The Role of ITK in the Development of Gamma Delta NKT Cells: A Dissertation

THE ROLE OF ITK IN THE DEVELOPMENT OF GAMMA DELTA NKT CELLS

A Dissertation Presented

By

Catherine Chih-Tzu Yin

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Abstract

The immune system is a complex network of interacting cells and tissues that is designed to protect the body from pathogens and other foreign substances. T cells are a major component of the immune system and consist of two distinct lineages distinguished by the expression of $\alpha\beta$ or $\gamma\delta$ T cell receptors (TCR). The Tec family kinase, Itk is an important mediator of signaling downstream of the TCR. Past studies on Itk has focused on how Itk regulates development, activation and differentiation of conventional $\alpha\beta$ T cells and more recently how Itk regulates the development of innate-like $\alpha\beta$ T cells. However, very little is known about the influence of Itk on $\gamma\delta$ T cells. My studies show a previously unknown role for Itk in the development and function of $\gamma\delta$ T cells. We report in the absence of Itk, $\gamma\delta$ T cells were responsible for the spontaneously elevated levels of serum IgE and *Itk*$^{-/-}$ mice $\gamma\delta$ T cells produced high levels of T_H2 cytokines. Furthermore, there was an increase in $\gamma\delta$ T cells specifically in the $V\gamma1.1^+V\delta6.3^+$ (V6) subset that represents the dominant population of $\gamma\delta$ NKT cells in *Itk*$^{-/-}$ mice. In addition, the V6 subset had increased expression of PLZF, a transcription factor normally required for $\alpha\beta$ *i*NKT cell development. We further show that V6 cells develop and mature similar to $\alpha\beta$ *i*NKT cells. Similar to defects previously seen in the terminal differentiation of *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cell, V6 cells also had impaired maturation in the thymus in the absence of Itk. This data

demonstrates a previously unknown role of Itk for the terminal maturation of V6 cells that has been shown to be the cell population that led to spontaneous dermatitis in mice. Given that drug companies have targeted Itk as a potential allergy drug due to Itk's role in T_H2 development and function, our data suggests that further studies on Itk are warranted.

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Chapter I: Introduction

Immunoglobulin E-mediated diseases

The last four decades has seen an increase in the prevalence of immunoglobulin E (IgE)-mediated diseases including hyper IgE syndrome (HIES) and allergies in the western world. A similar increase in IgE-mediated diseases has also manifested in developing countries such as India and China. Found in most mammals and marsupials, IgE normally plays a role in the defense against multicellular parasites (1). However, fatal anaphylaxis or severe asthma can develop 5-10 minutes after exposure to allergens during an IgE-mediated response. This IgE mediated response is normally mounted when dendritic cells differentiate and promote the formation of allergen-specific T helper type 2 (T_H2) cells which produce the cytokines IL-4 and IL-13. These cytokines are necessary for B cells to class switch and become IgE-producing plasma cells (2). Secreted IgE is able to bind to the high affinity IgE receptor, FcεR1, on mast cells and eosinophils. Activation of these cells in response to specific allergens can result in systemic anaphylaxis (3). Therefore, It has been suggested that synthesizing drugs to regulate the development and/or function of T_H2 cells may be an attractive target for asthma (4). In addition, studies in mouse models lacking molecules critical for proper T cell function such as Itk (inducible T-cell kinase) and Itch mutants, have hyper IgE syndrome making them ideal models to study. Furthermore, a recent case study involving a patient with HIES demonstrated that another T cell subset, $\gamma\delta$ T cells, was able to generate high levels of IL-4 that contributed to elevated IgE in the serum (5). Various studies performed in mice have also indicated that γδ T cells are able to provide help to B cells that can lead to IgE class switching (6-8). Taken together this data indicates that T_H2 cells may not be the only T cell type able to promote B cell IgE class switching. The work presented here will focus on the development and function of γδ T cells with respect to the signaling molecule Itk.

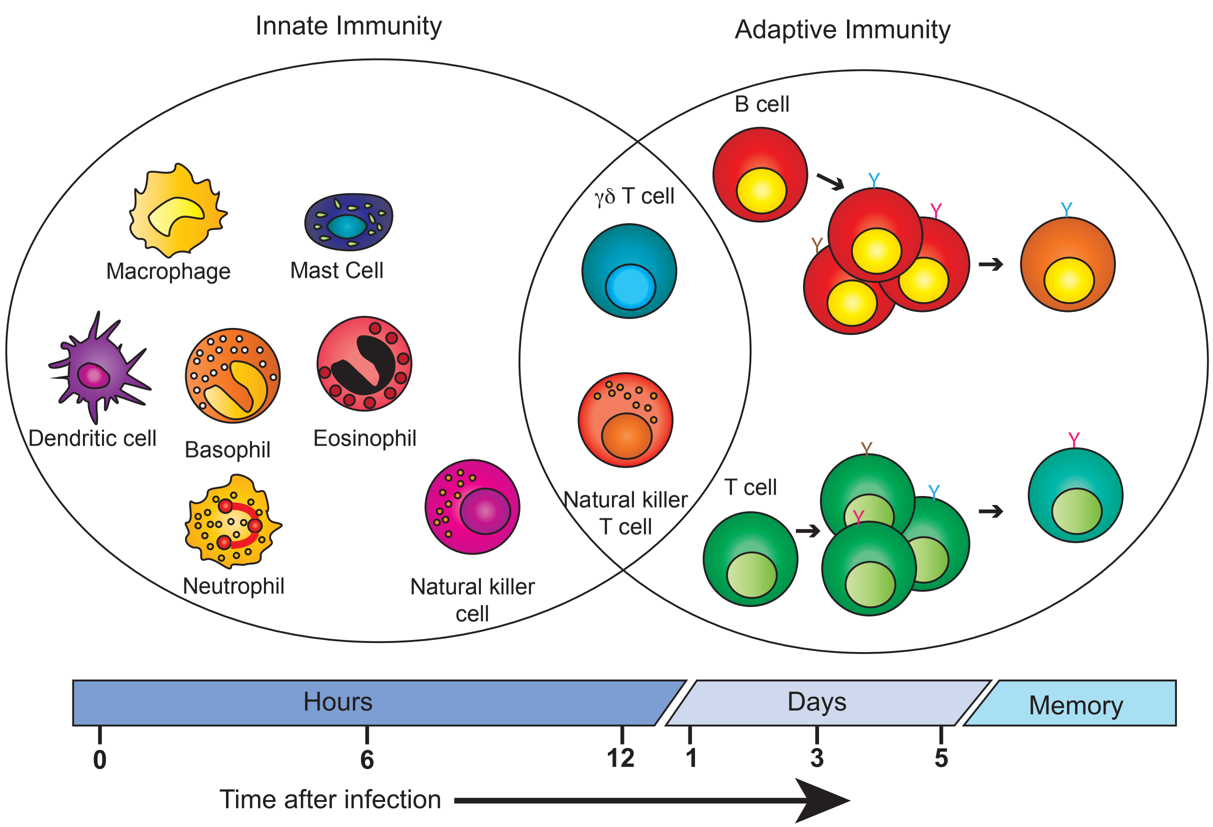
γδ T cells: interface between adaptive and innate immunity

γδ T cells are a subset of T cells constituting 1-5% of lymphocytes in the blood and peripheral organs yet they comprise over half of the lymphocytes found in the reproductive tract, skin and intestine. Within the epithelial tissues, γδ T cells are the first line of defense and can assist in wound repair (9). αβ T cells are another subset of T cells and are part of the adaptive immune system. Although T cells are commonly associated with the adaptive immune system, γδ T cells only share a handful of features with their αβ T cell counterparts (Figure 1.1). For example, cells that comprise the adaptive immune system are characterized by somatic rearranged receptors that require the enzyme recombination activating gene (RAG). RAG mediated recombination joins the variable (V) gene segments with joining (J) segments and diversity (D) segments thereby allowing for diverse receptors and recognition of very specific antigens for T cells. αβ T cells respond with delayed kinetics to pathogens and can form

Figure 1.1 Cells of the adaptive and innate immune system

The two categories of the immune system are the innate and adaptive immune system. Innate cells have germ line encoded receptors and respond within the first few hours upon infection. Adaptive cells have somatic rearranged receptors thereby producing few cells that can recognize specific pathogens during infection. During primary infection adaptive cells have a delayed response because only a few cells can recognize a specific pathogen and need to expand, but upon clearance of the infection adaptive cells can form memory. Therefore upon secondary infection with the same pathogen adaptive cells can respond much faster. Innate-like lymphocytes such as $\gamma\delta$ T cells and $\alpha\beta$ *i*NKT cells have characteristics of both innate and adaptive immune cells where they have an invariant receptor, respond to infection rapidly and have a memory phenotype.

Figure 1.1


Innate Immunity
Adaptive Immunity
Macrophage
Mast Cell
Dendritic cell
Basophil
Eosinophil
Neutrophil
Natural killer cell
γδ T cell
Natural killer T cell
B cell
T cell
Hours
Days
Memory
0
6
12
1
3
5
Time after infection

memory that allow for faster response kinetics upon secondary infection. However, not all T cells, such as αβ *i*NKT cells and γδ T cells fall into the adaptive branch of the immune system. Human γδ T cells are similar to $CD4^+$ T cells, another subset of T cells, in that they are able to up-regulate co-stimulatory molecules involved in B cell help, a characteristic of the adaptive immune response (10). Both αβ *i*NKT cells and γδ T cells have an invariant receptor that requires RAG mediated VDJ recombination but they cannot form memory, although they display an activated or memory-like phenotype meaning that they share features normally associated with memory or activated cells such as high expression of CD44. Unlike αβ T cells, αβ *i*NKT cells and γδ T cells respond rapidly to infection which is a characteristic of cells belonging to the second branch of the immune system, the innate immune system. In contrast to the adaptive immune system, cells belonging to the innate immune system have germ line encoded receptors and recognize microbes by conserved components. Cells of the innate system respond to a broad set of pathogens. In addition, innate immune cells cannot form memory and respond to primary and secondary infection with equally fast kinetics (11). γδ and αβ *i*NKT cells bridge the adaptive and innate immune system and plays an important role in providing a fast primary response to antigen specific pathogens and priming the adaptive immune system.

T cell development

All T cells develop from a common precursor during hematopoiesis before differentiating into distinct cell types. Thymocyte development can be tracked through stages by alterations in various cell surface markers such as the T cell receptor (TCR), CD4, CD8, CD25, CD44 and CD117 (c-kit) with each successive stage decreasing the potential to differentiate into other lineages. The model for T cell development begins with the thymic seeding progenitor (TSP), which are lymphoid committed (12), migrate to the thymus and become $CD4^-CD8^-$ double negative (DN) cells. This population can be further subdivided into four populations (DN1-4) based upon the surface expression of CD44 and CD25 (DN1: $CD44^+CD25^-$, DN2: $CD44^+CD25^+$, DN3: $CD44^-CD25^+$, DN4: $CD44^-CD25^-$) (13, 14). The DN populations can also be further subdivided based upon CD117 expression. DN cells then become $CD4^+CD8^+$ double positive (DP) cells, which are committed to the $\alpha\beta$ T cell lineage before further maturing into either $CD4^+$ single positive (SP) or $CD8^+$ SP $\alpha\beta$ T cells (15). During transition through the DN stages, rearrangement of TCRγ, TCRδ, and TCRβ gene is initiated at the DN2 stage (16, 17) and is completed at the DN3 stage (17, 18). Cells that express a functional pre-TCR, which consists of a correctly rearranged TCRβ chain and pre-Tα chain, transit to DN4 stage where the TCRα chain begins to rearrange. This process of selecting for a correctly rearranged TCRβ chain is called β-selection. Upon rearrangement of the TCRα chain during the DP stage $\alpha\beta$ T cells undergo positive selection where cells are selected by ligands presented by the major histocompatibility complex (MHC). Cells then transition to become SP cells (19-21) where negative selection occurs which deletes self-reactive αβ T cells (20). γδ T cells diverge from αβ T cells development during the DN stages though, the exact point where the separation occurs is unclear due to DN2-DN4 cells retaining the ability to develop into γδ T cells (22-24).

γδ T cell development

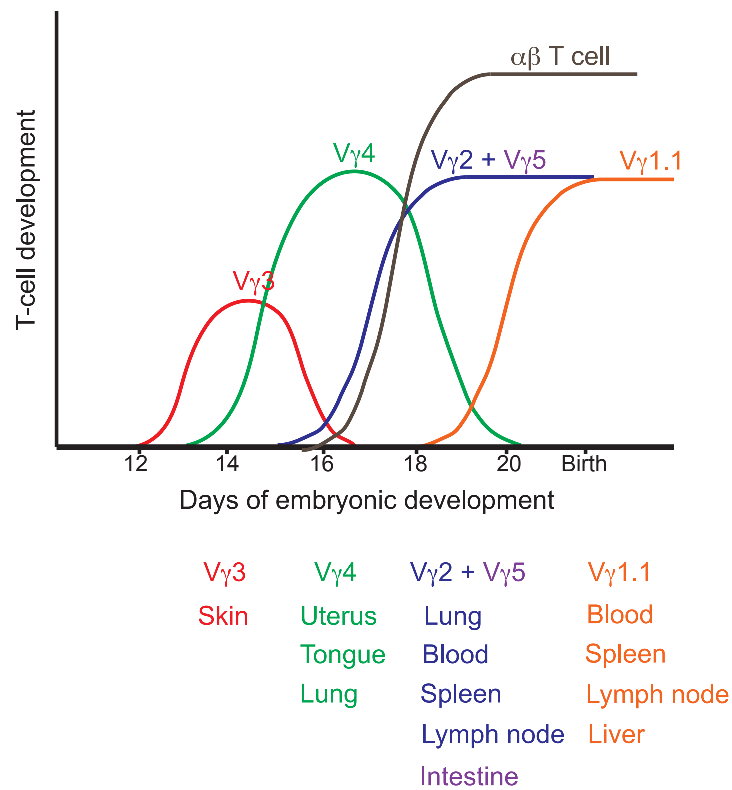
The mechanism of how γδ and αβ T cells diverge is unclear. Two models have been suggested to explain γδ and αβ T cell lineage commitment: the signal strength model and the stochastic model. The signal strength model proposes that the T cell receptor determines lineage fate. In this model, stronger signals are thought to favor γδ T cell development where weaker signals favor αβ T cell development (25-27). In contrast, the stochastic model proposes that the TCR enforces lineage decision but is not the main determinant. Instead, in this model commitment occurs prior to TCR expression and molecular programs decide γδ versus αβ lineage fate (23, 24, 28). The main difficulty in resolving which model is correct is due to the lack of known selecting ligands for γδ T cells. Even though it is unclear the exact moment αβ and γδ T cell development diverges more is known about the generation of γδ T cell.

A single Vγ and Vδ gene encodes the receptor repertoires of γδ T cells. Murine γ genes are located on chromosome 13 while the Vδ genes comprising of the constant (C)δ, Jδ, and Dδ gene segments are located on chromosome 14 between the Vα and Jα gene segments. The Vγ genes are often organized as clusters with the Cγ genes where Vγ5,Vγ2,Vγ4,Vγ3/Jγ1Cγ1 comprise one cluster while Vγ1.1/Jγ4Cγ4 is another cluster (29). γδ T cells begin to develop during embryonic day 14 (E14), which is three days before αβ T cells begin to develop. Certain γδ subsets encoded by specific Vγ gene segments are exported from the fetal and adult thymus at defined periods as shown in figure 1.2 (30). At E14 through E17, $V\gamma3^+$ cells are the first lymphocytes to develop. After E17, development of $V\gamma3^+$ cells is completed and can no longer be found in the fetal or adult thymus but can only be found in the skin. Following the development of $V\gamma3^+$ T cells, $V\gamma4^+$ T cells develop around E14 through E20 and home to the uterus, lung and tongue. $V\gamma2^+$ and $V\gamma5^+$ cells arise around E16 and migrate to the lungs and gut, respectively. Interestingly a small population of $V\gamma5^+$ cells are thought to also develop extra thymically (31) and they consist of half of the cells in the gut (32). Finally $V\gamma1.1^+$ cells begin to develop near the end of gestation at E18 and continue to develop in the adult thymus along with the $V\gamma2^+$ and $V\gamma5^+$ cells. $V\gamma1.1^+$ cells preferentially home to the liver, but can also be found in the spleen and lymph nodes (33-35). γδ T cells play a diverse role in the immune system including wound healing, tumor surveillance along with contributing

Figure 1.2 Generation of $\alpha\beta$ and $\gamma\delta$ T cells

Representation of the generation of $\gamma\delta$ T cells and $\alpha\beta$ T cells in the thymus. $\gamma\delta$ T cell receptors are encoded by specific Vγ- gene segments and leave the thymus in waves. As illustrated, $\gamma\delta$ T cells are the first lymphocytes to develop in the thymus around embryonic day 14 where as $\alpha\beta$ T cells do not begin to develop until around embryonic day 17. Each $\gamma\delta$ T cell subset preferentially migrates to specific organs upon leaving the thymus as indicated underneath the graph.

Figure 1.2


αβ T cell
Vγ4
Vγ2 + Vγ5
Vγ1.1
Vγ3
T-cell development
12
14
16
18
20
Birth
Days of embryonic development
Vγ3
Skin
Vγ4
Uterus
Tongue
Lung
Vγ2 + Vγ5
Lung
Blood
Spleen
Lymph node
Intestine
Vγ1.1
Blood
Spleen
Lymph node
Liver

to clearance of infections from viral, bacterial and fungal infections (36-39).

