal strength, and therefore they are just not selected, while the innate $CD8^+$ T cells, which are selected on the hematopoietic cells, require less signal strength and the gap generated by the lack of conventional $CD8^+$ T cells allows for their expansion. There are three problems with this line of reasoning. First, given how small the innate $CD8^+$ T cell population is usually, it seems more likely that another subset of T cells would be able to expand more efficiently. Second, selection of innate $CD8^+$ T

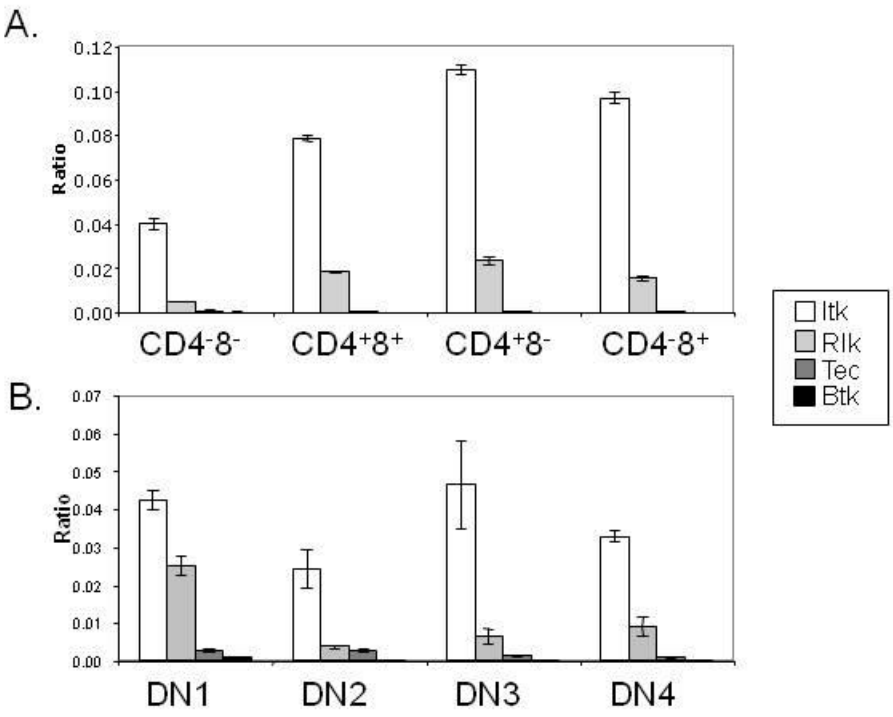
Figure 30: Expression of Tec family kinases in thymocyte subsets

[A] Thymocytes were isolated from WT mice and were sorted into DN, DP, and SP subsets based on expression of CD4 and CD8. Levels of Itk, Rlk, and Tec mRNA were determined by real-time quantitative PCR. Data are normalized to β-actin mRNA levels in each sample.

[B] Thymocytes isolated from WT mice were enriched for DN cells by complement depletion with anti-CD4 and anti-CD8 antibodies and sorted into DN1-DN4 subsets based on CD25 and CD44 expression. Levels of Itk, Rlk and Tec mRNA were determined by real-time quantitative PCR. Data shown are normalized to β-actin mRNA levels in each sample.

Results shown are representative of three independent experiments.

Figure 30


A.
Ratio
0.12
0.10
0.08
0.06
0.04
0.02
0.00
CD4-8-
CD4+8+
CD4+8-
CD4-8+
Itk
Rlk
Tec
Btk
B.
Ratio
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
DN1
DN2
DN3
DN4

cells is enhanced [both in percentages and numbers] in Itk$^{-/-}$H-2Kb$^{-/-}$H-2Db$^{-/-}$ mice when compared to H-2Kb$^{-/-}$H-2Db$^{-/-}$ mice, a system in which only innate and not conventional, CD8^{+} T cells can give rise (71, 73). Third, and most importantly, selection of the conventional CD8^{+} T cells is rescued in a large part [about half of them] by forcing selection on the thymic stroma or ablating expression of SAP, which is needed for selection of certain innate subsets like NKT cells (54-56, 73). If the decrease in conventional CD8^{+} T cells was governed alone by the decrease in signal strength in the Itk$^{-/-}$ mice, then absence of SAP shouldn't have any influence in the selection of these cells [i.e.: selection of conventional CD8^{+} T cells should still be poor in the Itk$^{-/-}$ SAP deficient mice].

The information just argued points to Itk having a qualitative role in the lineage choice of conventional CD8^{+} T cells rather than a quantitative role. Particularly, the dependence on SAP for generation of the innate CD8^{+} T cells in the Itk$^{-/-}$ mice seems to indicate that Itk might have a negative role in the SLAM family signaling pathway in early CD8^{+} T cells, preventing selection on hematopoietic cells and promoting it in thymic epithelial cells. Alternatively, Itk could be critical in dampening SLAM family mediated signals, which are geared to prevent positive selection of conventional CD8^{+} T cells. One big caveat in all of this is that SAP mediated signaling is needed for the generation of NKT cells, therefore if Itk is inhibitory to this pathway absence of Itk should promote expansion of NKT cells, but in the Itk$^{-/-}$ mice we see decreased numbers of NKT cells (174). More regarding the negative role for Tec family kinases will be