Heterogeneity in selection, function and surface expression within Vγ subsets

Unlike αβ T cells, which require ligand dependent signaling through MHC complexes (40) few ligands have been discovered for γδ T cells and it is unclear if ligands are required for γδ T cell development. The few selecting ligands that are known for γδ T cells include the γδ T cell clones KN6 and G8 which are recognized by the nonclassical MHC class 1b molecules T10 and T22 (41-43). T10/T22 specific γδ T cells, consists of 0.1%-1% of the total γδ T cell population (44) and express different Vγ chains (42) Many studies use KN6 γδ T cell transgenic mice to examine γδ T cell lineage fate (26, 44) but several caveats to this model system exist including T10/T22 specific γδ T cells consisting of only a minor portion of γδ T cells and therefore may not be representative of the entire population. In addition, one study demonstrated with the T10/T22 model that the function depended on if cells were selected. This study showed that γδ cells that were selected by the nonclassical MHC molecules T10/T22 were ligand-experienced and secreted IFNγ and γδ cells that were not selected were ligand-naïve and secreted IL-17 (44). Another population of γδ T cells that is known to require ligand selection are the dendritic epidermal T cells (DETC), which express the invariant $V\gamma3V\delta1$ (V3) receptor (45, 46). Recent studies have shown that V3 cells require the immunoglobulin-like protein Skint1, which is expressed on thymic epithelial cells and in the skin, for selection (47, 48). Overall, it seems that different subsets of $\gamma\delta$ T cells may have different selection requirements that may or may not require ligand for selection. In addition to certain subsets of $\gamma\delta$ T cells requiring ligand for selection, there also seems to be a substantial amount of heterogeneity in $\gamma\delta$ T cell function.

$\gamma\delta$ T cell function can be divided into subsets based upon cytokine secretion and this tends to correlate with the expression of the TCR. As demonstrated by Narayan *et. al.,* the $V\gamma1.1^{+}V\delta6.3^{-}$ (V1), $V\gamma1.1^{+}V\delta6.3^{+}$ (V6) and $V\gamma2^{+}$ (V2) population are three distinct subsets based upon high transcriptome analysis (49). V1 and V6 cells predominantly secrete IFNγ and IL-4 while V2 cells can secrete both IFNγ and IL-17 (50-52). Aside from cytokine production, transcription factors are also associated with $\gamma\delta$ T cell subsets based upon the types of cytokines that are secreted. For example, IL-17 producing $\gamma\delta$ T cells express the transcription factor Rorγt with most of the these cells belonging to the V2 subset (53). Classification of the $\gamma\delta$ T cell subsets based upon their TCR, cytokine profile and defining transcription factor is similar to how $\alpha\beta$ T cell subsets are defined. For example, $\alpha\beta$ $CD4^{+}$ T cells can differentiate into different T helper (T_H) subsets depending on the infection. Each subset is associated with a specific transcription factor and secretes specific cytokines. For example T_H1

cells produce IFNγ, T_H2 cells predominantly secrete IL-4 and IL-13 and T_H17 cells secrete IL-17 and IL-22. Furthermore, differentiation of each subset is regulated by a specific transcription factor. T_H1 cells require T-bet, T_H2 cells require GATA-3 and T_H17 cells require Rorγt (54). Upon differentiation of $CD4^+$ T cells into T_H cells, they can further the immune response by further activating other immune cells through the cytokines they secrete and cell-cell interactions.

For example, T_H2 cells upon differentiation secrete IL-4 and IL-13, which can affect IgG1 and IgE B cell isotype switching in germinal centers (GC) (55). Within the GC, B cell isotype-switching, somatic hypermutation and affinity maturation occurs. IgG1 and IgE are both considered T cell dependent B cell responses (56). In order for IgG1 and IgE isotype-swtiching to occur, several molecules have been identified to be important such as CD40 and ICOS, where lack of signaling downstream of these molecules in $CD4^+$ T cells result in defected GC formation (57). In addition to cell-cell interactions, the cytokines IL-4 and IL-13 are thought to enhance the gene transcription of the IgG1 and IgE constant regions (56, 58). Recent studies have begun to further elucidate $CD4^+$ T cell help for B cells and a new $CD4^+$ T cell subset has begun to emerge called the T follicular helper (T_{FH}) (59). The T_{FH} cells express the chemokine receptor CXCR5, localize to the B cell follicles, highly express CD40 and ICOS (59-62). It still remains unclear as to whether T_H2 and T_{FH} cells is more important in B cell class switching however, $CD4^+$ T cells are thought to be the main cell type responsible for T cell dependent B cell responses.

Unlike $\alpha\beta$ $CD4^+$ T cells, each $\gamma\delta$ T cell subset can be further subdivided based upon surface expression of other molecules. V2 cells that migrate to the skin secrete IL-17. Aside from the TCR receptor defining IL-17 secreting cells, they are also $CD44^+CD122^-$ and express the chemokine receptor CCR6 and scavenger receptor SCART2 (63, 64). In contrast, IFNγ secreting $\gamma\delta$ T cells are not $CCR6^+$, but instead express NK1.1 and CD122 (44, 64). These data taken together demonstrates that even within specific Vγ subsets there is quite a bit of heterogeneity in selection and development. Therefore, future studies can no longer examine $\gamma\delta$ T cells as a single entity.

Vγ1.1Vδ6.3 $\gamma\delta$ T cell subset generation and function

The focus of this thesis will be on the V6 cell subset of $\gamma\delta$ T cells. Originally defined as $Thy1.1^{dull}$ cells, V6 cells can simultaneously secrete IL-4 and IFNγ and reside in the adult thymus, spleen and liver, though they are most abundant in the liver (65-69) and are not dependent upon MHC class I or II for development (65). V6 cells express the activation marker CD44 and half the population expresses CD4 and NK1.1 (66). These cells are rare in newborn mice and expand during the first 2 weeks of postnatal life before reaching homeostasis 3 weeks after birth (66). Adult V6 cells have limited junctional sequence diversity within the Vδ6Cδ chains characterized by minimal to no N region diversity along with almost an invariable Dδ2-Jδ1 junction. These features are a characteristic of TCR rearrangements normally found in the fetal thymus (66, 67, 69, 70) due to the absence of terminal deoxytnucleotidyl transferase (TdT). TdT adds N-nucleotides during TCR recombination and is not expressed until three to five days after birth (71-75). Subsequent studies demonstrated that when adult bone marrow was grafted into lethally irradiated mice, the V6 population did not fully develop. In contrast, transplantation of fetal thymi into syngenic hosts resulted in V6 cell development suggesting that a majority of V6 cells arise from fetal precursors (76).

Several molecules have been implicated in V6 cell development. This includes the adaptor protein signaling lymphocyte activation molecule associated protein (SAP) a protein consisting of a single Src homology-2 domain and a short C-terminus. SAP was first shown to play an important role in the immune system when mutations in SAP were found in cases of X-linked lymphoproliferative-1 (XLP) diseases (77). SAP is found down stream of signaling lymphocyte activation molecule (SLAM) receptors and binds to SLAM receptors through their immunoreceptor tyrosine based switch motif (ITSM), a signaling motif found in the cytoplasmic domain (77, 78). SAP has recently been found to not only regulate positive signals through SLAM but also negative signals as well (77, 79). Lack of SAP has been shown to negatively affect V6 cells (80) thereby suggesting that SAP may modulate the development of V6 cells.

Another molecule important for V6 cells is the transcription factor PLZF (promyelocytic leukemia zinc finger, *Zbtb*16) a member of the BTB/POZ-ZF (broad complex, trimtrack, bric-a-brac or poxvirus and zinc finger) family of transcription factors. PLZF is defined by the presence of BTB-POZ and zinc finger domains, which was thought to be expressed exclusively in αβ *i*NKT and mucosal associated invariant T (MAIT) cells, an innate-like cell found in the gut lamina propria (80-83). Although frequency, number and localization of V6 cells were not affected in *PLZF*$^{-/-}$ mice V6 cells were incapable of dual IL-4 and IFNγ secretion following stimulation (80, 83). This data indicates that PLZF is required for the innate-like phenotype of V6 cells. Another member of the BTB-POZ family member, ThPOK, was also found to be expressed in the V6 subset of γδ T cells (80, 84) and that as V6 cells mature, ThPOK expression increases. Lack of ThPOK was shown to result in a decrease in V6 cells whereas overexpression of ThPOK resulted in an increase of V6 cells (84). In addition, these studies showed that V6 cells highly expressing ThPOK were also PLZF^{+} and that in the absence of ThPOK, PLZF expression was reduced along with decreased IL-4 secretion (80). It is still unclear the exact role ThPOK has in V6 cells but it is possible that PLZF may have a role in regulating or modulating ThPOK.

Though lack of SAP, PLZF and ThPOK negatively affect V6 cells, lack of several other molecules has been shown to positively influence V6 cells. Inhibitor of DNA binding 3 (Id3) belongs to the extracellular signal related kinase (ERK), early growth response gene (Egr), Id3 signaling pathway and Id3 inhibits E protein activity during T cell development (85). Several groups have shown that lack of Id3 results in the expansion of the V6 population thereby suggesting that Id3 negatively regulates the development of V6 cells (86, 87) though the exact mechanism is unclear. The role of these cells needs to be further elucidated but one study demonstrated that V6 transgenic mice on a *RAG*$^{-/-}$ background developed spontaneous dermatitis in the tail. Closer examination of the tail in these mice showed massive infiltration by lymphocytes and granulocytes (83). This suggests that the role of V6 cells may include the recruitment of other lymphocytes.

In recent years, the discovery that SAP and PLZF are required for the maturation and function of V6 cells have sparked interest in this T cell subset. Interestingly, the localization and function of V6 cells resembles another innate-like lymphocyte, the αβ *i*NKT cells.

αβ iNKT cells

αβ *i*NKT cells are considered a classic example of an innate-like lymphocyte. They constitute less than 1% of mouse T lymphocytes but promote cell-mediated immunity to bacterial and viral pathogenesis (88, 89). αβ *i*NKT cells are primarily found in the thymus, spleen, bone marrow and liver where they preferentially home. A small population of αβ *i*NKT cells can also be found in the lymph nodes and intestines. The majority of mouse αβ *i*NKT cells express the invariant T cell receptor (TCR) Vα14-Jα18 with a limited Vβ pairing that are positively selected by CD1d recognizing lipid antigens (90). In addition, SLAM and its downstream adaptor molecule, SAP are required for $\alpha\beta$ *i*NKT cell development, which is exemplified by the lack of SAP leading to a decrease in $\alpha\beta$ *i*NKT cells (91, 92). The transcription factor PLZF, a member of the BTB/POZ-ZF family of transcription factors, is required for $\alpha\beta$ *i*NKT cell dual secretion IL-4 and IFNγ (81, 82). PLZF also allows $\alpha\beta$ *i*NKT cells to acquire a memory phenotype during development (93-97). Based upon mouse studies, four stages of $\alpha\beta$ *i*NKT cell development have been defined (Figure 1.3).

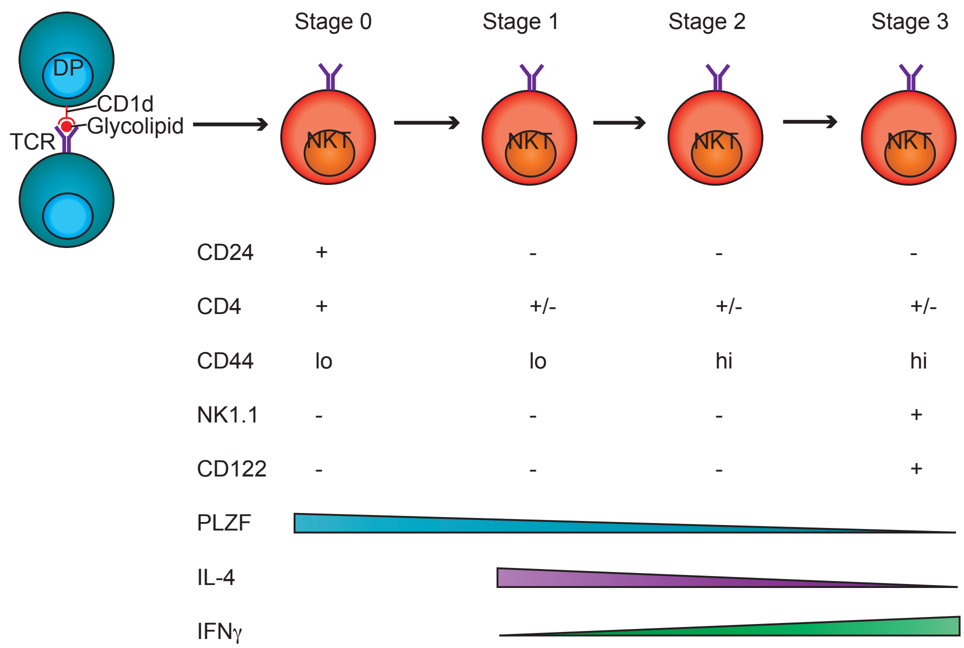
The earliest population of $\alpha\beta$ *i*NKT cells to emerge are termed stage 0 and are defined by expression of $CD24^{+}$ also known as heat stable antigen (HSA), $CD44^{lo}NK1.1^{lo}$. These cells all express CD4, are extremely rare and do not proliferate. As CD24 is down regulated and cells enter stage 1 ($CD24^{lo}CD44^{lo}NK1.1^{lo}$), they enter a highly proliferative phase thereby expanding the NKT cell pool. As $\alpha\beta$ *i*NKT cells progress to stage 2 they become $CD24^{lo}CD44^{hi}NK1.1^{lo}$. Stage 3 of $\alpha\beta$ *i*NKT cell maturation includes the up-regulation of NK cell receptors such as NK1.1 thereby becoming $CD24^{lo}CD44^{hi}NK1.1^{hi}$ and is accompanied by much less proliferation (98, 99).

Other markers can also be used to delineate the stages of $\alpha\beta$ *i*NKT cell maturation including the up regulation of the IL-2/IL-15 receptor β-chain (CD122) during stage 3 (93). Along with surface markers defining the maturation stages of $\alpha\beta$ *i*NKT cells, there are several other important changes in $\alpha\beta$ *i*NKT cell function that accompany this transition. Stage 1 and stage 2 $\alpha\beta$ *i*NKT cells

1.3 αβ *i*NKT cell development and maturation

αβ *i*NKT cells arise in the thymus from $CD4^{+}CD8^{+}$ double-positive (DP) thymocytes which have randomly rearranged their TCR. Thymocytes that are selected by CD1d bound to self-glycolipid expressed by other DP thymocytes become αβ *i*NKT precursors. αβ *i*NKT cell precursors undergo further differentiation characterized by the expression of cell surface markers (CD24, CD44, CD4, CD122 and NK1.1), changes in transcription factor (PLZF) expression and functional potential (IL-4 and IFNγ).

Figure 1.3


DP
CD1d
Glycolipid
TCR
Stage 0
Stage 1
Stage 2
Stage 3
NKT
NKT
NKT
NKT
CD24 + - - -
CD4 + +/- +/- +/-
CD44 lo lo hi hi
NK1.1 - - - +
CD122 - - - +
PLZF
IL-4
IFNγ

acquire the ability to secrete IL-4 and make little IFNγ, whereas stage 3 $\alpha\beta$ *i*NKT cells produce abundant IFNγ but little IL-4 (100, 101). Stages 0, 1 and 2 are thought to be thymus dependent but most $\alpha\beta$ *i*NKT cells emigrate from the thymus before stage 3 and maturation continues out in the periphery (102, 103).

Thus far the development and function of V6 and $\alpha\beta$ *i*NKT cells, two different innate-like lymphocyte populations, have been described. To better understand the molecular mechanisms of V6 cells, the T cell signaling pathway in $\gamma\delta$ T cells should be further examined.

TCR signaling

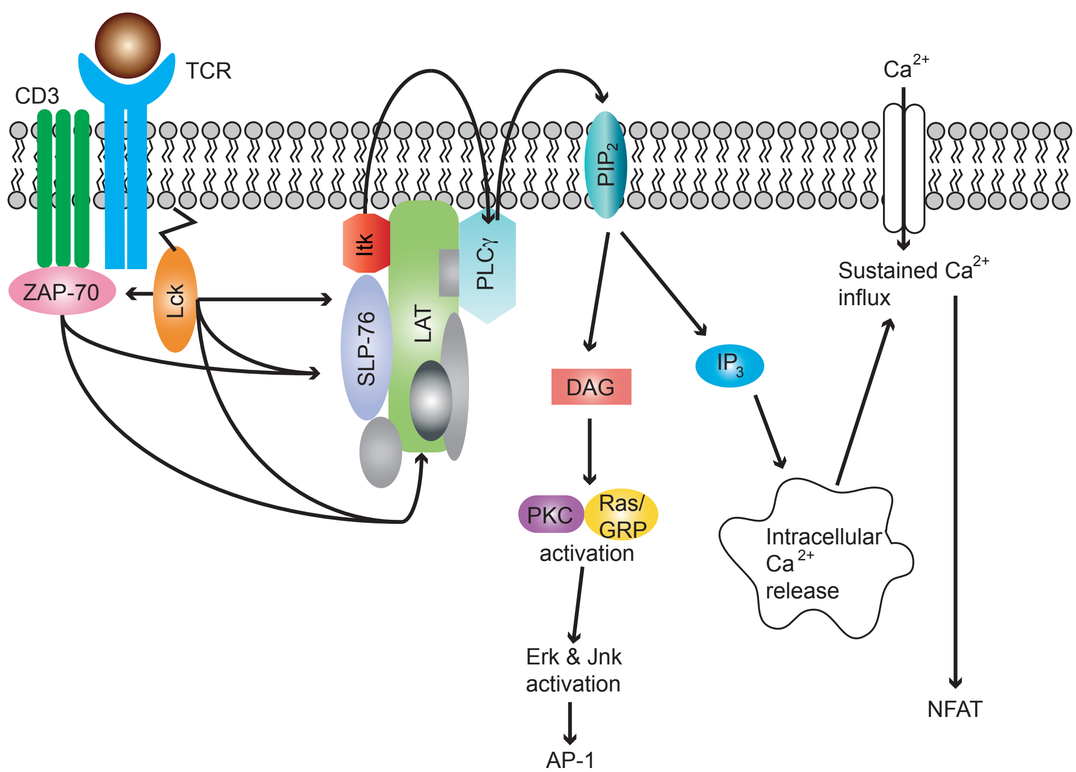
Activation of the $\alpha\beta$ T cell receptor (TCR) is initiated after TCR interaction with peptide-MHC complexes on antigen presenting cells (APC), leading to a cascade of signaling events. Following TCR stimulation, the Src-family kinase Lck is activated leading to the phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) on the intracellular domains of CD3 resulting in the recruitment of ZAP-70. ZAP-70 binds to the phosphorylated ITAMS and is phosphorylated by Lck, which goes on to phosphorylate the adaptors linker for activation of T cells (LAT) and SH2-domain-containing leukocyte protein of 76 kDa (SLP-76) (104-108). Activation and recruitment of the Tec family kinase Itk, to the membrane through its pleckstrin homology (PH) domain occurs following phosphoinositide-3-kinase (PI3K) activation and accumulation of phosphatidylinositol (3,4,5) triphosphate (PIP_3) in the plasma membrane (109-111). Phosphorylated LAT and SLP-76 serves as a platform for recruitment of Itk through Itk's Src homology (SH)-3 and SH2 domains (112-118) allowing for Lck phosphorylation of Itk on its activation loop (Y511) (119). Upon activation and recruitment of Itk to the membrane, Itk phosphorylates and activates phospholipase Cγ1 (PLCγ1)(19, 21) which hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PI_{(4,5)}P_2$) into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (120). Following cleavage of $PI_{(4,5)}P_2$, DAG activates members of the protein kinase C (PKC) family and Ras-GRP which leads to the activation of JUN amino-terminal kinase (JNK) and ERK1/2 thereby regulating the transcription factor activator protein-1 (AP-1) (121-124). In addition, ERK can also go on to activate the Id3 (125). IP_3 enables intracellular Ca^{2+} release and sustained calcium influx results in activation of downstream effectors including nuclear factor of activated T cells (NFAT) transcription factor (126, 127). TCR signaling results in transcription activation (126), cytokine release (128, 129) and actin reorganization (130) (Figure 1.4)

Deletion of the proximal components of TCR signaling such as Itk, have defects downstream of the TCR. *Itk*$^{-/-}$ T cells exhibit reduced but not absent responses to TCR stimulation. This includes defects in PLCγ phosphorylation, IP_3 production, Ca^{2+} influx, ERK and JNK activation along with decreased downstream activation of transcription factors, NFAT and AP1, and decreased cytokine production (121, 123, 129, 131, 132). This indicates that Itk plays an

Figure 1.4 T cell receptor signaling

Stimulation through the T cell receptor results in the recruitment and activation of both Lck and ZAP-70. Lck and ZAP-70 phosphorylates and activates Slp-76 and LAT resulting in the formation of a signaling complex which recruits Itk. Lck phosphorylates and activates Itk where Itk goes on to phosphorylate PLCγ. Activated PLCγ catalyzes PIP_2 to IP_3 and DAG. IP_3 induces Ca^{2+} mobilization and DAG induces mitogen-associated protein kinase (MAPK) activation resulting in the transcription of nuclear factor for activated T cells (NFAT) and activator protein-1 (AP-1), respectively.

Figure 1.4


TCR
CD3
Ca^{2+}
ZAP-70
Lck
Itk
SLP-76
LAT
PLCγ
PIP_2
Sustained Ca^{2+} influx
DAG
IP_3
PKC
Ras/ GRP
activation
Intracellular Ca^{2+} release
Erk & Jnk activation
AP-1
NFAT

important role in $\alpha\beta$ TCR signaling.

Tec family kinases

The Tec family kinases are a small group of nonreceptor tyrosine kinases consisting of Tec, Btk, Itk/Emt/Tsk, Rlk/Txk and Bmx/Etk. These kinases are primarily found in hematopoieic cells, including B cells (133-136), T cells (133, 137-141) and mast cells (142-144), although Tec and Bmx are also found in endothelial cells. Each cell subset may express more than one Tec kinase but may favor the use of one Tec kinase over the other. For example even though T cells can express Itk, Rlk and Tec, Itk is predominantly expressed. Similar to Src family tyrosine kinases, Tec kinases are comprised of an SH2 domain which recognizes phosphorylated tyrosines, an SH3 domain that binds proline rich motifs, and a C-terminal kinase domain that transfers phosphates onto tyrosine residues on substrate proteins (145). Unlike Src kinases, at the amino-terminal side of the SH3 domain, the Tec family kinase members, with the exception of Bmx, have a proline rich region (PRR). Within the amino-terminal end of the PRR in Itk, Btk and Tec, there is also a Zn^{2+}-binding region called the Btk homology (BH) motif. The Tec homology (TH) domain consists of both the BH domain and the PRR. With the exception of Rlk, all the Tec family kinases contain a pleckstrin homology (PH) domain that binds to phosphatidylinositol ligands in cell membranes. Rlk contains a cysteine-string motif at the amino-terminal region allowing Rlk to be constitutively localized at the cell membrane (Figure 1.5) (146) .

Tec Family Kinase, Itk

Tec kinases were first found to be relevant in humans in 1993 when mutations in Btk were found to play a role in immunodeficiences such as human B-cell immunodeficiency, X-linked agammaglobulinemia (XLA) and the murine mutant X-linked immunodeficiency (*xid*) (134-136, 147). The importance of Tec kinases in lymphocytes and antigen receptor signaling in humans was emphasized by XLA being the first example of a mutation in a tyrosine kinase found in a human immunodeficiency. In T cells, Itk, Rlk and Tec are the three Tec family kinases expressed with Itk having the predominant role in TCR signaling. Itk is expressed in mature T cells, mast cells and $\alpha\beta$ *i*NKT cells, with the maximal level of expression found in the mature adult thymus. These studies demonstrate that upon TCR stimulation and treatment with IL-2, Itk is induced (138, 140, 141, 148, 149) while Rlk mRNA and protein levels are reduced upon activation (139, 144). In addition, in mature resting T cells Rlk mRNA levels are already 3-10 fold lower compared to Itk mRNA levels (139, 144, 150, 151). Furthermore, Itk levels are up regulated 2-3 fold in T_H2 cells compared to T_H1 cells during naïve $CD4^+$ T cell helper differentiation (150, 151). These data suggests that Itk is the predominant Tec kinase in T cells and plays an important

Figure 1.5 Organization of the Tec family kinase domains

Tec family kinases are structurally similar to Src family kinases. Tec kinases include a catalytic C terminal kinase domain, followed by two Src homology (SH2 and SH3) domains. Btk and Tec contain 2 PRR regions, while Itk and Rlk only contain one. All Tec family kinases have a Btk homology domain (BH) or a N-terminal pleckstrin homology region (PH), except Rlk. Instead Rlk has N-terminal cysteine repeats.

Figure 1.5

role in T cell differentiation.

Until recently, mutations in Itk were not shown to be associated with diseases in humans. Itk was first associated with human diseases during examination of two patients who died after developing fatal Epstein Barr virus (EBV)-associated lymphoproliferative disorder. This disorder clinically resembled XLP, and the patients were found with homozygous missense mutations in the SH2 domain of Itk. This mutation in Itk led to protein destabilization, lack of Itk in lymph node biopsies along with an absence of NKT cells (152). Additional studies in patients with EBV-driven lymphoproliferative disease uncovered a range of mutations in Itk including missense, nonsense and indel mutations resulting in varying degrees of loss of function in Itk (153). In addition to loss of function mutations, altered expression of Itk has been found in pathological states such as atopic dermatitis. Analysis of genes in patients with atopic dermatitis, a T_H2-mediated disease, revealed elevated Itk expression in T cells (154). These studies indicate that Itk contributes to human diseases affecting T cell activation and cytokine production.

Itk regulation of T cell function

Itk deficiency in TCR signaling lead to defects in activation of downstream signaling components, such as PLCγ. Therefore, it was interesting that T cells were still able to develop in *Itk*$^{-/-}$ mice. However, differentiation of T cells, including $CD4^+$ $\alpha\beta$ T cells, was altered. Numerous studies have addressed T cell function *in vivo* during pathogen infections and found that lack of Itk affects T_H2 responses. The first study to demonstrate Itk's importance in T_H2 cells was performed in *Itk*$^{-/-}$ Balb/c mice. Infection with *Leishmania major* in *Itk*$^{-/-}$ Balb/c mice, which normally elicits a T_H2 response, showed that *Itk*$^{-/-}$ Balb/c mice were unable to mount a T_H2 response compared to wild type mice. Instead *Itk*$^{-/-}$ Balb/c mice generated a protective T_H1 response (131). Defects in T_H2 responses In *Itk*$^{-/-}$ mice were further confirmed with *in vivo* infections with *Nippostrongylus brasiliensis* (131) and *Schistosoma mansoni* (132). These two parasitic infections are normally eliminated by T_H2-dependent recruitment and activation of granulocytes, but closer analysis of cytokine responses of the parasite-specific T cells in *Itk*$^{-/-}$ mice revealed decreased IL-4, IL-5, IL-10 and IL-13 production (132). *In vitro* experiments with $CD4^+$ T cells from *Itk*$^{-/-}$ mice also demonstrated reduced levels of T_H2 cytokines under T_H2 polarizing conditions compared to wild type $CD4^+$ T cells further confirming the *in vivo* experiments (131, 132, 151). These experiments establish that in *Itk*$^{-/-}$ mice T_H2 cells are defective in IL-4 and IL-13 production and as mentioned previously, these cytokines are required for IgE class switching by B cells which can go on to further activate mast cells to release histamine (3) leading to allergies. Therefore, it is interesting that *Itk*$^{-/-}$ mice have elevated IgE levels in the serum (155).

In addition to the requirement for Itk in effector cytokine production for $CD4^+$ T_H2 cells, Itk is also required for $\alpha\beta$ *i*NKT cell maturation and effector cytokine production. Studies by numerous labs have shown that *Itk*$^{-/-}$ mice exhibit a reduction in the number of $\alpha\beta$ *i*NKT cells. Furthermore, the remaining $\alpha\beta$ *i*NKT cells display an immature phenotype, show poor response to TCR stimulation and produced little to no IL-4 and IFNγ following *in vivo* activation (156-158). Given the importance of Itk in the generation of T_H2 cytokines such as IL-4 and IL-13, Itk has become an attractive target for anti-asthmatic and allergy drugs. Yet, human studies have demonstrated that further research needs to be done to understand the effect of Itk in cells other then T_H2 cells. The experiments that follow in this thesis further examine the role of Itk on the innate like population $\gamma\delta$ T cells.

Chapter II: Tec kinase Itk in $\gamma\delta$ T cells is pivotal for controlling IgE production *in vivo*

Chapter II Attributions and Copyright information

The material in this chapter consists of a collaboration between Martin Felices [MF] and I. The material is derived from the PNAS publication (155). Specific contributions for each figure are as follows: figure 2.1 [CY and MF], figure 2.2 [MF], figure 2.3 [CY], figure 2.4 [CY and MF], figure 2.5 [CY], and figure 2.6 [CY and MF], figure 2.7 [CY], figure 2.8 [CY and MF], figure 2.9 [CY and MF], figure 2.10 [CY], figure 2.11 [MF].

Felices, M., C. C. Yin, Y. Kosaka, J. Kang, and L. J. Berg. 2009. Tec kinase Itk in gamma delta T cells is pivotal for controlling IgE production in vivo. *Proc. Natl. Acad. Sci. USA* 106: 8308–8313.

Introduction

The Tec family tyrosine kinase Itk is important for signaling downstream of the T cell receptor (146). In particular, Itk-deficient T cells have defects in PLC-γ phosphorylation, calcium mobilization, MAP kinase activation, and AP-1 and NFAT activation following TCR stimulation. Itk is also critical for conventional $\alpha\beta$ T cell development, selection, and function. Of particular importance, Itk signaling regulates $CD4^+$ T helper cell differentiation, playing a key role in the development of T_H2 responses (159). Based on this well-documented defect *of* *Itk*$^{-/-}$ mice in generating T_H2 effector responses and cytokine production, it was surprising to discover that these mice had spontaneously-elevated levels of serum IgE (4, 132), as B cell isotype switching to IgE is highly dependent on T_H2 cytokines IL-4 and IL-13 (2). As our previous studies had indicated that *Itk*$^{-/-}$ $\alpha\beta$ TCR^+ NKT cells (referred to as $\alpha\beta$ NKT cells) were also highly defective in producing effector cytokines such as IL-4 (158), we considered the possibility that $\gamma\delta$ TCR^+ NKT cells were the major source of T_H2 cytokines in *Itk*$^{-/-}$ mice.

$\gamma\delta$ T cells are a highly conserved subset of T cells that constitutes 1-5% of the lymphocytes in the blood and peripheral organs of mice, but can account for up to 50% of the lymphocytes in the mucosal epithelia. As with other subsets of 'innate' T cells, $\gamma\delta$ T cells express memory cell surface markers (160), and are capable of rapidly secreting effector cytokines (34). Among the many functions attributed to $\gamma\delta$ T cells, a great deal of recent interest has focused on their ability to modulate adaptive immune responses, specifically the humoral response (161).

A variety of studies have indicated that γδ T cells are able to provide help for B cell responses. Initial studies performed in mice lacking αβ T cells showed that B cell expansion, differentiation, and secretion of ‘T-dependent’ antibody isotypes, IgE and IgG_1, were all intact in these mice (7). Further, *TCRβ*$^{-/-}$ mice, challenged repeatedly with parasitic infections, could produce germinal centers and generate increased antibody production (6). Utilizing a model of pulmonary allergic inflammation, decreased production of IgE and IgG_1 was seen in mice lacking γδ T cells compared to wild type mice (162). γδ T cells have also been shown to directly induce germinal center formation and Ig hypermutation (163). Interestingly, even though the γδ T cells expressing CD4 account for only 5-10% of all γδ cells, it is this subset that appears to be responsible for inducing germinal centers (164). Human γδ T cells have also been found in germinal centers; these cells were found to up-regulate B-cell costimulatory molecules such as CD40L, OX40, CD70, and ICOS in response to TCR stimulation (10, 165). Together, these data indicate that γδ T cells can promote, either directly or indirectly, the humoral immune response.

Here we show that, in the absence of Itk, γδ T cell differentiation and effector function are dramatically altered. *Itk*$^{-/-}$ mice contain increased numbers of $CD4^+$ and $NK1.1^+$ γδ T cells that normally constitute the γδ NKT population

(166). These cells express PLZF, a transcription factor that uniquely defines $\alpha\beta$ NKT cells and is essential for normal $\alpha\beta$ NKT cell development (81, 82).   As a consequence, *Itk*$^{-/-}$ $\gamma\delta$ T cells produce robust amounts of T_H2 cytokines when stimulated, accompanied by enhanced expression of cell surface receptors associated with B cell help, such as ICOS and CD40L, and thus, promote a spontaneous elevation in serum IgE levels. These data indicate a surprising role for Itk in regulating the lineage development and effector function of $\gamma\delta$ T cells, particularly in controlling the PLZF$^+$ subset.

Results

$\gamma\delta$ T cells promote the hyper IgE and enriched GC phenotype seen in Itk$^{-/-}$ mice

In an effort to identify the cell type producing Th2 cytokines and driving IgE class-switching and secretion in unimmunized *Itk*$^{-/-}$ mice, we considered $\gamma\delta$ T cells. To test this possibility, *Itk*$^{-/-}$ mice were crossed to *Tcrd*$^{-/-}$ mice (167) that lack $\gamma\delta$ T cells. As shown in Figure 2.1A and reported previously (4, 132), *Itk*$^{-/-}$ mice have elevated concentrations of serum IgE compared to wild type controls. Strikingly, in *Itk/Tcrd*$^{-/-}$ double-deficient mice, serum IgE levels drop significantly compared to Itk$^{-/-}$ mice. Similar results were seen upon analysis of the proportion of germinal center phenotype B cells (B220$^+$PNA$^+$) (Figure 2.1B). Individual cohorts of mice were tested at 2 months of age, 3.5 months of age, and 5 months of age, with similar results (Figure 2.2 A and B).

Figure 2.1. $\gamma\delta$ T cells in *Itk*$^{-/-}$ mice are responsible for the spontaneous elevation in serum IgE levels.

(A) Serum obtained from wild type*, Tcrd*$^{-/-}$*, Itk*$^{-/-}$, and *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice were analyzed for IgE by ELISA. (*B)* Splenocytes from wild type, *Tcrd*$^{-/-}$, *Itk*$^{-/-}$, and *Itk*$^{-/-}$ *Tcrd*$^{-/-}$ mice were stained with α-B220 Ab and PNA to identify germinal center B cells. Differences between wild type and *Tcrd*$^{-/-}$ (p=0.14) and between *Itk*$^{-/-}$ and *Tcrd*$^{-/-}$ (p=0.54) were not statistically significant. (*C)* Cells were prepared from thymus, spleen, mesenteric lymph nodes (mLN), intestinal epithelium (iIELs), and liver and were stained with anti-TCRδ and anti-TCRβ Abs. (*D-F)* Percentages and absolute numbers of $\gamma\delta$ T cells were compiled for the thymus *(D)* and spleen *(E)*; percentages only were compiled for liver *(F)*.