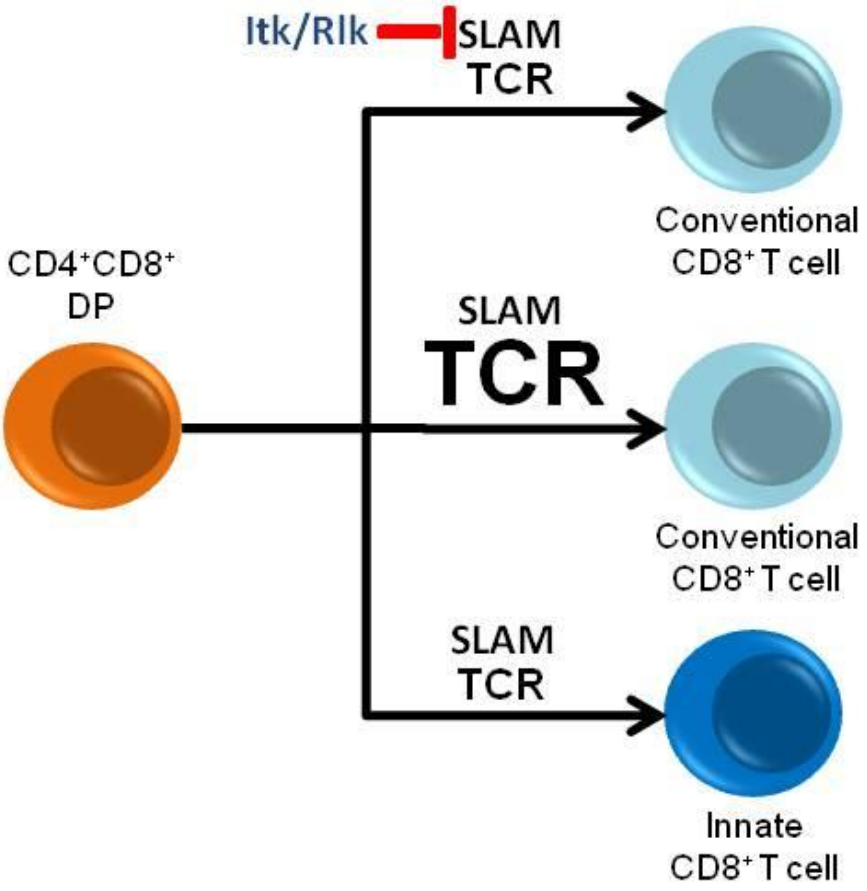
discussed in a later discussion section. It should however be pointed out that signal strength might also have some role in the generation of conventional $CD8^+$ T cells as boosting signaling through use of a transgenic TCR in these cells can rescue the conventional phenotype [as described by the OT-1 experiments in chapter II] (69). Another indication that signal strength has a role in conventional $CD8^+$ T cell development is drawn from crosses of the Itk deficient mice to mice containing a hyperactive Erk mutant (71). In this model system, where TCR signal strength is enhanced due to increased Erk activity, the conventional $CD8^+$ T cell phenotype is also rescued, albeit only partially. Most likely, the outcome of $CD8^+$ T cell lineage selection is dependent on both quality and quantity of signals [Figure 31]. Further work needs to be carried out in order to clarify this issue, and make these speculations more convincing.

A lot less is known about the role of Itk in γδ and NKT lineage decision. One should point out that its mere conjecture that there is a lineage decision between these two subsets, and very little data exists on the subject. It seems clear that selection of the NKT subset is impaired in $Itk^{-/-}$ mice as the proportion of NKT cells in the thymus is decreased from about 1% to 0.4% (174). There is also a very large decrease in the proportion of NK1.1 positive NKT cells. In contrast, selection of γδ T cells is promoted as proportions increase in the thymus from 0.3% to about 1-1.5% in the $Itk^{-/-}$ mice. Proportions of NK1.1 positive γδ T cells are also increased in the Itk deficient mice. Of interest too is that where in NK1.1 positive NKT cells Tec family kinases are slightly

Figure 31: Tec family kinase influence on $CD8^+$ T cell lineage decision

For the majority of $CD8^+$ T cells generated normally, Itk and Rlk might have a role in blocking signaling downstream of SLAM family receptors, inhibiting innate $CD8^+$ T cell selection and promoting conventional $CD8^+$ T cell development [top]. Absence of Itk and Rlk under normal signaling circumstances allows for signaling downstream SLAM receptors and selection of innate $CD8^+$ T cells [bottom]. SLAM family signaling in the absence of Itk can be thwarted by enhanced TCR signal strength, leading to the generation of conventional $CD8^+$ T cells [middle].

Figure 31


Itk/Rlk
SLAM
TCR
Conventional
CD8+ T cell
CD4+CD8+
DP
SLAM
TCR
Conventional
CD8+ T cell
SLAM
TCR
Innate
CD8+ T cell

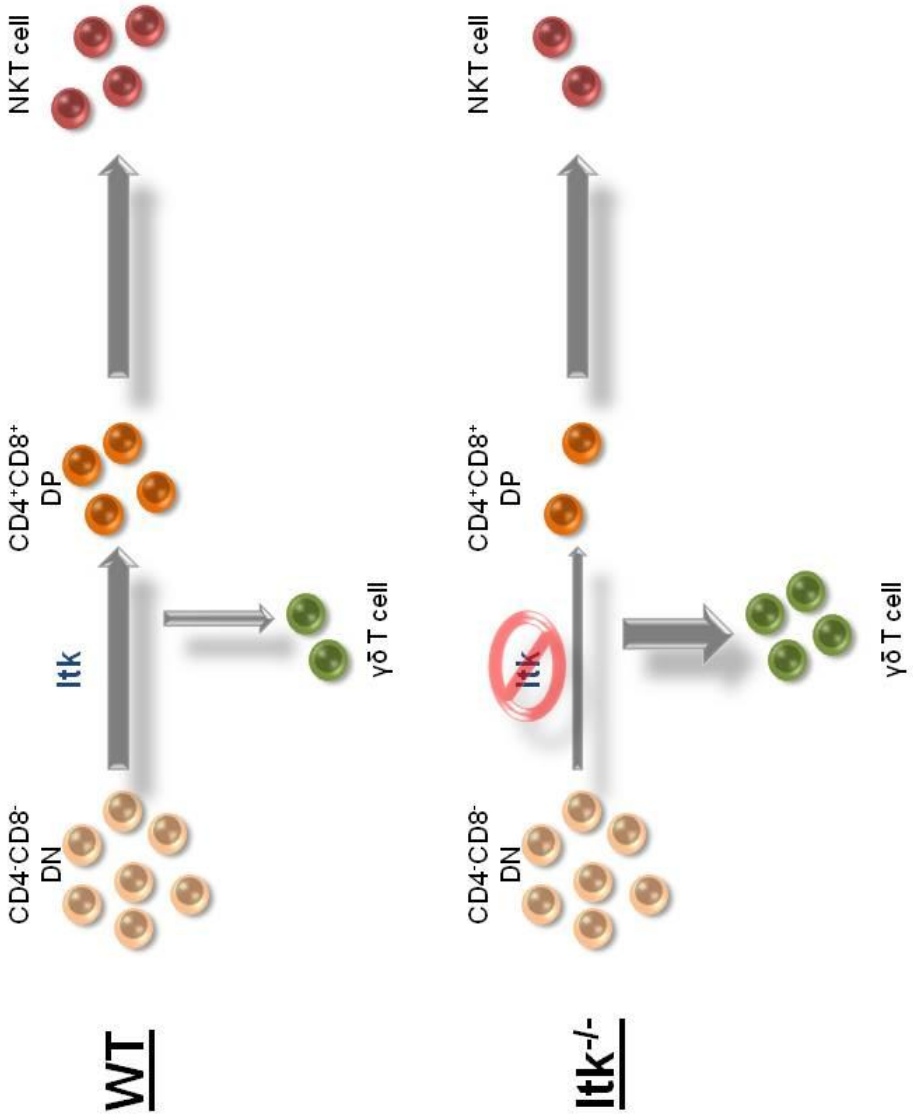
upregulated [Figure 15B], the opposite is true in NK1.1 positive γδ T cells [Figure 24A-C]. These opposing phenotypes prompt the question of whether absence of Itk causes a lineage diversion from NKT cells into γδ T cells. This notion is not entirely unheard of as one study showed that ablation of selection of NK1.1^{+} αβ T cells which produce IL-4 [in other words NKT cells] gives rise to an increased proportion of NK1.1^{+} γδ T cells (215). Another set of studies indicates that there could be a lineage decision between γδ T cells and another innate subset, NK cells (217). Finally, albeit somewhat controversial due to the stage where enhanced signaling occurs, a third set of studies has shown a role for TCR signal strength in lineage decision between γδ and αβ T cells (81). This data is at best speculative, but this information makes the possibility that Itk is influencing NKT versus γδ lineage decision somewhat plausible [Figure 32]. Present technology is not advanced enough to properly investigate this possibility, but some experiments are mentioned in the future directions section that might shed some light on the subject.