Figure 2.1

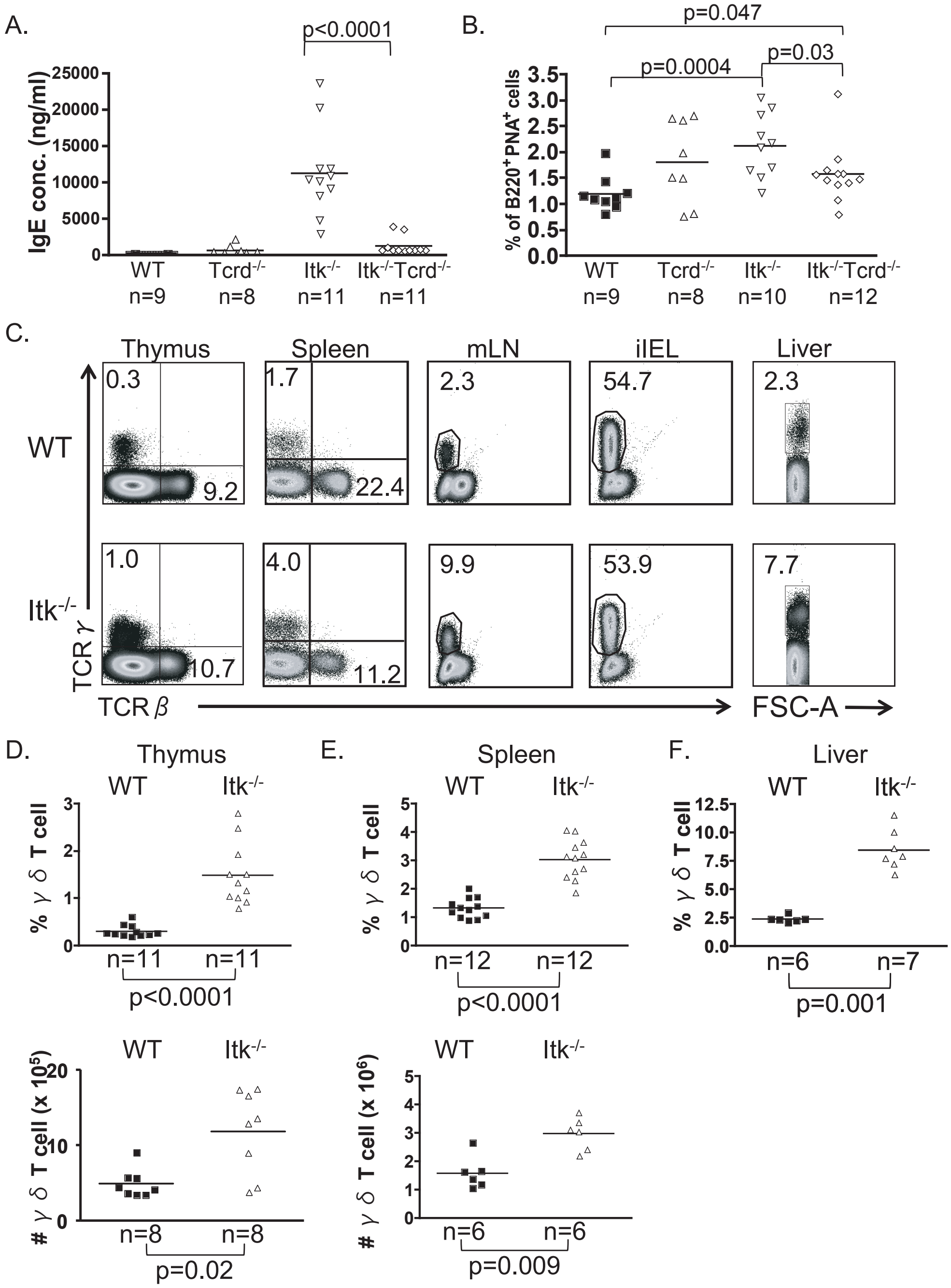
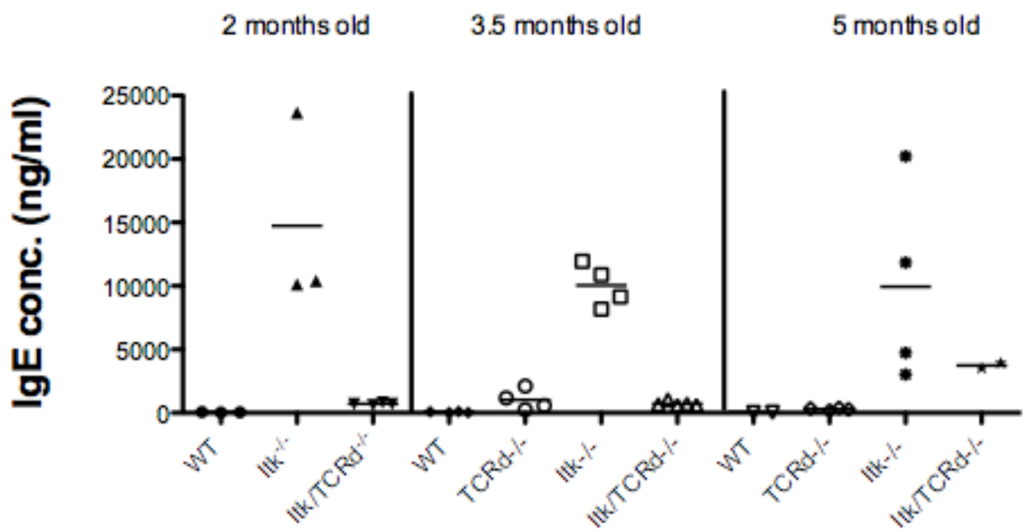

A.
p<0.0001
IgE conc. (ng/ml)
25000
20000
15000
10000
5000
0
WT n=9
Tcrd-/- n=8
Itk-/- n=11
Itk-/-Tcrd-/- n=11
B.
p=0.047
p=0.0004
p=0.03
% of B220+PNA+ cells
WT n=9
Tcrd-/- n=8
Itk-/- n=10
Itk-/-Tcrd-/- n=12
C.
Thymus
Spleen
mLN
iIEL
Liver
WT
Itk-/-
0.3
9.2
1.7
22.4
2.3
54.7
2.3
1.0
10.7
4.0
11.2
9.9
53.9
7.7
TCR γ
TCR β
FSC-A
D.
Thymus
% γ δ T cell
WT n=11
Itk-/- n=11
p<0.0001
E.
Spleen
WT n=12
Itk-/- n=12
p<0.0001
F.
Liver
WT n=6
Itk-/- n=7
p=0.001
# γ δ T cell (x 10^5)
n=8
n=8
p=0.02
# γ δ T cell (x 10^6)
n=6
n=6
p=0.009

Figure 2.2. Hyper IgE syndrome can be seen in *Itk*$^{-/-}$ mice as early as 2.5 months.

(A) Serum obtained from 2.5, 3 and 5 month old wild type*, Tcrd*$^{-/-}$*, Itk*$^{-/-}$, and *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice were analyzed for IgE by ELISA. (*B)* Splenocytes from 2.5, 3 and 5 month old wild type, *Tcrd*$^{-/-}$, *Itk*$^{-/-}$, and *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice were stained with α-B220 Ab and PNA to identify germinal center B cells.

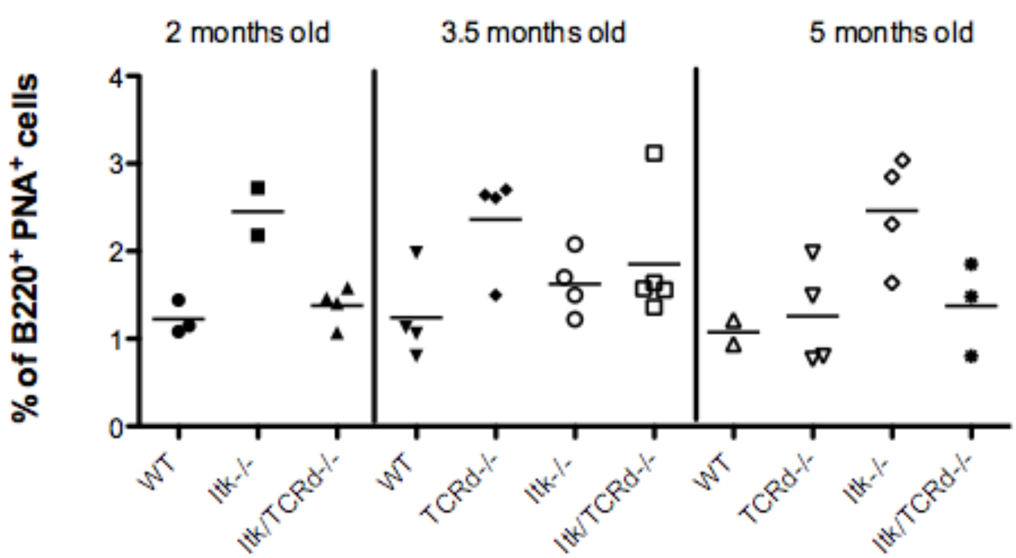
Figure 2.2

A



2 months old
3.5 months old
5 months old
IgE conc. (ng/ml)
25000
20000
15000
10000
5000
0
WT
Itk-/-
Itk/TCRd-/-
WT
TCRd-/-
Itk-/-
Itk/TCRd-/-
WT
TCRd-/-
Itk-/-
Itk/TCRd-/-

B



2 months old
3.5 months old
5 months old
% of B220+ PNA+ cells
4
3
2
1
0
WT
Itk-/-
Itk/TCRd-/-
WT
TCRd-/-
Itk-/-
Itk/TCRd-/-
WT
TCRd-/-
Itk-/-
Itk/TCRd-/-

Itk-deficient mice have increased numbers of γδ T cells

The finding that elevated serum IgE levels in *Itk*$^{-/-}$ mice were dependent on γδ T cells suggested that the γδ T cells present in *Itk*$^{-/-}$ mice might be altered relative to their wild type counterparts. We first examined γδ T cell numbers. As seen in Figure 2.1C, *Itk*$^{-/-}$ mice have increased proportions of γδ T cells in the thymus, the spleen, the mesenteric lymph nodes, and the liver, but not in the intestinal intraepithelial lymphocyte (iIEL) compartment. A summary of data indicates significant increases in the proportion and absolute numbers of γδ T cells in both the thymus and the spleen, as well as an increase in the percentage of γδ T cells in the liver, in the absence of Itk (Figure 2.1D-F).

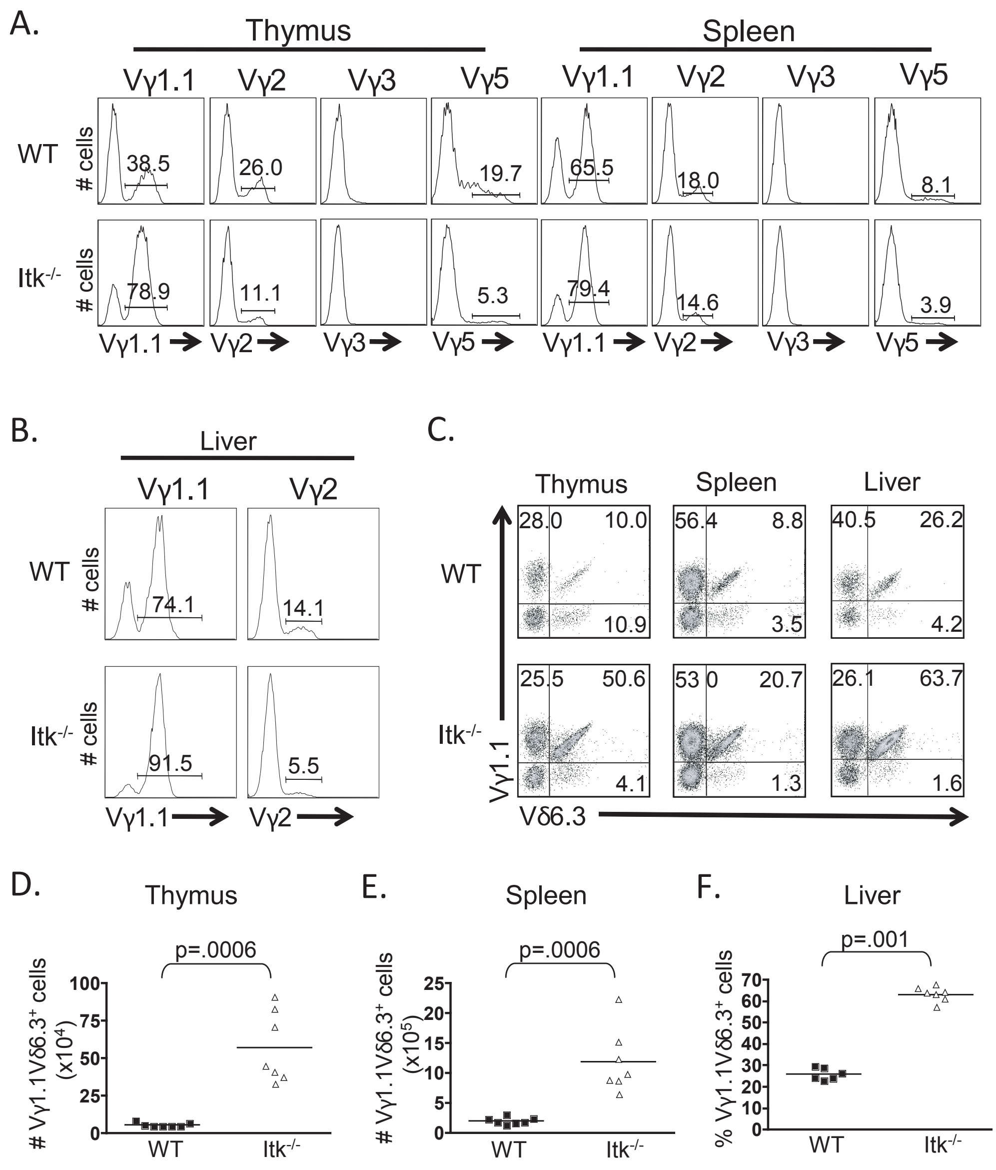
Itk$^{-/-}$ *mice contain increased proportions of γδ T cells subsets expressing Vγ1.1*$^{+}$*Vδ6.3*$^{+}$*, CD4 and NK1.1*

Analysis of TCRγ chain repertoires indicated an increased proportion in Vγ1.1^{+} γδ T cells in Itk-deficient versus wild type mice, and a concomitant decrease in the other major subsets (Vγ2^{+} and Vγ5^{+}) (Figure 2.3A, B). Further, the majority of Vγ1.1^{+} γδ T cells in the thymus and liver of *Itk*$^{-/-}$ mice also expressed Vδ6.3 (Figure 2.3C). Overall, we find a significant increase in the absolute numbers of this γδ T cell subset in thymus and spleen of *Itk*$^{-/-}$ mice compared to controls (Figure 2.3D, E), as well as an increased proportion of Vγ1.1^{+}Vδ6.3^{+} γδ T cells in *Itk*$^{-/-}$ liver (Figure 2.3F). Interestingly, Vγ1.1^{+}Vδ6.3^{+}

Figure 2.3. *Itk*$^{-/-}$ mice have an increased population of Vγ1.1Vδ6.3^{+} $\gamma\delta$ T cells.

(A) Thymocytes and splenocytes were stained with antibodies to TCRδ and either Vγ1.1, Vγ2, Vγ3, and Vγ5. Histograms show Vγ staining on gated TCRδ^{+} cells. (*B)* Lymphocytes were isolated from the liver and stained with antibodies to TCRδ and Vγ1.1 or Vγ2. Histograms show Vγ staining on gated TCRδ^{+} cells. (*C)* Thymocytes, splenocytes, and liver cells were stained with antibodies to TCRδ, Vγ1.1 and Vδ6.3. Dot plots show Vγ1.1 versus Vδ6.3 staining on gated TCRδ^{+} T cells. (*D)* Absolute numbers of Vγ1.1Vδ6.3^{+} $\gamma\delta$ T cells in the thymus and spleen, and percentages of Vγ1.1Vδ6.3^{+} $\gamma\delta$ T cells in the liver were compiled.

Figure 2.3



A.
Thymus
Spleen
Vγ1.1
Vγ2
Vγ3
Vγ5
WT
Itk-/-
cells
38.5
26.0
19.7
65.5
18.0
8.1
78.9
11.1
5.3
79.4
14.6
3.9
B.
Liver
74.1
14.1
91.5
5.5
C.
Thymus
Spleen
Liver
28.0
10.0
10.9
56.4
8.8
3.5
40.5
26.2
4.2
25.5
50.6
4.1
53.0
20.7
1.3
26.1
63.7
1.6
Vδ6.3
D.
Thymus
p=.0006
# Vγ1.1Vδ6.3+ cells (x10^4)
E.
Spleen
p=.0006
# Vγ1.1Vδ6.3+ cells (x10^5)
F.
Liver
p=.001
% Vγ1.1Vδ6.3+ cells

$\gamma\delta$ T cells are the predominant subset of $\gamma\delta$ NKT cells, and readily produce effector cytokines (66, 166, 168).

In contrast to wild type $\gamma\delta$ T cells, the *Itk*$^{-/-}$ $\gamma\delta$ T cell population showed a striking increase in the proportion of CD4^{+} cells in the thymus, spleen and liver (Figure 2.4A, B). *Itk*$^{-/-}$ mice also had elevated proportions of thymic NK1.1^{+} $\gamma\delta$ T cells (Figure 2.4C, D). Interestingly, the majority of the thymic *Itk*$^{-/-}$ CD4^{+} $\gamma\delta$ T cells and virtually all of the thymic *Itk*$^{-/-}$ NK1.1^{+} $\gamma\delta$ T cells express high levels of the memory cell marker CD44 (Figure 2.4E), as well as the activation marker CD69. As previously reported, the majority of $\gamma\delta$ T cells in the liver of wild type mice are NK1.1^{+} (i.e., $\gamma\delta$ NKT cells) (166), and this is also the case in *Itk*$^{-/-}$ liver (Figure 2.4C, D).

Focusing on the Vγ1.1^{+}Vδ6.3^{+} subset of $\gamma\delta$ T cells, we find that wild type and *Itk*$^{-/-}$ mice each have a similar proportion of NK1.1^{+} CD4^{-} cells, whereas NK1.1^{+} CD4^{+} cells are increased substantially in *Itk*$^{-/-}$ mice (Figure 2.5). Furthermore, outside the thymus, the Vγ1.1^{+}Vδ6.3^{+} subset is found predominantly in the liver, rather than the spleen (Figure 2.4F, G), as has been reported previously for $\gamma\delta$ NKT cells (166). The $\gamma\delta$ T cells not expressing Vγ1.1Vδ6.3 correspond to the "conventional" NK1.1^{-}CD4^{-}CD8^{-} subset; this conventional $\gamma\delta$ T cell subset is not altered in numbers in *Itk*$^{-/-}$ versus wild type mice, indicating that Itk controls the generation of "nonconventional" CD4^{+} or NK1.1^{+}, but not conventional, $\gamma\delta$ T cells.

Figure 2.4. Altered γδ T cell subsets in the spleen and thymus of *Itk*$^{-/-}$ mice. Cells were prepared from thymus, spleen, and liver of wild type and *Itk*$^{-/-}$ mice and analyzed by flow cytometry. (*A)* CD4 versus CD8 expression on gated TCRδ^{+}TCRβ^{-} cells. The percentages of each subpopulation are indicated, and the mean fluorescence intensities are shown in parentheses. (*B*) The percentages of CD4^{+}TCRδ^{+} cells in the thymus, spleen, and liver are shown. (*C)* CD4 versus NK1.1 expression on gated TCRδ^{+}TCRβ^{-} cells in the thymus, spleen, and liver. (*D)* The percentages of NK1.1^{+}TCRδ^{+} cells in the thymus, spleen, and liver. (*E)* Thymic TCRδ^{+}TCRβ^{-} cells were analyzed for CD44 versus CD4 *(top)* or NK1.1 *(bottom)* expression. Data are representative of 2 independent experiments. (*F)* TCRδ^{+}TCRβ^{-} cells from the liver were analyzed for Vγ1.1, Vδ6.3, NK1.1, and CD4 expression. Dot-plots show NK1.1 versus CD4 expression on gated Vγ1.1^{+}Vδ6.3^{+} cells. (G) The percentages of total cells in the thymus, spleen, and liver that represent the Vγ1.1^{+}Vδ6.3^{+}NK1.1^{+} subset were calculated. Differences between the wild type and *Itk*$^{-/-}$ were statistically significant (thymus, p=0.0006; spleen, p=0.0006; liver, p=0.001).

Figure 2.4

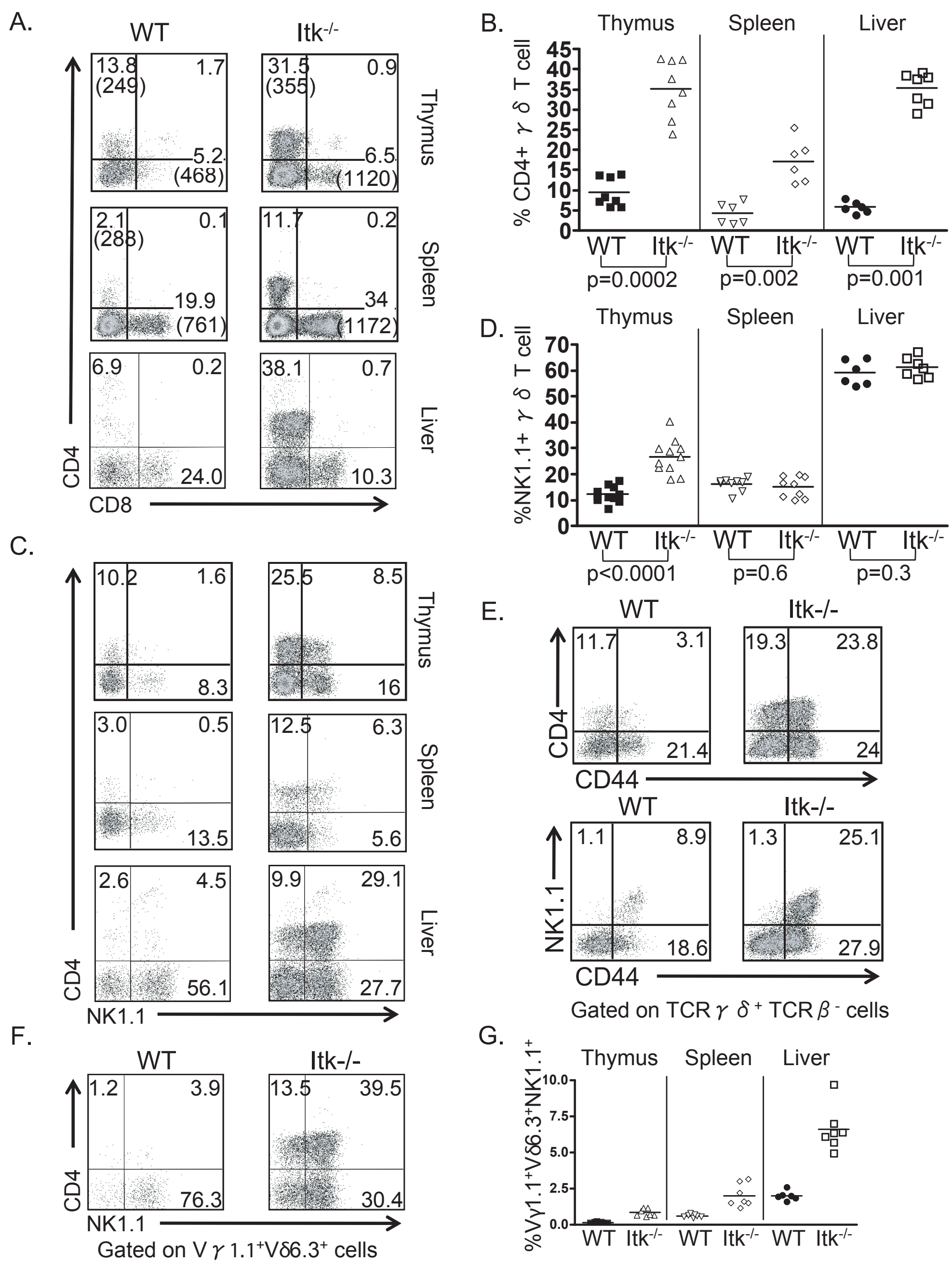
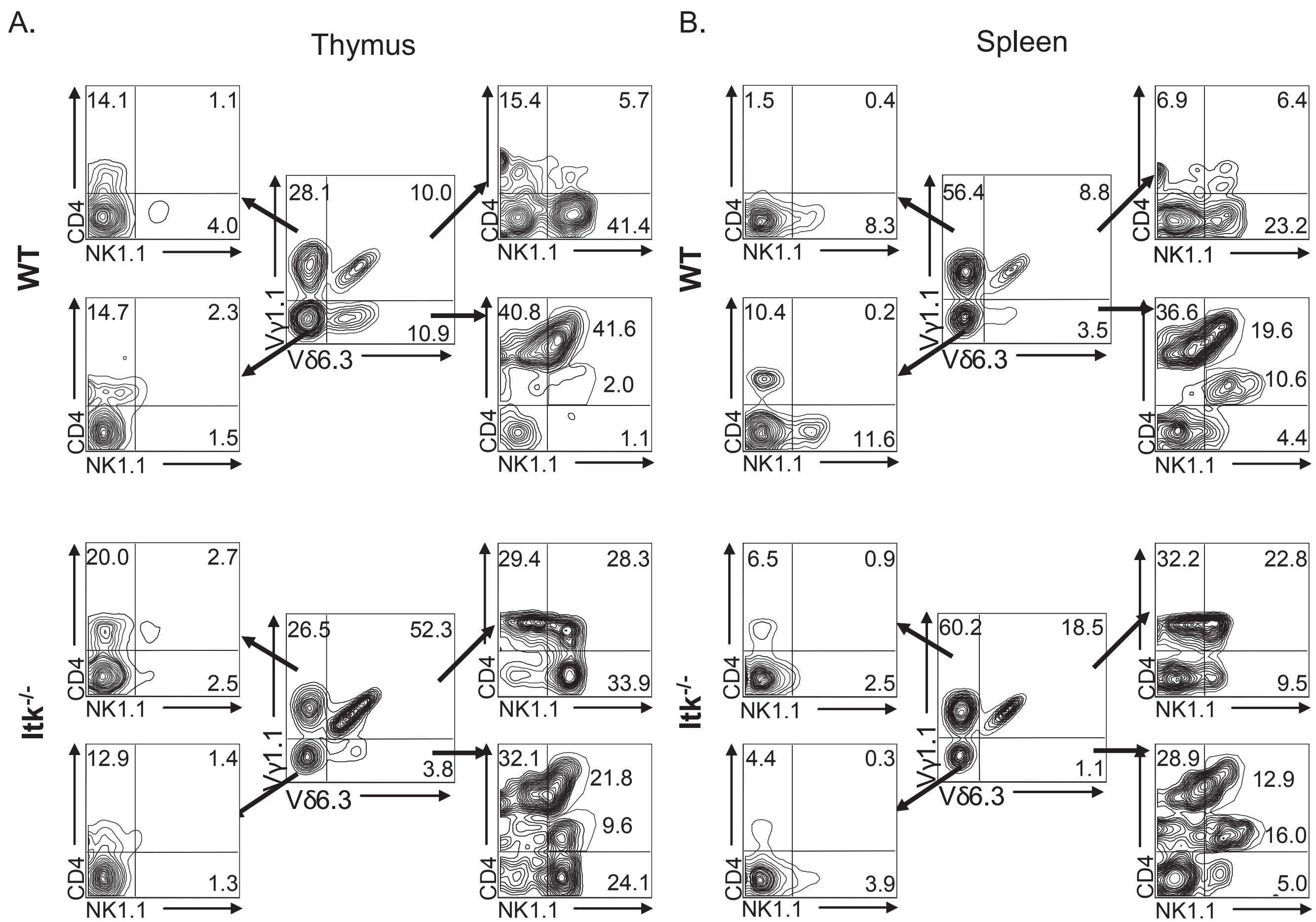

A.
WT
Itk-/-
Thymus
Spleen
Liver
CD4
CD8
B.
% CD4+ γ δ T cell
p=0.0002
p=0.002
p=0.001
C.
NK1.1
D.
%NK1.1+ γ δ T cell
p<0.0001
p=0.6
p=0.3
E.
CD44
Gated on TCR γ δ+ TCR β- cells
F.
Gated on V γ 1.1+Vδ6.3+ cells
G.
%Vγ1.1+Vδ6.3+NK1.1+

Figure 2.5 Enhanced population of CD4^{+} NK1.1^{+} Vγ1.1^{+} Vδ6.3^{+} $\gamma\delta$ T cells in *Itk*$^{-/-}$ mice.

Thymocytes (A) and splenocytes (B) were isolated from wild type and *Itk*$^{-/-}$ mice and were stained with antibodies to TCRδ, CD4, NK1.1, Vγ1.1, and Vδ6.3. TCR$\gamma\delta$ T cells were analyzed for Vγ1.1 vs. Vδ6.3 staining. Each of the four subpopulations was then examined for CD4 vs. NK1.1 staining, as shown. Numbers on dot-plots indicate the percentage of cells in the quadrant. Data are representative of two independent experiments with three mice per group.

Figure 2.5



A.
Thymus
B.
Spleen
WT
Itk-/-
CD4
NK1.1
Vγ1.1
Vδ6.3
14.1
1.1
4.0
14.7
2.3
1.5
28.1
10.0
10.9
15.4
5.7
41.4
40.8
41.6
2.0
1.1
20.0
2.7
2.5
12.9
1.4
1.3
26.5
52.3
3.8
29.4
28.3
33.9
32.1
21.8
9.6
24.1
1.5
0.4
8.3
10.4
0.2
11.6
56.4
8.8
3.5
6.9
6.4
23.2
36.6
19.6
10.6
4.4
6.5
0.9
2.5
4.4
0.3
3.9
60.2
18.5
1.1
32.2
22.8
9.5
28.9
12.9
16.0
5.0

Expanded populations of $CD4^+$ $\gamma\delta$ T cells have been previously demonstrated in mice lacking expression of both TCRβ and CD5 (164). We therefore examined CD5 expression on *Itk*$^{-/-}$ $\gamma\delta$ T cells, to determine if altered levels of CD5 might be contributing to the increased number of $CD4^+$ $\gamma\delta$ T cells in these mice, but detected no differences between wild type and *Itk*$^{-/-}$ mice (Figure 2.6A). However, like a previously-reported subset of activated $\gamma\delta$ T cells specific for self-ligands, the MHC class Ib molecules, T10/T22 (23), the $NK1.1^+$ subset of thymic Itk$^{-/-}$ $\gamma\delta$ T cells are all CD122-positive (Figure 2.6B).

To determine whether the alterations we observed in *Itk*$^{-/-}$ mice are intrinsic to the hematopoietic cells or to the environment, we generated bone marrow chimeras using wild type or *Itk*$^{-/-}$ bone marrow cells. These experiments demonstrated that the increased proportions of both $\gamma\delta$ T cells and germinal center B cells seen in *Itk*$^{-/-}$ mice are intrinsic to *Itk*$^{-/-}$ hematopoietic cells (Figure 2.7). Consistent with these data, we also find that the predominant Tec kinase expressed in wild type $\gamma\delta$ T cells is Itk (Figure 2.8A).

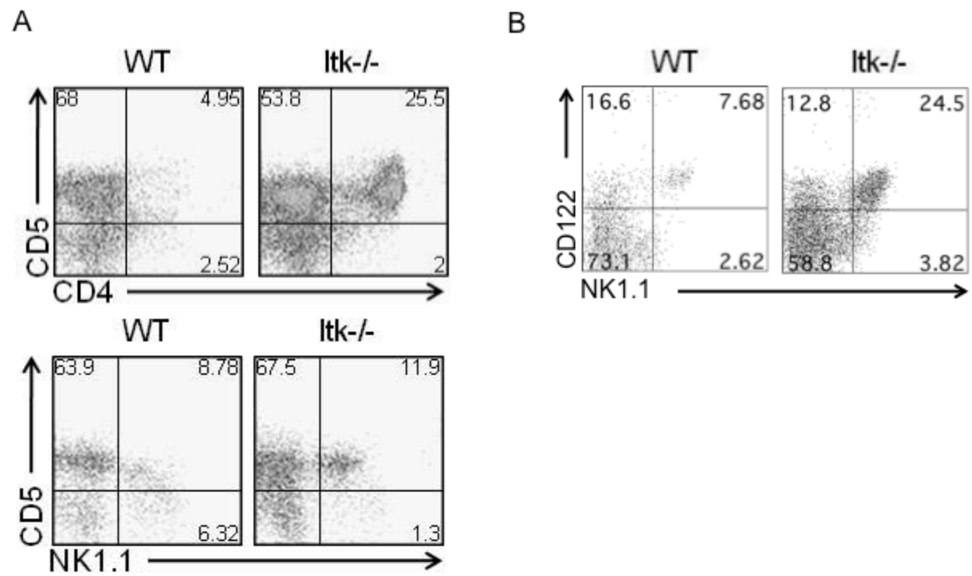
Enhanced expression of IL-4 and PLZF in Itk-deficient $\gamma\delta$ T cells

To determine the basis for the altered function of *Itk*$^{-/-}$ $\gamma\delta$ T cells, we examined mRNA levels for T-bet, Eomesodermin, and GATA-3 in wild type versus *Itk*$^{-/-}$ $\gamma\delta$ T cells. We found that *Itk*$^{-/-}$ $\gamma\delta$ T cells consistently expressed higher levels of GATA-3 mRNA and protein compared to wild type $\gamma\delta$ T cells,

Figure 2.6: Itk$^{-/-}$ $\gamma\delta$ T cells express CD5 and CD122

Cells were prepared from thymi and/or spleens of wild type and itk$^{-/-}$ mice, stained and gated on TCRγ^{+}TCRβ^{-} cells. (A) Splenic TCRγ^{+}TCRβ^{-} cells were analyzed for CD5 and CD4 expression [top panel] and CD5 and NK1.1 expression [bottom panel]. (B) Thymic TCRγ^{+}TCRβ^{-} cells were analyzed for CD122 and NK1.1 expression. Data is representative of 2 independent experiments with 3 mice per group.

Figure 2.6



A
WT
Itk-/-
68
4.95
2.52
53.8
25.5
2
CD5
CD4
WT
Itk-/-
63.9
8.78
6.32
67.5
11.9
1.3
CD5
NK1.1
B
WT
Itk-/-
16.6
7.68
73.1
2.62
12.8
24.5
58.8
3.82
CD122
NK1.1

Figure 2.7 The γδ T cell phenotype in *Itk*$^{-/-}$ mice is intrinsic to the hematopoietic cells.