Figure 32: γδ versus NKT cell differentiation model

The model depicted here shows a pool of early DN thymocytes either destined to become a γδ T cell or a NKT cell depending on the signals they receive. When Itk is present [top], Itk mediated signals causes a lineage choice away from γδ T cells to DP thymocytes which eventually become NKT cells. This decision must occur early on during development before the transition to the DP stage.

Absence of Itk [bottom] allows for a lineage decision towards γδ T cells, decreasing the pool of cells that can eventually become NKT cells. This decision again occurs early on in development prior to DP transition.

Figure 32


WT
CD4-CD8-
DN
Itk
CD4+CD8+
DP
NKT cell
γδ T cell
Itk-/-
CD4-CD8-
DN
Itk
CD4+CD8+
DP
NKT cell
γδ T cell

NK1.1 expression on Itk$^{-/-}$ T cells

Expression of NK1.1 seems to correlate well with the effects of the absence of Itk on T cell subsets. Where presence of NK1.1 is enhanced on a number of Itk$^{-/-}$ innate CD8^{+} T cells and γδ T cells, which are developmentally favored in these mice, the majority of NKT cells in the Itk deficient mice lack NK1.1 expression, correlating with the developmental disadvantage seen in the NKT cells of these mice. It is not known exactly what is controlling NK1.1 expression in these subsets of cells and whether or not expression of NK1.1 has a role in their developmental outcome.

As discussed in chapter III, in NKT cells expression of NK1.1 is linked to terminal maturation of the cells and the upregulation of T-bet (68, 174). However, how T-bet influences NK1.1 upregulation or what influences decreased expression of T-bet in the Itk deficient NKT cells remains a mystery. Perhaps expression of T-bet is somewhat controlled by its phosphorylation by Itk or interaction with it (116), explaining the decreased levels seen in NKT cells and γδ T cells [Figure 20 and 24]. However this hypothesis is contradicted by the role Itk has in conventional differentiating CD4^{+} T cells in which absence of Itk seems to promote expression of T-bet (102).

What regulates expression of NK1.1 on γδ T cells and innate CD8^{+} T cells is much greater of a mystery. A previous study has identified regulatory

elements controlling expression of NK1.1 in different lineages, however it is not known how these elements are influenced (253). Another study utilizing fetal thymic and fetal liver cultures has shown that induction of NK1.1 might be predetermined by exposure to the thymic micro-environment (254). Given the differences in signaling caused by absence of Itk, it is entirely possible that the thymic microenvironment in Itk deficient mice is altered. More experiments must be carried out to verify this possibility.

A negative role for Itk in other innate subsets

Tec family kinases are typically thought to have a positive role in signaling (1, 98), granted the data utilized to make such a conclusion was derived from conventional T cell studies. Given the developmental advantages seen in the γδ T cells and innate $CD8^+$ T cells of Itk deficient mice, it is tempting to speculate that Tec family kinases might have a role in a negative signaling pathway, inhibiting development of these two subsets.

One subset of innate T cells where both positive and [especially] negative roles of Tec family kinases can be studied is mast cells. Unlike T cells or B cells, mast cells express Btk, Itk, Rlk, and Tec together (93-95) [and data not shown]. This prompts the question of whether or not all Tec family kinases share the

same role in this subset of cells. Btk seems to have a positive role in mast cell signaling, as $Btk^{-/-}$ mast cells have defects in effector function (255-258). A previous study seemed to indicate a positive role for Itk in mast cell signaling (259). However, more recent [not yet published] work from two postdocs in our lab [Yoko Kosaka and Markus Falk] seems to indicate that Itk has predominantly a negative role in mast cell signaling. Itk deficient mast cells show increased PLCγ activation and cytokine secretion capabilities. Of importance, Markus Falk has shown that Itk can associate with SHIP-1 and can phosphorylate SHIP-1, Dok-1, and Dok-2. Furthermore, he has demonstrated that Itk can co-localize with a negative signaling complex upon antigen mediated stimulation. Evidence also exists indicating that Btk might also perform a negative regulatory role, although in dendritic cells, indicating that the ability to function in negative regulatory pathways is not restricted to Itk alone (260).

Research I performed on NK cells, albeit of a more preliminary nature, seems to indicate that Tec family kinase Rlk is involved in a negative regulatory pathway in this innate subset. Expression of Rlk is much higher in NK cells than in αβ or γδ T cell subsets, but it still remains lower than expression of Itk [Figure 33]; albeit the difference is much smaller now [2.5 fold versus 10 fold]. It should be noted that in these experiments NK1.1 positive γδ T cells were not excluded, but given that they express even less Rlk than αβ T cells, this can only dilute out the total expression of Rlk in NK cells; in other words, if the NK1.1 positive γδ T cells were taken out of the equation chances are that expression of Rlk in NK

cells would even be higher. Phenotypic analysis of NK cells in $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice did not reveal any significant maturation defects in the periphery [data not shown], however activating and inhibitory NK cell receptor expression was altered particularly in the $Itk^{-/-}Rlk^{-/-}$ NK cells when compared to the WT or $Itk^{-/-}$ NK cells [Figure 34]. Of note expression of the activating receptor Ly49D was increased, while inhibitory receptors Ly49C/I and Ly49G2 were decreased. Again, this data should be taken with a grain of salt as $NK1.1^{+}$ γδ T cells are included in this analysis.

Functional in vitro assays utilizing platebound anti-NK1.1 to activate enriched NK cells showed increased IFNγ secretion on the cells from the $Rlk^{-/-}$ mice [data not shown]. To study *in vivo* function of NK cells in the different mice a previously described *in vivo* cytotoxicity assay was utilized (261, 262). In order to specifically look at NK cell killing, CD8 T cells or NK cells were depleted a day before the experiment to verify that the killing seen in this experiment was NK cells mediated. As it can be seen on Figure 35, $Rlk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ NK cells showed similarly high killing, when compared to the WT and the $Itk^{-/-}$ alone, of the alloreactive targets [Balb-c splenocytes labeled with a high dose of CFSE] while no self targets were lysed [B10 splenocytes labeled with a low dose of CFSE].