Thymocytes (A) and splenocytes (B) were isolated from wild type, *Itk*$^{-/-}$ and wild type➔ wild type and *Itk*$^{-/-}$➔ wild type chimeric mice, and were analyzed by flow cytometry for γδ T cells. γδ T cells were further examined for expression of CD4, CD8, NK1.1, and CD44. Numbers on dot-plots indicate the percentage of cells in the quadrant. Data are representative of four mice per group.

Figure 2.7

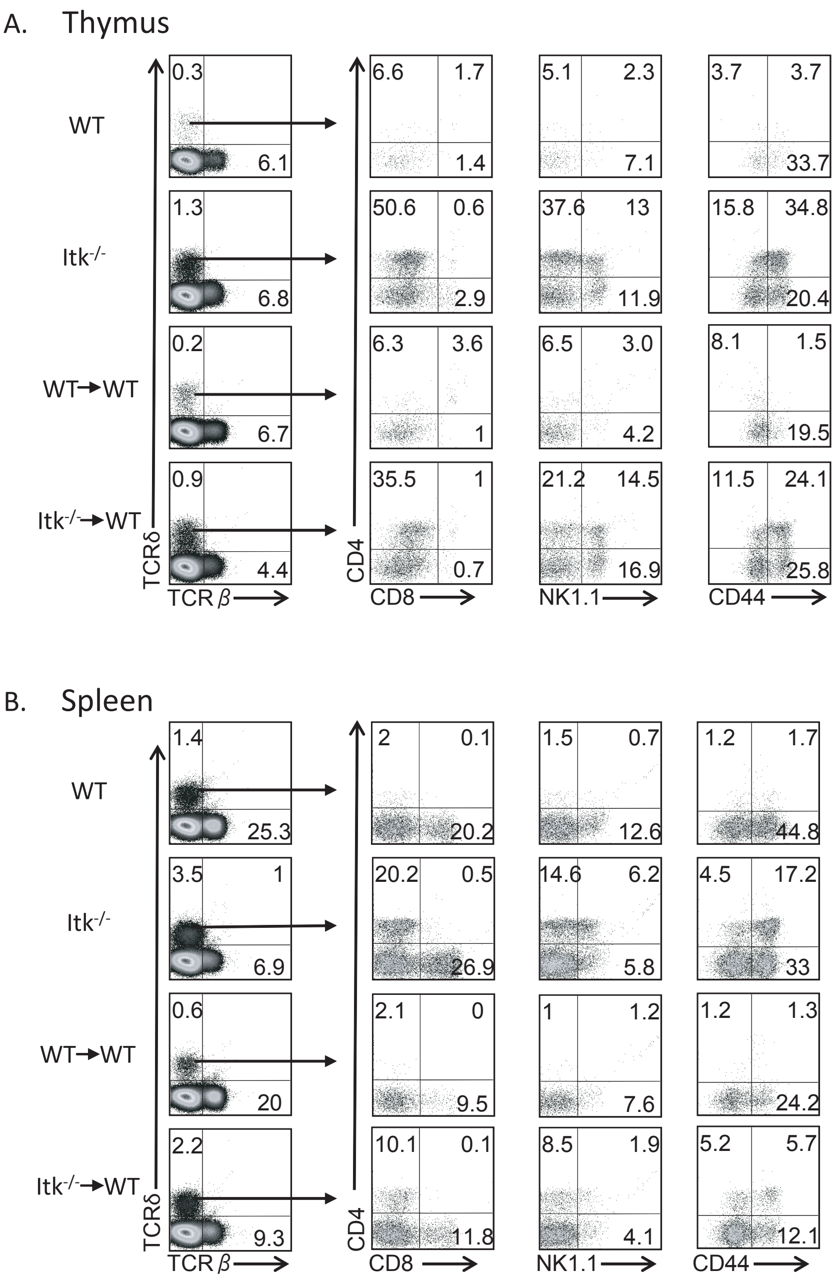
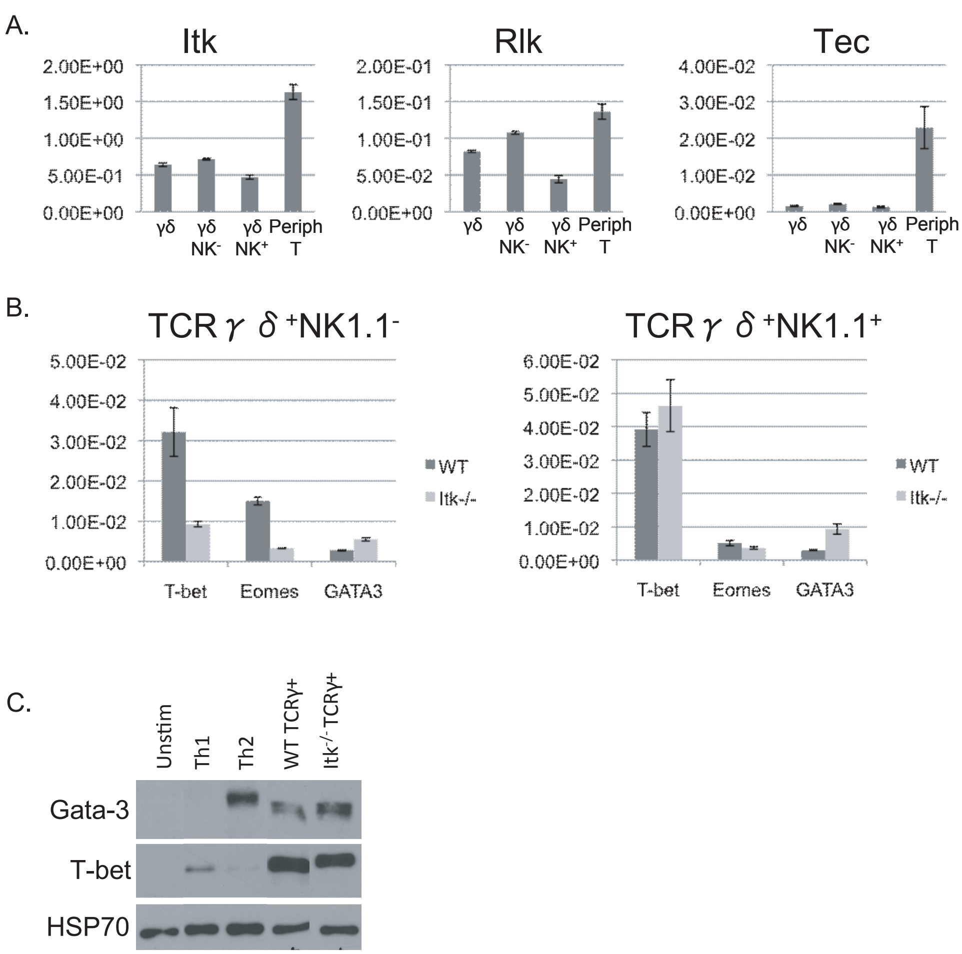

A. Thymus
WT
Itk-/-
WT→WT
Itk-/-→WT
TCRδ
TCRβ
CD4
CD8
NK1.1
CD44
0.3
6.1
6.6
1.7
1.4
5.1
2.3
7.1
3.7
3.7
33.7
1.3
6.8
50.6
0.6
2.9
37.6
13
11.9
15.8
34.8
20.4
0.2
6.7
6.3
3.6
1
6.5
3.0
4.2
8.1
1.5
19.5
0.9
4.4
35.5
1
0.7
21.2
14.5
16.9
11.5
24.1
25.8
B. Spleen
1.4
25.3
2
0.1
20.2
1.5
0.7
12.6
1.2
1.7
44.8
3.5
1
6.9
20.2
0.5
26.9
14.6
6.2
5.8
4.5
17.2
33
0.6
20
2.1
0
9.5
1
1.2
7.6
1.2
1.3
24.2
2.2
9.3
10.1
0.1
11.8
8.5
1.9
4.1
5.2
5.7
12.1

Figure 2.8 Expression of Tec kinases and transcription factors in $\gamma\delta$ T cells. (A) Pooled splenocytes and lymph node cells were isolated from WT mice and total $\gamma\delta$ T cells, NK1.1$^-$ and NK1.1$^+$ $\gamma\delta$ T cells subsets were isolated by cell sorting, and RNA was prepared. Peripheral CD8$^+$ $\alpha\beta$ T cells were analyzed for comparison. Levels of Itk (left panel), Rlk (middle panel), and Tec (right panel) mRNA were determined by real-time quantitative RT-PCR. Graphs show data normalized to β-actin mRNA levels in each sample and are representative of two independent experiments. (B) NK1.1$^-$ (left panel) and NK1.1$^+$ (right panel) $\gamma\delta$ T cells were isolated from spleens of wild type and *Itk*$^{-/-}$ mice, and RNA was prepared. Levels of T-bet, eomesodermin, and GATA-3 mRNA were determined by real-time quantitative RT-PCR. Graphs show data normalized to β-actin mRNA levels in each sample and are representative of two independent experiments. (C) $\gamma\delta$ T cells were isolated from spleens of wild type and *Itk*$^{-/-}$ mice, and protein lysates were analyzed for T-bet and GATA-3 levels by Western blot analysis. As controls, CD4$^+$ $\alpha\beta$ T cells were isolated, protein lysates were prepared from unstimulated cells, and cells cultured in vitro for 7 days in T_H1 or T_H2 polarizing conditions. Levels of HSP70 are shown as a loading control.

Figure 2.8


A.
Itk
Rlk
Tec
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
2.00E-01
1.50E-01
1.00E-01
5.00E-02
4.00E-02
3.00E-02
2.00E-02
1.00E-02
γδ
γδ NK-
γδ NK+
Periph T
B.
TCRγδ+NK1.1-
TCRγδ+NK1.1+
5.00E-02
6.00E-02
WT
Itk-/-
T-bet
Eomes
GATA3
C.
Unstim
Th1
Th2
WT TCRγ+
Itk-/- TCRγ+
Gata-3
T-bet
HSP70

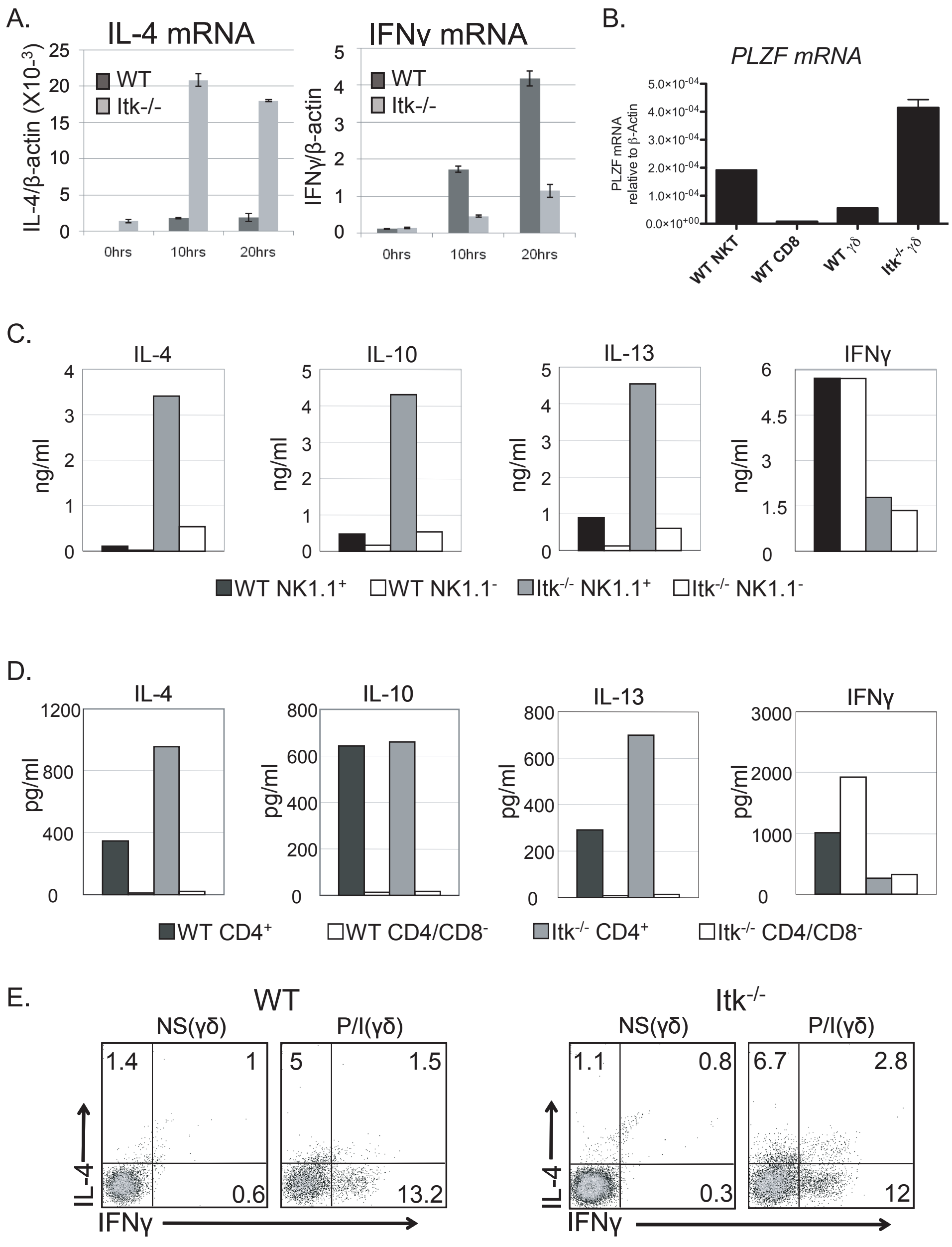
while the NK1.1$^-$ subset of *Itk*$^{-/-}$ $\gamma\delta$ T cells have reduced levels of mRNA for both T-bet and Eomesodermin (Figure 2.8B, C). These findings suggested that *Itk*$^{-/-}$ $\gamma\delta$ T cells might produce a distinct pattern of cytokines compared to wild type $\gamma\delta$ T cells. We first examined mRNA levels for the signature cytokines, IL-4 and IFNγ, following $\gamma\delta$ T cell activation *in vitro*. As shown in Figure 2.9A, in response to $\gamma\delta$ TCR stimulation, *Itk*$^{-/-}$ $\gamma\delta$ T cells have constitutively elevated levels of IL-4 mRNA prior to stimulation, and show dramatically enhanced induction of IL-4 mRNA at 10h and 20h post-stimulation compared to wild type $\gamma\delta$ T cells. Basal levels of IFNγ mRNA were similar between the wild type and *Itk*$^{-/-}$ $\gamma\delta$ T cells, and following stimulation, both cell types produced IFNγ, although wild type $\gamma\delta$ T cells show higher levels of IFNγ mRNA compared to $\gamma\delta$ T cells lacking Itk.

Based on the high proportion of Vγ1.1$^+$Vδ6.3$^+$ $\gamma\delta$ T cells present in *Itk*$^{-/-}$ mice, and the previous association of this $\gamma\delta$ T cell subset with dual production of IL-4 and IFNγ (66, 168), we also examined wild type and *Itk*$^{-/-}$ $\gamma\delta$ T cells for expression of the transcription factor PLZF. PLZF has recently been found to be critical for the development and effector function of TCR$\alpha\beta^+$ NKT cells, where it promotes the simultaneous production of IL-4 and IFNγ (81, 82). Interestingly, we found that splenic *Itk*$^{-/-}$ $\gamma\delta$ T cells express substantially higher amounts of PLZF mRNA than do WT $\gamma\delta$ T cells (Figure 2.9B). Furthermore, among *Itk*$^{-/-}$ $\gamma\delta$ T cells, the NK1.1$^+$ fraction expresses particularly high levels of PLZF mRNA (Figure 2.10).

Figure 2.9. *Itk*$^{-/-}$ γδ T cells produce IL-4 plus IFN-γ and express the transcription factor PLZF.

Lymph nodes and spleens from wild type and *Itk*$^{-/-}$ mice were pooled and total TCRγδ^{+} cells *(A-B)* or sorted subpopulations *(C-D)* were analyzed. *(A)* 2 x 10^{5} cells were stimulated with 10 μg/ml of anti-TCRδ for 0, 10 and 20 hours. IL-4 *(left panel)* and IFNγ *(right panel)* mRNA expression levels normalized to β-actin were determined by real-time quantitative RT-PCR. Data shown are representative of two independent experiments. *(B)* Levels of PLZF mRNA normalized to β-actin were determined by real-time quantitative RT-PCR. WT peripheral CD8^{+} αβ T cells and αβ NKT cells were analyzed for comparison. Data are representative of two independent experiments. *(C)* 5x10^{4} cells were stimulated with 10 μg/ml of anti-TCRδ for 72 hours and supernatants were analyzed for the presence of IL-4, IL-10, IL-13, and IFNγ by cytometric bead array (CBA). Data are representative of three independent experiments. *(D)* 3x10^{4} cells were stimulated as in *C*. Supernatants were analyzed for the presence of IL-4, IL-10, IL-13, and IFNγ by CBA. Data are representative of two independent experiments. *(E)* Unstimulated (NS) and stimulated (P/I) wild type *(left)* and *Itk*$^{-/-}$ *(right)* γδ T cells were analyzed for intracellular IL-4 and IFN-γ production. Cells were stimulated with 10 ng/ml PMA and 2 μg/ml Ionomycin (P/I) for 4 hours. Data are representative of four independent experiments.