Figure 33: Tec family kinase expression in NK cells

NK cells were sorted as $TCR\beta^{-}NK1.1^{+}$ and $CD8^{+}$ T cells were sorted as $TCR\beta^{+}CD8^{+}CD4^{-}$ from splenocytes. Levels of Itk, Rlk, and Tec mRNA were determined by real-time quantitative PCR. Data are normalized to β-actin mRNA levels in each sample. Representative of two independent experiments.

Figure 33

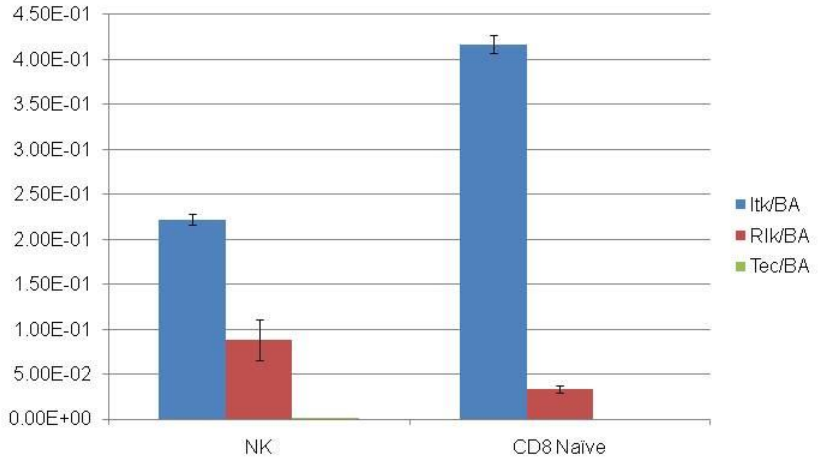
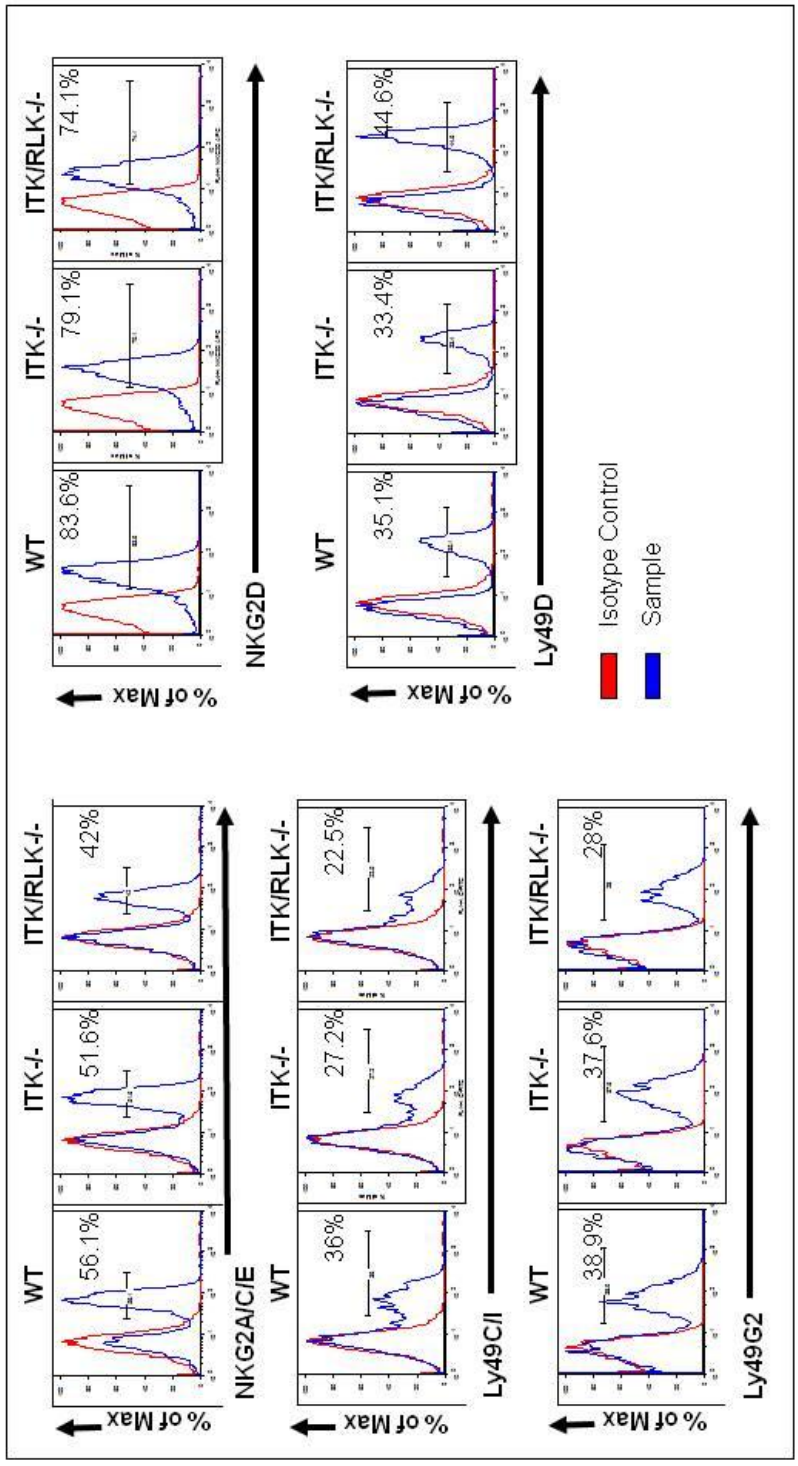

4.50E-01
4.00E-01
3.50E-01
3.00E-01
2.50E-01
2.00E-01
1.50E-01
1.00E-01
5.00E-02
0.00E+00
NK
CD8 Naïve
Itk/BA
Rlk/BA
Tec/BA

Figure 34: Expression of NK receptors on Tec family kinase deficient NK cells is altered

Splenocytes from WT, $Itk^{-/-}$, and $Itk^{-/-}Rlk^{-/-}$ mice were harvested, stained and gated on $TCR\beta^{-}NK1.1^{+}$ cells. Expression of several NK cell inhibitory [left column] and activating [right column] receptors was assessed on these cells. Red line represents the Isotype control while the blue represents the antibody mentioned in the X-axis. Representative of at least 4 different mice.