Figure 2.9

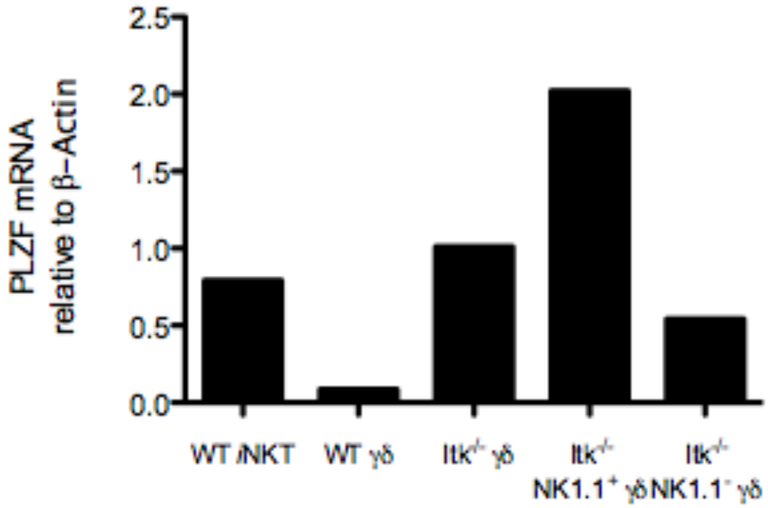


A.
IL-4 mRNA
IL-4/β-actin (X10-3)
WT
Itk-/-
0hrs
10hrs
20hrs
IFNγ mRNA
IFNγ/β-actin
B.
PLZF mRNA
PLZF mRNA relative to β-Actin
WT NKT
WT CD8
WT γδ
Itk-/- γδ
C.
IL-4
IL-10
IL-13
IFNγ
ng/ml
WT NK1.1+
WT NK1.1-
Itk-/- NK1.1+
Itk-/- NK1.1-
D.
IL-4
IL-10
IL-13
IFNγ
pg/ml
WT CD4+
WT CD4/CD8-
Itk-/- CD4+
Itk-/- CD4/CD8-
E.
WT
Itk-/-
NS(γδ)
P/I(γδ)
1.4
1
0.6
5
1.5
13.2
1.1
0.8
0.3
6.7
2.8
12
IL-4
IFNγ

Figure 2.10. *Itk*$^{-/-}$ NK1.1^{+} $\gamma\delta$ T cells express elevated levels of the transcription factor PLZF.

Lymph nodes and spleens from wild type and *Itk*$^{-/-}$ mice were pooled and total TCR$\gamma\delta^{+}$ cells and sorted subpopulations were analyzed for PLZF expression. PLZF mRNA expression was determined by real-time quantitative RT-PCR and normalized to β-actin.

Figure 2.10


PLZF mRNA
relative to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
WT iNKT
WT γδ
Itk-/- γδ
Itk-/- NK1.1+ γδ
Itk-/- NK1.1- γδ

These findings support for the conclusion that $\gamma\delta$ T cell development is altered in the absence of Itk.

To assess levels of cytokine protein secretion, individual subsets of *Itk*$^{-/-}$ and wild type $\gamma\delta$ T cells were purified and stimulated. As previous studies have found that NK1.1^{+} $\gamma\delta$ T cells and CD4^{+} $\gamma\delta$ T cells produce the highest levels of cytokines, particularly 'T_H2' cytokines (65, 169-172), we compared NK1.1^{+} to NK1.1^{-}, and CD4^{+} to CD4^{-} $\gamma\delta$ T cell subsets. After 72h of stimulation, wild type NK1.1^{+} $\gamma\delta$ T cells secreted more IL-4, IL-10, and IL-13 than their NK1.1^{-} counterparts; in contrast, both NK1.1^{+} and NK1.1^{-} subsets of wild type $\gamma\delta$ T cells secreted large amounts of IFNγ (Figure 2.9C). Consistent with the analysis of cytokine mRNA levels, we observed elevated secretion of the T_H2 cytokines IL-4, IL-10, and IL-13 by the *Itk*$^{-/-}$ NK1.1^{+} $\gamma\delta$ T cells when compared to WT NK1.1^{+} cells; furthermore *Itk*$^{-/-}$ NK1.1^{+} $\gamma\delta$ T cells secreted much higher amounts of these cytokines per cell relative to the Itk$^{-/-}$ NK1.1^{-} subset. In addition, we found that both subsets of *Itk*$^{-/-}$ $\gamma\delta$ T cells secreted IFNγ, but at a lower level than the wild type cells.

Comparison of the CD4^{+} versus CD4^{-} subsets of $\gamma\delta$ T cells confirmed previous data (171, 172) that $\gamma\delta$ T cells expressing CD4 are the major cytokine producing population, particularly for the 'T_H2' cytokines. As shown in Figure 2.9D, *Itk*$^{-/-}$ CD4^{+} $\gamma\delta$ T cells produce elevated levels of IL-4 and IL-13 compared to wild type CD4^{+} $\gamma\delta$ T cells, but similar levels of IL-10. None of these cytokines were detected in supernatants of stimulated CD4$^-$ γδ T cells from either *Itk*$^{-/-}$ or wild type mice. As noted above, wild type γδ T cells (both CD4$^+$ and CD4$^-$ subsets) secrete higher levels of INFγ than *Itk*$^{-/-}$ γδ T cells.

On a population basis, *Itk*$^{-/-}$ γδ T cells secreted both IL-4 and IFNγ following stimulation. To determine whether individual cells were dual producers of both effector cytokines, we stimulated wild type and *Itk*$^{-/-}$ γδ T cells *in vitro* with PMA and ionomycin and then examined IL-4 and IFNγ production by intracellular cytokine staining (Figure 2.9E). As expected, a larger proportion of wild type γδ T cells produce IFNγ in response to stimulation compared to those that produce IL-4, and few cells produce both cytokines. In contrast, *Itk*$^{-/-}$ γδ T cells include a significantly larger population that produces IL-4 than is seen in the wild type γδ T cell subset (*Itk*$^{-/-}$, 6.9±1.1; wild type, 3.5±0.8; n=7; p=0.04); additionally, a greater proportion of *Itk*$^{-/-}$ γδ T cells produces both IL-4 plus IFNγ compared to wild type γδ T cells (*Itk*$^{-/-}$, 3.9±0.8; wild type, 1.7±0.3; n=7; p=0.02). Since this pharmacological stimulation bypasses the need for Itk in TCR-mediated signaling, these data indicate that a larger proportion of *Itk*$^{-/-}$ γδ T cells are programmed to produce IL-4, as well as IL-4 plus IFNγ, prior to their activation. This latter finding, together with the data demonstrating increased numbers of CD4$^+$ and NK1.1$^+$ γδ T cells in the *Itk*$^{-/-}$ mice, strongly suggest that γδ T cell development is altered in the absence of Itk leading to a striking increase in a PLZF-positive, IL-4-producing γδ T cell population.

$Itk^{-/-}$ γδ T cells up-regulate surface receptors that promote B cell help

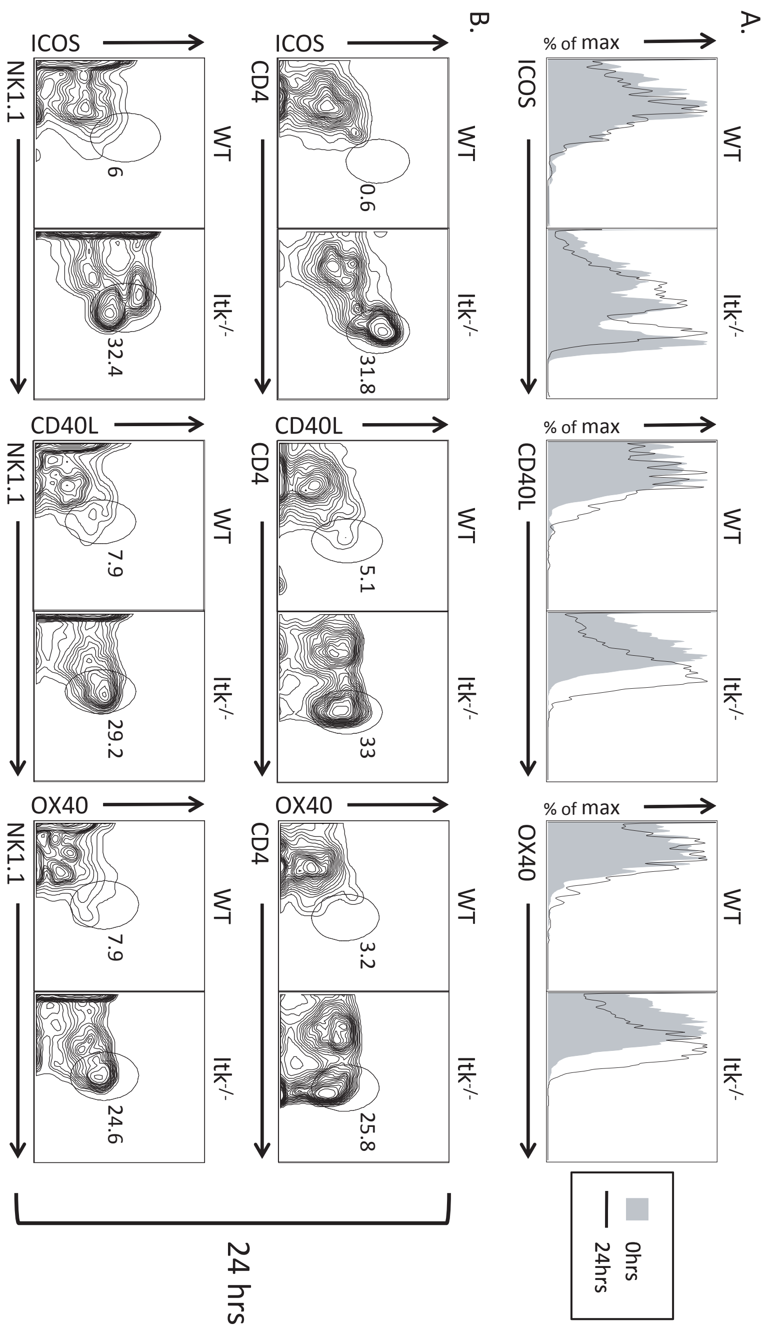
We next examined γδ T cells for the expression of co-stimulatory molecules that provide B cell help, such as CD40L, CD70, OX40, and ICOS (10). For these experiments, wild type and *$Itk^{-/-}$* thymocytes and splenocytes were evaluated directly *ex vivo*, and in addition, were cultured for 24h in the presence of stimulatory anti-TCRδ antibodies. While analysis of splenic γδ T cells from wild type and *$Itk^{-/-}$* mice did not reveal any major changes in co-stimulatory markers, we did see a small increase in the proportion of constitutively ICOS-positive γδ T cells in the spleens of *$Itk^{-/-}$* mice. Since *$Itk^{-/-}$* mice have a two-fold increase in the absolute number of total γδ T cells in the spleen compared to wild type, this difference amounts to a ~10-fold increase in ICOS-positive splenic γδ T cells, and thus could account for the enhanced B cell activation observed in *$Itk^{-/-}$* mice.

More strikingly, levels of ICOS were increased on a large proportion of the thymic *$Itk^{-/-}$* γδ T cells compared to controls, but remained unaltered following stimulation (Figure 2.11A). Evaluation of the $ICOS^{hi}$ fraction of *$Itk^{-/-}$* thymic γδ T cells indicated that nearly all of these cells were $CD4^+$, and a substantial proportion were also $NK1.1^+$ (Figure 2.11B). Little to no difference was seen in the basal levels of CD40L and OX40 when comparing thymic $Itk^{-/-}$ γδ T cells to wild type γδ T cells directly *ex vivo* (Figure 2.11A). However, after 24h of *in vitro*

Figure 2.11. *Itk*$^{-/-}$ $\gamma\delta$ T cells up-regulate costimulatory molecules involved in B cell help.

(A) Thymocytes from wild type and *Itk*$^{-/-}$ mice were pooled, and left unstimulated (0 hrs) or were stimulated with 10 μg/ml α-TCRδ for 24 hours. Unstimulated and stimulated TCRδ^{+}TCRβ^{-} T cells were then analyzed for the expression of ICOS, CD40L, and OX40. (*B)* Wild type and Itk$^{-/-}$ thymocytes stimulated for 24 hours with 10 μg/ml α-TCRδ antibodies were stained for CD4, ICOS, CD40L, OX40, and NK1.1 expression.

Figure 2.11



A.
B.
% of max
ICOS
CD40L
OX40
WT
Itk-/-
0hrs
24hrs
CD4
NK1.1
0.6
31.8
5.1
33
3.2
25.8
6
32.4
7.9
29.2
7.9
24.6
24 hrs

stimulation on anti-TCRδ-coated plates, *Itk*$^{-/-}$ cells up-regulated both CD40L and OX40, whereas wild type γδ T cells did not. This up-regulation of CD40L and OX40 was detected on all subsets of *Itk*$^{-/-}$ γδ T cells (Figure 2.11B). Finally, we could not detect expression of CD70 on either wild type or *Itk*$^{-/-}$ thymic γδ T cells.

Discussion

Overall, our data indicate that γδ T cell development is significantly altered in the absence of Itk, yielding increased numbers of γδ T cells and a shift in the major effector functions mediated by these cells. Most strikingly, *Itk*$^{-/-}$ mice have elevated numbers of γδ T cells expressing CD4 and NK1.1. Both CD4$^+$ and NK1.1$^+$ γδ T cells have been previously described to secrete IL-4 (65, 171, 172), which is thought to be a major "switch factor" cytokine required for IgG1 and IgE class switching (58). Further, unlike the γδ T cells in wild type mice, the *Itk*$^{-/-}$ γδ T cells secrete large quantities of the T_H2 cytokines, IL-4, IL-10, and IL13, in addition to the IFNγ typically secreted by activated wild type γδ T cells, correlating with high levels of PLZF, a transcription factor required for the function of αβ *i*NKT cells. These findings strongly suggest that Itk signaling plays a key role in regulating γδ T cell lineage development.

Itk$^{-/-}$ mice have previously been shown to have spontaneously elevated serum levels of IgG1 and IgE though the increase in IgG1 is not as severe as IgE (132). Surprisingly, these altered γδ T cells are responsible for promoting significant levels of spontaneous IgE secretion in *Itk*$^{-/-}$ mice as $\gamma\delta$ T cell deficient *Itk*$^{-/-}$ mice do not have highly elevated IgE levels. Based on the findings presented here, it is likely that the high levels of IL-4 and IL-13 produced by the activated *Itk*$^{-/-}$ $\gamma\delta$ T cells are a major factor in the hyper IgE phenotype. Interestingly a study by Huang *et. al.* determined that Vγ1.1^{+} cells, which include V6 cells, are able enhance IgE responses upon immunization with OVA-alum (173) thereby supporting the possibility that V6 cells are the main $\gamma\delta$ T cell subset responsible for the hyper IgE in *Itk*$^{-/-}$ mice. However, further experiments are required with IL-4 and IL-13 deficient mice to determine if V6 cells are required to produce both of these cytokines for B cell activation and Ig class switching. In addition, studies are required to determine if V6 cells directly promote the humoral response by examining if they are located within the germinal center where Ig class switching occurs. CXCR5 has been shown to be important for T_{FH} cells to migrate to germinal centers (60-62) and it would be interesting to see if *Itk*$^{-/-}$ V6 cells express CXCR5 similarly to T_{FH} cells. Overall our data indicate that activated *Itk*$^{-/-}$ $\gamma\delta$ T cells express elevated amounts of B cell co-stimulatory molecules, such as ICOS, CD40L, and OX40, further suggesting that the $\gamma\delta$ T cells may be directly providing help to the B cells, leading to B cell activation and Ig class switching.

In humans, a variety of studies have implicated $\gamma\delta$ T cells in allergic airway inflammation (8, 174), and specifically, in promoting B cell activation and IgE class switching (175, 176). One interesting clinical report found that IL-4-

producing γδ T cells were likely responsible for a case of hyper IgE syndrome (5). Studies performed *in vitro* with human γδ T cells showed that these cells, in combination with IL-4, can induce B cell activation, Ig isotype switching, and secretion of IgE (177). Further, these findings correlate well with observations that human γδ T cells can express ICOS, CD40L, OX40, and CD70 (10). Our data indicate that the Tec family tyrosine kinase, Itk, plays a key role in regulating this potentially highly detrimental function of γδ T cells. As mentioned previously, *Itk*$^{-/-}$ mice also have spontaneously elevated levels of IgG1 in addition to IgE. We have shown that the γδ T cells in *Itk*$^{-/-}$ mice are responsible for the hyper-IgE but we did not examine IgG1 in *Itk/TCRd*$^{-/-}$ mice. It is possible that γδ T cells do not affect IgG1 isotype switching as one study with allergic airway inflammation showed that lack of γδ T cells resulted in decreased IgE production but not IgG1 (8). In contrast, another study determined that γδ T cells were required for both IgE and IgG1 (178). Therefore, further studies are required to determine if γδ T cells in *Itk/TCRd*$^{-/-}$ mice affect IgG1.

Recently, γδ T cells have also been implicated in the elevated IgE concentrations seen in mice carrying mutations in additional T cell signaling proteins. For instance, in the absence of the E3-ubiquitin ligase, Itch, γδ T cells secrete IL-4 and promote IgE production in unimmunized mice (174). More strikingly, mice expressing a mutant allele of the adapter protein LAT, which lacks the three c-terminal tyrosines, succumb to a fatal lymphoproliferative disorder that is mediated by γδ T cells (179). In these LAT mutant mice, the γδ T cells accumulate to large numbers, and show a phenotype remarkably similar to those lacking Itk. For instance, the LAT mutant γδ T cells secrete IL-4, rather than IFN-γ, and many of them express the CD4 co-receptor; in addition, the mice also have elevated levels of serum IgE. As this mutant LAT protein does not support αβ T cell development, these altered γδ T cells are the only source of T cell help for B cell activation and IgE class switching. As Itk and LAT interact in a TCR-dependent signaling complex in αβ T cells, the similarities in the γδ T cell phenotype in these two lines of mice strongly suggest that these proteins are also in the same signaling pathway downstream of the γδ TCR, and further, that this pathway regulates the development and effector function of γδ T cells. As Itk has previously been shown to suppress the development of innate αβ lineage T cells and to promote the development of conventional αβ T cells (180), a similar function for Itk may be required in γδ T cells; thus, in the absence of Itk, enhanced development of innate (e.g., $PLZF^+$, $NK1.1^+$) γδ T cells occurs, leading to increased numbers of effector cytokine-producing γδ T cells in $Itk^{-/-}$ mice.