Figure 34


WT
ITK-/-
ITK/RLK-/-
56.1%
51.6%
42%
36%
27.2%
22.5%
38.9%
37.6%
28%
% of Max
NKG2A/C/E
Ly49C/I
Ly49G2
83.6%
79.1%
74.1%
35.1%
33.4%
44.6%
NKG2D
Ly49D
Isotype Control
Sample

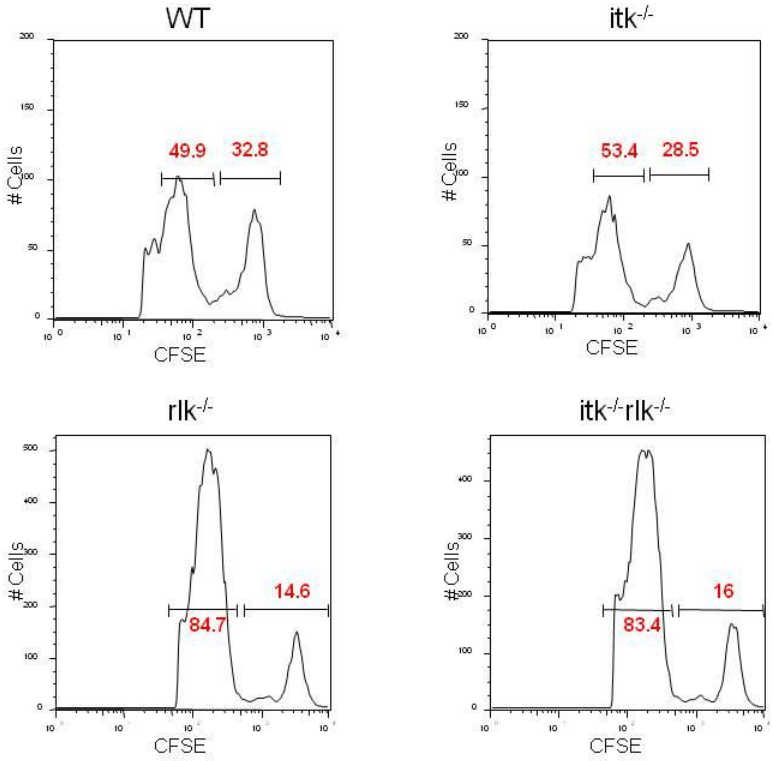
When quantitated and normalized to a double depletion of both NK and CD8 T cells, NK cells from the $Rlk^{-/-}$ and $Itk/Rlk^{-/-}$ mice lysed about 60-70 percent of the target population in the assay time, while WT cells lysed from 30-40% and $Itk^{-/-}$ cells lysed just a bit more. These results indicate to us that Rlk, and not Itk, has a negative role in NK cell signaling since its absence leads to increased cytokine secretion and lytic activity.

Interestingly the functional phenotype of the Rlk deficient mice matches well that of the EAT-2 or ERT deficient mice quite well (263). NK cells in EAT-2 and ERT deficient mice have increased potential for cytotoxicity and IFNγ secretion, much like that seen in the Rlk deficient mice. Both EAT-2 and ERT are members of the SAP family of adaptors, and in NK cells EAT-2 transduces signals downstream of 2B4, a SLAM family member (264, 265). Unlike SAP, however, EAT-2 and ERT are actually negative regulators of NK cell function. Of interest, negative activity of EAT-2 [and presumably ERT] requires tyrosine phosphorylation of its C-terminus. It is quite tempting to speculate, given the functional similarities in phenotype, that Rlk might be responsible for this phosphorylation event. This, along with the presumed negative role of Itk in SAP signaling for the development of conventional $CD8^{+}$ T cells [chapter II], would further link Tec family kinases in SLAM family mediated signaling.

Figure 35: NK cells from Rlk$^{-/-}$ mice lyse target cells more efficiently than those from WT or Itk$^{-/-}$ mice

The in vivo cytotoxicity assay was performed as previously described (261, 262). Briefly, spleens were harvested from B10 or Balb-c mice and single-cell suspensions were prepared. Splenocytes were incubated with either 2 µM [B10] or 0.4 µM [Balb-c] CFSE [Sigma] for 15 minutes at 37°C. Splenocytes were washed and the populations were combined at equal ratios. Cells [2 x 10^7] were adoptively transferred I.V. into WT, Itk$^{-/-}$, Rlk$^{-/-}$, or Itk$^{-/-}$Rlk$^{-/-}$ recipient mice. Spleens from recipient mice were harvested 20 hours later, and the survival of each transferred population was assessed by flow cytometry. To quantify alloreactive NK cell cytotoxicity in the absence of CD8$^+$ T cell killing, CD8$^+$ T cells were depleted 1 day prior to adoptive transfer of CFSE-labeled cells using anti-CD8 antibody [not shown]. To quantify alloreactive CD8$^+$ T-cell cytotoxicity in the absence of natural killer [NK] cell killing, NK cells were depleted 1 day prior to adoptive transfer of CFSE-labeled cells using anti-NK1.1 antibody [not shown]. At the time points shown, roughly about 95% of the killing seen is NK cell mediated [not shown]. Data is representative from 2 experiments with three mice each [only one experiment with the Rlk$^{-/-}$ mice].

Figure 35


WT
49.9
32.8
Cells
CFSE
itk-/-
53.4
28.5
Cells
CFSE
rlk-/-
14.6
84.7
Cells
CFSE
itk-/-rlk-/-
16
83.4
Cells
CFSE

Concluding remarks

The data presented in this thesis illustrates a substantial role for Tec family kinases in innate T cell development. In the case of $CD8^+$ T cells Tec family kinases Itk and Rlk are needed in order to maintain conventional development, as their absence ensues in an innate population of $CD8^+$ T cells. For NKT cells, Itk and Rlk are critical for terminal maturation of these cells, and seem to be quite important for an initial selection step. Finally, for γδ T cells Itk seems to be involved in maintaining the Th1 phenotype of these cells and keeping their development in check. Given all the data presented, it is quite tempting to speculate about a role for these kinases in signaling downstream of SLAM family receptors, and how that might affect lineage decision of different conventional as well as innate T cells subsets. Continuing these lines of research might help solidify instructive versus stochastic models in the field of innate T cell research.

Future directions

A great deal of work needs to be undertaken in order to describe a mechanism and solidify a role for Tec family kinases in innate T cell subsets. Where some of the projects described here are probably reaching the end of the rope, others will start to ask the most interesting questions.

Currently efforts are underway by a post-doc [Megan Enos] in the lab that could be utilized to look at lineage decision and generation of different innate T cell subsets. She is setting up an OP9-DL1 system to look at the role of Itk in CD8 lineage decision, albeit this system [as described later] could be utilized to study NKT cells and γδ T cells. Itk expression can be manipulated in these cultures in order to ascertain if indeed expression of Itk is necessary for conventional $CD8^+$ T cell lineage commitment. Furthermore the cultures could be used to study possible interactions between Itk and the SLAM-SAP-Fyn axis [through immuno precipitations [IP]]. To follow up on this work kinase assays [or insect cell expression assays] could be utilized to determine if Itk can phosphorylate members of this axis. Finally, although quite tough, FRET could be used to look at direct interactions between these molecules. This work could elucidate more conclusively if Itk is involved in dampening of the SLAM family axis in order to promote conventional T cell development.

Biochemical studies could help elucidate the role of Itk and Rlk in NKT cell development and function. In order to get around the cell numbers issue Itk deficient mice could be crossed to RAG deficient Vα14Jα18 transgenic mice. Activation assays could then help describe specifically the components of the signaling cascade that are particularly affected [PLCγ, ERK, JNK, Ca^{2+}, etc.]. Furthermore, NKT cells from Itk sufficient, RAG deficient, Vα14Jα18 transgenic mice could be used to look at interactions [through IPs] between Itk and Fyn [perhaps elucidating a role for Tec family kinases early on NKT cell development] or interactions between Itk and T-bet [to study the later role of Itk in NKT cell terminal maturation].

The OP9-DL1 system could also be utilized to look at the possibility of Itk having a role in a theoretical lineage decision between NKT cells and γδ T cells. By over-expressing or ablating expression of Itk in the bone marrow derived T cell progenitors one could study if expression levels shift the balance of lineage decision between γδ and NKT cells. It should be noted that the role of Itk in lineage decision might not just be restricted downstream of the TCR but could be involved downstream of a co-stimulatory pathway required for the selection of a specific subset [as implied with speculation of its role in SAP signaling]. What seems apparent is that if Itk is indeed involved in the lineage decision between γδ and NKT cells, its function has to be very early on in development [DN2-DN3 stage]. Research utilizing Itk deficient mice to look at γδ versus NKT cell lineage

decision could perhaps be used to shed some light on the complicated stochastic versus instructive models present in both γδ and NKT cell fields.

Use of bone-marrow chimeras will be crucial in future experiments in order to determine if the altered development of NKT and γδ T cells is caused by an altered thymic microenvironment in the Itk deficient mice, and whether or not this might also cause differences in NK1.1 expression. As discussed in chapter II, it seems clear that the altered development of $CD8^+$ T cells is cell intrinsic (71, 73), but to date we do not know for sure if this is the case for NKT cells and γδ T cells.

One interesting question which is yet to be answered is whether γδ T cells need SLAM-SAP-Fyn signaling for their development. Given the importance this signaling axis has on the development of innate $CD8^+$ T cells in the Itk deficient mice, it would be interesting to investigate if the increase in γδ T cells can also be ablated by knocking out SAP in these mice. If so, the OP9-DL1 system could be utilized to generate enough γδ T cells to look at interactions between Itk and members of this axis.

Other experiment s also need to be carried out to address the Th2 phenotype more clearly. For instance, we need to insure that the IL-4 produced by the γδ T cells is responsible for causing the class switching to IgE, as increases in IL-4 and IL-13 production are also seen in Itk deficient γδ T cells. Albeit not the most conclusive experiment, verification that there are no systemic

levels of IL-4 [by looking at the blood] could help infer that the γδ T cells are responsible for the phenotype [just by virtue of localization]. It would also be great to verify that the γδ T cells in the Itk deficient mice are indeed entering the germinal centers and interacting with B cells [confocal microscopy]. Confirmation of interaction between Itk and T-bet in this subset of cells would help sell the hypothesis that this is what is maintaining the Th1 phenotype in this type of cells. Albeit some of these experiments are not completely conclusive, and other experiments cannot be performed yet due to technological limitations, this future work will help describe the role of Tec family kinases in the development and function of innate T cells to a much greater extent.

Chapter VI: Materials and methods

Mice:

Itk$^{-/-}$ mice were generated previously in our lab (131) and were backcrossed >13 times to the C57BL/10 background. *Itk*$^{-/-}$*Rlk*$^{-/-}$ mice (139) were a kind gift of Dr. Pamela Schwartzberg [NIH], and were backcrossed >10 times to the C57BL/6 background**.** Rlk$^{-/-}$ mice were derived from Itk$^{-/-}$*Rlk*$^{-/-}$ mice. Wild-type littermates and non-littermate C57BL/10 mice were used as controls. *Itk*$^{-/-}$ mice were crossed to IL-15$^{-/-}$ [C57BL/6] mice obtained from Dr. Joonsoo Kang with permission from The Immunex Corporation. OT-1 TCR [C57BL/6] transgenic mice were obtained from Dr. Kenneth Rock with permission from Dr. Frank Carbone. CD45.1 congenic mice were purchased from Charles River Laboratories at NCI-Fredrick Animal Prodution Area [Fredrick, Maryland]. γTCR$^{-/-}$ mice on the B57BL/6 background were purchased from Jackson Laboratories. γTCR$^{-/-}$ mice were crossed to Itk$^{-/-}$ mice to obtain γTCR$^{-/-}$Itk$^{-/-}$ mice. Wildtype mice used in the studies were γTCR$^{+/-}$Itk$^{+/-}$ littermates or C57BL/10 mice purchased from Jackson Laboratories. All mice used were between 6-12 weeks of age, with the exception of the IgE and GC study in which the mice were 2-3 months of age [unless otherwise specified], and were maintained at the University of Massachusetts Medical Center animal facility under specific pathogen free [SPF] conditions after review and approval by the institutional animal care and use committee.

Antibodies, tetramer and flow cytometric analysis:

Thymocytes and peripheral lymphocytes were obtained and stained as described previously [Lucas, 2002 #162]. The following antibodies and secondary reagents were purchased from BD Pharmingen [San Diego, CA]: CD8α-FITC, CD8α-allophycocyanin, CD8α-PerCP-Cy5.5, CD4-PE, CD4-allophycocyanin, CD4-PerCP-Cy5.5, CD5- Chrome™, CD44-Cy-Chrome™, CD62L-PE, TCRβ-FITC, TCRβ-Cy- Chrome™, TCRβ-allophycocyanin, TCRβ-PE, TCRβ-biotin (188), CD122-PE, HSA-biotin, HSA-FITC, NK1.1-PE, NK1.1-allophycocyanin, NK1.1-PE-Cy7, CD3-biotin, CD8-Cy-Chrome™, CD8-allophycocyanin, IFNγ-PE, IFNγ-FITC, IFNγ- allophycocyanin, B220- allophycocyanin, Vβ2-PE, Vβ5-FITC, strepavidin-allophycocyanin, Bcl-2-FITC, CD69-FITC, CD1d-PE, SLAM-FITC, OX40-biotin, NKG2D-allophycocyanin, Ly49C/I-FITC, Ly49G2-FITC, Ly49-F-biotin, IL-4-PE, AnnexinV-FITC, and 7AAD. Anti-Bcl-xL-PE was purchased from Southern Biotechnology Associates [Birmingham, AL]. Anti-BrdU-FITC was purchased from BD Biosciences [Mountain View, CA]. ICOS-PE and CD40L-allophycocyanin were purchased from eBioscience. PNA-FITC was purchased from Vector Laboratories, Inc. Strep-Cascade Yellow was purchased from Invitrogen Molecular Probes. CFSE was purchased from Molecular Probes. CD1d-PBS44-PE Tetramer used for preliminary experiments was provided by Albert Bendelac [University of Chicago, Chicago, IL]. CD1d-PBS57-PE and CD1d-PBS57-allophycocyanin Tetramers were provided by the NIAID Tetramer

Facility [Atlanta, GA]. Antibody staining was analyzed using a FACSCalibur or LSRII [BD Biosciences] and data analyzed using both Cell Quest [BD Pharmingen, San Jose, CA] and Flowjo software [Treestar, Ashland, OR]. Thymocyte and splenocyte subsets were sorted on either a MoFlo [Cytomation] or a FACSVantage [BD Biosciences] cell sorter.

Ex vivo stimulation and intracellular staining for IFNγ and Bcl-xL:

Thymocytes were stimulated with PMA [5ng/ml] and ionomycin [50ng/ml] in media containing golgi-plug and golgi-stop [BD Pharmingen] for 5 h or with IL-15 [40ng/ml, R&D Systems, Minneapolis, MN] for 36 h at 37^{o}. Following stimulation, the cells were stained for surface antigens and then intracellulary for IFNγ or Bcl-xL using the intracellular stain Cytofix/Cytoperm kit protocol [BD Pharmingen].

NKT in vivo stimulation assays:

NKT cells were activated *in vivo* for cytokine analysis by ELISA as previously described (191). Briefly, wild type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice were injected i.v. with 1 µg anti-CD3 antibody [BD Pharmingen]. Spleens were harvested after 90 minutes and a suspension of 10^{7} cells was incubated at 37^{o}C for 2hrs, at which time supernatants were collected and IL-4 and IFNγ were measured by ELISA [BD Biosciences]. For intracellular cytokine staining, mice were injected with 2

μg α -Galactosylceramide [αGAL; Alexis Biochemicals [distributed by Axxora]] i.v. in 0.025% PBS Tween, and spleens were harvested after 2 and 3 hrs. Cells were then surface stained for either anti-NK1.1 and anti-TCRβ or anti-HSA and CD1d-tetramer, fixed and permeabilized using the Cytofix/Cytoperm Kit [BD Pharmingen], and stained for anti-IFNγ and anti-IL-4. Splenocytes were also activated with PMA [10 ng/ml [SIGMA Chemicals]] and Ionomycin [2,000 ng/ml [CalTech]] for one hour and incubated with Golgi-stop [BD Pharmingen] for three hours, after which they were stained for cytokines as described above.

γδ in vitro stimulation assays:

Wild-type and $Itk^{-/-}$ $TCR\gamma^{+}NK1.1^{+}$, $TCR\gamma^{+}NK1.1^{-}$, $TCR\gamma^{+}CD4^{+}$ and $TCR\gamma^{+}CD4^{-}$ subsets from were activated *in vitro* with 10 μg/ml of anti-TCRγ biotin [BD Pharmingen]. Cells were incubated at 37° for 72 hours, at which time supernatants were collected and IL-4, IL-10, IL-13 and IFNγ were measured by cytometric bead array [CBA [BD Pharmingen]]. Cells used for quantitative PCR were stimulated for 10 and 20 hours and examined for IL-4 and IFNγ message. For intracellular cytokine staining, cells from wild type and $Itk^{-/-}$ mice were stimulated and stained as previously described in the *in vivo* stimulations. In brief cells were stimulated with 10 ng/ml PMA [Sigma-Aldrich] and 2 μg/ml ionomycin [Calbiochem] for 1 hour and incubated with Golgi-stop [BD Pharmingen] for 3 hours. Cells were then surface stained for anti-TCRγ and anti-NK1.1, fixed, and permeabilized using a Cytofix/Cytoperm kit [BD Pharmingen]

and stained for IL-4 and IFNγ. For ICOS, CD40L and OX40 expression on γδ T cells, cells were stimulated for 24 hours with 10μg/ml anti-TCRγ. Cells were then surface stained with anti-ICOS, anti-CD40L and anti-OX40.

BrdU incorporation:

Mice were injected intraperitoneally with 2 mg of BrdU [Sigma Aldrich, St. Louis, MO] in PBS 12 h before harvest. Cells were plated at 6 x 10^6/well and then stained for surface antigens. Following extracellular staining the cells were washed in PBS and then fixed and permeabilized with Cytofix/Cytoperm for 20 min at 4^o C. The cells were then washed with PBS and fixed again in 1%/0.1% paraformaldehyde/Tween-20. To stain for BrdU, cells were spun down and washed twice in PBS at room temperature, and subsequently resuspended in 200μl of DNase solution [750μl 5M NaCl, 105μl 1M $MgCl_2$, 250μlμ1mM HCl, 24mg DNase [Roche Applied Sciences, Indianapolis, IN], 23.9ml dH_2O] and incubated at RT for 10 – 30 min. The cells were then washed and incubated in 50μl of a 1:10 dilution of anti-BrdU-FITC for 30 min. At the end of the incubation period, the cells were washed twice and analyzed by flow cytometry.

Adoptive Transfer of $CD8^+$ thymocytes:

Single cell suspensions were generated from thymi isolated from wild-type and *Itk*$^{-/-}$ mice. $CD4^+$ and DP thymocytes were depleted by incubating with a

complement-fixing anti-CD4 antibody followed by incubation with rabbit complement [Cedarlane Laboratories, Inc. Hornby, Ontario, Canada]**.** Live cells were isolated by Lympholyte®-M [Cedarlane Labs] gradient**,** stained for CD8 and CD44 expression and sorted into $CD8^{+}CD44^{lo}$ and $CD8^{+}CD44^{hi}$ subsets. These sorted populations were then adoptively transferred [$2x10^{6}$ wild-type and $Itk^{-/-}$ $CD8^{+}$ $CD44^{lo}$ thymocytes and $4x10^{6}$ $CD8^{+}$ $Itk^{-/-}$ $CD44^{hi}$ thymocytes] into separate wild-type CD45.1 congenic mice. The presence of transferred CD45.2 donor cells in spleen, lymph node, and thymus of host mice was examined from day 1.5 post-transfer to approximately 16 days post-transfer.

Bone marrow chimeras:

Bone marrow was isolated from femurs of CD45.2 wild-type, $Itk^{-/-}$, and $Itk^{-/-}Rlk^{-/-}$ mice and depleted for T cells using complement fixing anti-Thy1.2 antibody and rabbit complement. 1×10^{6} cells were injected into lethally irradiated congenic [CD45.1] mice. Mice were analyzed 8 – 12 weeks after reconstitution.

Real-time quantitative PCR:

Cells were prepared from thymi and spleens isolated from wild type, $Itk^{-/-}$ or $Itk^{-/-}$ $Rlk^{-/-}$ mice and lysed for RBCs. For the analysis on Chapter II, total RNA was prepared from $0.2\text{-}1.0x10^{6}$ sorted thymocytes as previously described (138). For

the analysis on Chapter III thymic NKT cells were first enriched by depleting with anti-CD8α-magnetic microbeads [Miltenyi BioTec, Auburn, CA] on an AutoMACS machine. Splenic NKT cells were first enriched by labeling with anti-B220-biotin and anti-CD8α-biotin [BD Pharmingen] and depleting with anti-biotin-magnetic microbeads [Miltenyi BioTec, Auburn, CA] on an AutoMACS machine. Remaining cells were then sorted to >95% purity on either a Moflo [Cytomation] or a FACSVantage [BD Biosciences] cell sorter by gating on HSA^{lo} CD1d/α-Galactosylceramide [αGAL] $tetramer^{+}$ cells. For controls $CD8^{+}CD4^{-}$ conventional T cells were sorted on Moflo cell sorter [Cytomation]. For chapter IV splenocytes and lymph nodes from 6-10 C57BL/6 or $Itk^{-/-}$ mice were sorted for NK1.1 and CD4 subsets based on expression. TCRγ subsets were first enriched by labeling with anti-TCRβ biotin and anti-CD19 biotin [BD Pharmingen] and depleted with anti-biotin magnetic microbeads [Miltenyi BioTec] on an AutoMACS machine. Remaining cells were stained with anti-TCRγ-FITC, anti-NK1.1-PE and anti-CD4-PerCP-Cy5.5 and sorted into $TCR\gamma^{+}NK1.1^{+}$, $TCR\gamma^{+}NK1.1^{-}$, $TCR\gamma^{+}CD4^{+}$ or $TCR\gamma^{+}CD4^{-}$ populations on a FACSVantage cell sorter [BD Biosciences]. Data shown in the discussion section [Chapter V] was prepared as follows. For isolation of subsets based on CD4 and CD8 expression, thymocytes were stained with anti-CD4-PE and anti-CD8-CyCychrome and sorted into DN, DP, $CD4^{+}$ SP, and $CD8^{+}$ SP populations. For DN1-DN4 subsets, thymocytes from twenty-five C57Bl/6 mice were pooled and depleted with complement-fixing anti-CD4 and anti-CD8 antibodies, followed by incubation with rabbit complement

[Cedarlane Laboratories, Inc. Hornby, Ontario, Canada]. Live cells were isolated on a Lympholyte®-M [Cedarlane Labs] gradient and sorted as described above into DN1-DN4 subsets on a high-speed MoFlo cell sorter [Cytomation, Fort Collins, CO]. In order to sort the DN thymocyte subsets, lineage-positive cells were stained with biotinylated Abs to CD3, CD4, CD8, B220, IgM, Ter119, Pan-NK[DX5], Gr1[Ly6G], Mac1[CD11b], and CD11c, followed by staining with anti-CD25, anti-CD44, and strep- allophycocyanin. All strep- allophycocyanin $^{+}$ events were gated out and the CD25 vs. CD44 profiles of the remaining cells were determined. RNA and cDNA were prepared as described previously (138). Real-time quantitative PCR was performed on an i-Cycler [Bio-Rad]. Primers sequences were as follows, β-actin sense 5'- CGA GGC CCA GAG CAA GAG AG -3', antisense 5'-CGG TTG GCC TTA GGG TTC AG -3'; eomesodermin sense 5'- TGA ATG AAC CTT CCA AGA CTC AGA -3', antisense 5'- GGC TTG AGG CAA AGT GTT GAC A -3'; T-bet sense 5'- GGG CTG CGG AGA CAT GCT GA -3', antisense 5'– GGC TCG GGA TAG AAG AAA CG -3'; GATA-3 sense 5'- GAA GGC AGG GAG TGT GTG AA -3', antisense 5'- TGT CCC TGC TCT CCT TGC TG -3'; IFNγ sense 5'- CCT GCA GAG CCA GAT TAT CTC -3', antisense 5'- CCT TTT TCG CCT TGC TGT TGC -3'; IL-4 sense 5'- CGA AGA ACA CCA CAG AGA GTG AGC T -3', antisense 5'- GAC TCA TTC ATG GTG CAG CTT ATC G -3'; Itk sense 5'- CTC CGC TAT CCA GTT TGC TCC -3', antisense 5'- GTC CTT GTT GAG CCA GTA GCC -3'; Rlk sense 5'- TCA ATC CAA CAG AGG CGG G -3', antisense 5'- CCG CTC TCT TCA GTG CCA A -3'; and Tec sense 5'-

GTT TGG AGT GGT GAG GCT T -3', antisense 5'- GGT AAC GAT GTA GAT GGG C -3'. For the generation of standard curves, cDNA clones of each gene mentioned above were used.

In vitro survival and proliferation assay:

Cell preparations were made from spleens of wild type, $Itk^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ mice. Cells were then labeled with CFSE [5- [and 6-] carboxyfluorescein diacetate succininyl ester], then 5 X 10^5 cells were plated per well in quadruplicate for each genotype and condition in a 96-well round bottom plate. Cells were then incubated at 37^{o}C in RPMI 10% FBS with: no stimulus [NS], IL-2 [20 ng/ml], IL-2 [2 µg/ml], IL-7 [10 ng/ml [R&D]], αGAL [500 ng/ml], IL-2 [20 ng/ml] plus αGAL [500 ng/ml], and IL-2 [2 µg/ml] plus αGAL [500 ng/ml]. At day 2 post incubation, medium was replaced with RPMI 10% FBS containing no additions [for the NS group and αGAL group], or the appropriate cytokine alone [without αGAL]. Cells were then harvested at day 4 and day 6, quadruplicate samples were pooled, and cells were stained for anti-HSA and CD1d-tetramer and analyzed by flow cytometry.

Serum analysis:

Blood was collected from wild type, γTCR$^{-/-}$, Itk$^{-/-}$, and γTCR$^{-/-}$Itk$^{-/-}$ mice. Serum was obtained by spinning the blood at 5,000 rpm for 5 min. and removing the supernatant. Supernatant was analyzed by ELISA for IgE.

Statistical analysis:

Two-tailed non-parametric Mann-Whitney Tests were performed using In-Stat software [GraphPad].

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