Interestingly, a recent report by Jensen et al demonstrates that γδ T cells, like αβ T cells, are found as both naïve and effector subsets (44). Effector-type γδ T cells express higher levels of CD44, NK1.1, and CD122 relative to the naïve subset, and in addition, show an altered cytokine secretion profile. Furthermore, the presence of the effector γδ T cell population correlated with the expression of the TCR ligand for these $\gamma\delta$ T cells, indicating that ligand recognition was responsible for their activated phenotype. As a large population of *Itk*$^{-/-}$ $\gamma\delta$ T cells exhibit a similar effector cell phenotype, and produce effector cytokines such as IFN-γ and IL-4, these findings suggest that ligand recognition by *Itk*$^{-/-}$ $\gamma\delta$ T cells may contribute to their activation *in vivo*, and their role in promoting IgE production in unimmunized mice.

Furthermore, the increase in total $\gamma\delta$ T cells in *Itk*$^{-/-}$mice is due to the expansion of the V6 cells which have been shown to share similar characteristics to $\alpha\beta$ *i*NKT cells such as CD4, NK1.1 and PLZF expression along with the ability to secrete both IL-4 and IFNγ. Therefore, it is possible that Itk is affecting the maturation of the V6 cells. Previous studies have demonstrated that lack of Itk negatively affects the maturation stages of *i*NKT cells by causing a defect during progression through maturation (156-158).

These findings have substantial relevance to the potential effects of small molecule inhibitors of Itk. Due to the importance of Itk in T_H2 development and cytokine production by CD4$^+$ $\alpha\beta$ T cells, this kinase is currently being targeted for the development of drugs to treat asthma and other allergic diseases (2, 181). It would be unfortunate if Itk inhibition also led to the aberrant activation of $\gamma\delta$ T cells, and thus to enhanced production of IgE. Elevated levels of serum IgE would, in turn, lead to up-regulation of the FcεRI on mast cells (182), promoting hyper-responsiveness of these cells to IgE-mediated receptor aggregation. In light of the findings presented here, further studies on the role of Itk in human $\gamma\delta$ T cells is clearly warranted.

Chapter III: $\gamma\delta$NKT cell maturation phenocopies $\alpha\beta$ *i*NKT cells and requires the Tec Kinase Itk

Chapter III Attributions and Copyright information

The material in this chapter consists of collaboration between Ok Hyun Cho [OC], Kavitha Narayan [KN] and I. Specific contributions for each figure are as follows: figure 3.1 [CY], figure 3.2 [CY and OC], figure 3.3 [OC], figure 3.4 [CY], figure 3.5 [CY], figure 3.6 [KN and CY], figure 3.7 [CY], table 3.1 and 3.2 [KN], figure 3.8 [CY], figure 3.9 [CY], figure 3.10 [CY and OC] and figure 3.11 [OC]

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Introduction

The Tec family tyrosine kinase, Itk, regulates signal transduction in T cells via the T-cell receptor (TCR). In the absence of Itk, T cells have defects in phospholipase C-γ1 (PLCγ1) activation, calcium mobilization, mitogen-activated protein kinase (MAPK kinase) phosphorylation, and AP-1 and nuclear factor of activated T cells (NFAT) activity after stimulation (183). As a consequence, Itk plays a critical role in $\alpha\beta$ T cell development, influencing both positive and negative selection. During thymic development, conventional CD4$^+$ and CD8$^+$ T cells are the predominant populations that arise; these cells develop with a naïve phenotype (CD62L^{hi}/CD44^{lo}) and require a lengthy process of activation, proliferation, and differentiation prior to exhibiting effector cell characteristics (108, 184-186). In contrast, thymic development in *Itk*$^{-/-}$ mice produces populations of both CD4$^+$ and CD8$^+$ T cells that resemble memory cells and exhibit innate effector functions (187-190). These data indicate that TCR signaling via Itk is not simply regulating the efficiency of conventional CD4$^+$ and CD8$^+$ T cell maturation, but also required for the appropriate balance of naïve versus innate T cell subsets.

One unexpected phenotype observed in *Itk*$^{-/-}$ mice is a spontaneous elevation in levels of serum IgE (4, 132). Based on a wealth of data indicating that *Itk*$^{-/-}$ CD4+ T cells are unable to generate T_H2 effector responses (159), and the fact that *Itk*$^{-/-}$ $\alpha\beta$ TCR$^+$ *i*NKT ($\alpha\beta$ *i*NKT) cells are also defective in producing IL-

4 (157, 158) the source of type 2 cytokines responsible for promoting B cell isotype switching to IgE was initially a mystery. However, recent studies showed that the cell type responsible for the hyper-IgE syndrome seen in *Itk*$^{-/-}$ mice was a specific subset of $\gamma\delta$ TCR$^+$ T cells. These $\gamma\delta$ T cells, expressing the Vγ1.1$^+$Vδ6.3$^+$ (V6) TCR and the transcription factor PLZF (80, 83, 191) are greatly increased in number in *Itk*$^{-/-}$ mice, and secrete high levels of T_H2 cytokines (155). Previous studies have also shown V6 cells have a restricted TCR repertoire with limited junctional diversity and that they originate from fetal or postnatal precursors which is characterized by a few to no N nucleotide additions along with preferential use of Dδ2 (66-68, 76). The V6 cell subset, now known as $\gamma\delta$ NKT cells, is found in the thymus, spleen and liver (66, 166). Their preferential localization to the liver along with their expression of PLZF has linked them to $\alpha\beta$ *i*NKT cells (81, 82). In addition, transcriptome analysis of $\alpha\beta$ and $\gamma\delta$ NKT cells indicates a common molecular program among the two lineages (49).

$\alpha\beta$ TCR$^+$ NKT cells are an innate subset of $\alpha\beta$ T cells that have characteristics of both T and NK cells. These lymphocytes reside in the thymus, spleen and liver and are rarely detected in the lymph nodes or intestine. Unlike conventional T cells, $\alpha\beta$ *i*NKT cells can rapidly produce large amounts of cytokines such as IFNγ, IL-4, and IL-10 (88, 192). Murine $\alpha\beta$ *i*NKT cells predominantly express an invariant TCR Vα and Jα receptor (Vα14-Jα18) that preferentially pairs with a restricted TCR β-chain (Vβ8.2 or Vβ7); further, this TCR recognizes the glycolipid α-galactosyl ceramide (αGalcer) bound to the MHC class Ib molecule, CD1d (90).

Like conventional T cells, $\alpha\beta$ *i*NKT cells develop in the thymus from $CD4^{+}CD8^{+}$ double positive (DP) progenitors, and are dependent on the presence of CD1d (193-195). Following their initial positive selection, $\alpha\beta$ *i*NKT cell precursors undergo additional stages of differentiation in the thymus. Starting with the earliest detectable population $CD24^{+}$ (HSA), $CD44^{lo}NK1.1^{lo}$ (stage 0), $\alpha\beta$ *i*NKT cells proceed to down-regulate CD24 (stage 1), and then up-regulate CD44 ($CD24^{lo}CD44^{hi}NK1.1^{lo}$; stage 2). Finally, the cells up-regulate the IL-2/IL-15 receptor β-chain (CD122) and NK cell receptors, such as NK1.1 ($CD24^{lo}CD44^{hi}NK1.1^{hi}$; stage 3). Transition through these stages is also accompanied by a change in cytokine profile. During stage 1 and 2, $\alpha\beta$ *i*NKT cells are potent producers of IL-4, but make little IFNγ; this phenotype correlates with expression of high levels of PLZF. As they transition to stage 3, $\alpha\beta$ *i*NKT cells produce abundant amounts of IFNγ, but less IL-4, and PLZF expression decreases (9, 93, 99). Other molecules important for $\alpha\beta$ *i*NKT cell development include the SLAM family surface receptors and the adaptor protein SLAM-associated protein (SAP)(95).

Previous studies have shown that $Itk^{-/-}$ mice have reduced numbers of $\alpha\beta$ *i*NKT cells, and that the remaining subset of *i*NKT cells in these mice have a defect in progressing past stage 2 of development (156-158). Even though $\gamma\delta$ T cells develop in the thymus, they develop from $CD4^{-}CD8^{-}$ progenitors, rather than from DP thymocytes (22, 23). Thus, it was unclear whether γδ NKT cells would share similar intrathymic subsets with αβ *i*NKT cells. In addition, information on the development of *Itk*$^{-/-}$ γδ NKT cells was also lacking. Here we show both αβ and γδ NKT cells in the thymus include two populations expressing distinct levels of PLZF. Further, as with αβ *i*NKT cells, down-regulation of PLZF in γδ NKT cells correlates with changes in cytokine production, and up-regulation of maturation markers. We also find that γδ NKT cells in *Itk*$^{-/-}$ mice are impaired in this progression, leading to increased numbers of $PLZF^{+}$ IL-4-producing γδ NKT cells in the periphery of these mice relative to wild type mice. Finally, we investigated the TCR repertoire of γδ NKT cells in the thymus versus the periphery, and find that γδ NKT cells are generated from both fetal and adult precursors in two waves with *Itk*$^{-/-}$ thymus containing more γδ NKT cells being generated from fetal precursors. These data support a similar developmental progression for γδ NKT cells as for αβ *i*NKT cells, and indicate that Itk plays a parallel role in the functional and phenotypic maturation of both subsets of NKT cells.

Results

V6 cells display an immature phenotype in the spleens of Itk$^{-/-}$ *mice*

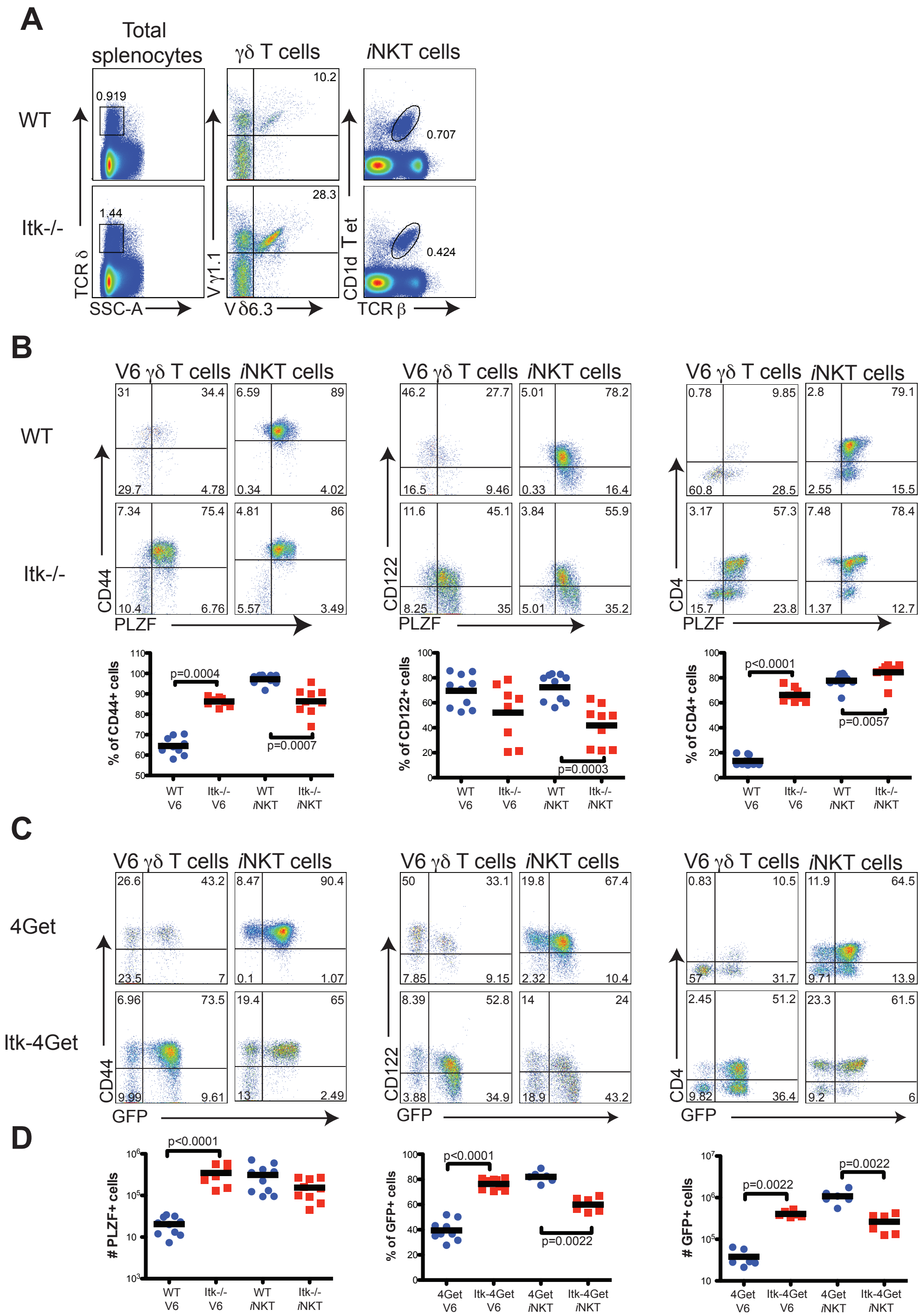
We have previously shown that the absolute number and proportion of $\gamma\delta$T cells are increased in the thymus, spleen and liver of *Itk*$^{-/-}$ mice. This defect can be attributed to a specific subset of $\gamma\delta$T cells expressing the Vγ1.1$^+$Vδ6.3$^+$ (V6) TCR (155) (Figure 3.1A). Numerous similarities between V6 cells and $\alpha\beta$ *i*NKT cells have been observed, we therefore hypothesized that V6 cells develop in a similar manner to $\alpha\beta$ *i*NKT cells and that lack of Itk affects splenic V6 cells similarly to $\alpha\beta$ *i*NKT cells. Splenic $\alpha\beta$ *i*NKT cells were compared side by side with V6 cells. Consistent with previous reports, $\alpha\beta$ *i*NKT cells from *Itk*$^{-/-}$ mice are reduced approximately two fold in both number and percent (156-158). To assess whether or not V6 cells in the spleen had similar characteristics to $\alpha\beta$ *i*NKT cells, CD1d tetramer and $\gamma\delta$ antibodies were used to identify $\alpha\beta$ *i*NKT cells and V6 cells. In addition $\alpha\beta$ *i*NKT and V6 cells were further stained with antibodies specific for cell surface markers and transcription factors associated with mature $\alpha\beta$ *i*NKT cells such as CD122, CD44, CD4 and PLZF (99). Phenotypic analysis of HSA$^-$ V6 cells and $\alpha\beta$ *i*NKT cells from the spleens of wild type mice show that the majority of these cells express the activation markers CD44 and CD122 (Figure 3.1B and C) which are up-regulated on mature $\alpha\beta$ *i*NKT cells (156, 196). In addition, there is a population of $\alpha\beta$ *i*NKT and V6 cells that are PLZF$^+$ along with a proportion of both populations that remained CD4 positive (Figure 3.1B).

In contrast, examination of $\alpha\beta$ *i*NKT and V6 cells from *Itk*$^{-/-}$ mice reveals a

Figure 3.1: Spleen V6 cells in *Itk*$^{-/-}$ mice have increased expression of PLZF and IL-4 transcription.

Cells were prepared from spleens of wild type and *Itk*$^{-/-}$ mice or 4Get and *Itk*-4Get mice and analyzed by flow cytometry. (A) Splenocytes were stained with anti-TCRδ for γδ T cells. γδ T cells were further stained with anti-Vγ1.1 and anti-Vδ6.3 to examine the V6 population. *i*NKT cells were also examined by staining with CD1d tetramer and anti-TCRβ. (B) HSA$^-$ V6 γδ T cells and *i*NKT cells were further analyzed for expression of CD44, CD122, CD4 and PLZF. Percentage of HSA$^-$ V6 γδ T cells and *i*NKT CD44$^+$, CD122$^+$ and CD4$^+$ are shown. Each data point represents one mouse with the black bar indicating the mean. (C) HSA$^-$ V6 γδ T cells and *i*NKT cells from 4Get and *Itk*-4Get mice were stained and analyzed for CD44 vs GFP, CD122 vs GFP and CD4 vs GFP. Data are representative of at least two independent experiments. (D) Absolute numbers of PLZF$^+$ cells, % and absolute numbers of GFP$^+$ from HSA$^-$ V6 γδ T cells and *i*NKT cells are shown.

Figure 3.1


A
Total splenocytes
γδ T cells
iNKT cells
WT
Itk-/-
TCR δ
SSC-A
Vγ1.1
Vδ6.3
CD1d Tet
TCR β
B
V6 γδ T cells
iNKT cells
CD44
CD122
CD4
PLZF
% of CD44+ cells
% of CD122+ cells
% of CD4+ cells
p=0.0004
p=0.0007
p=0.0003
p<0.0001
p=0.0057
C
4Get
Itk-4Get
GFP
D
PLZF+ cells
% of GFP+ cells
# GFP+ cells
p<0.0001
p=0.0022

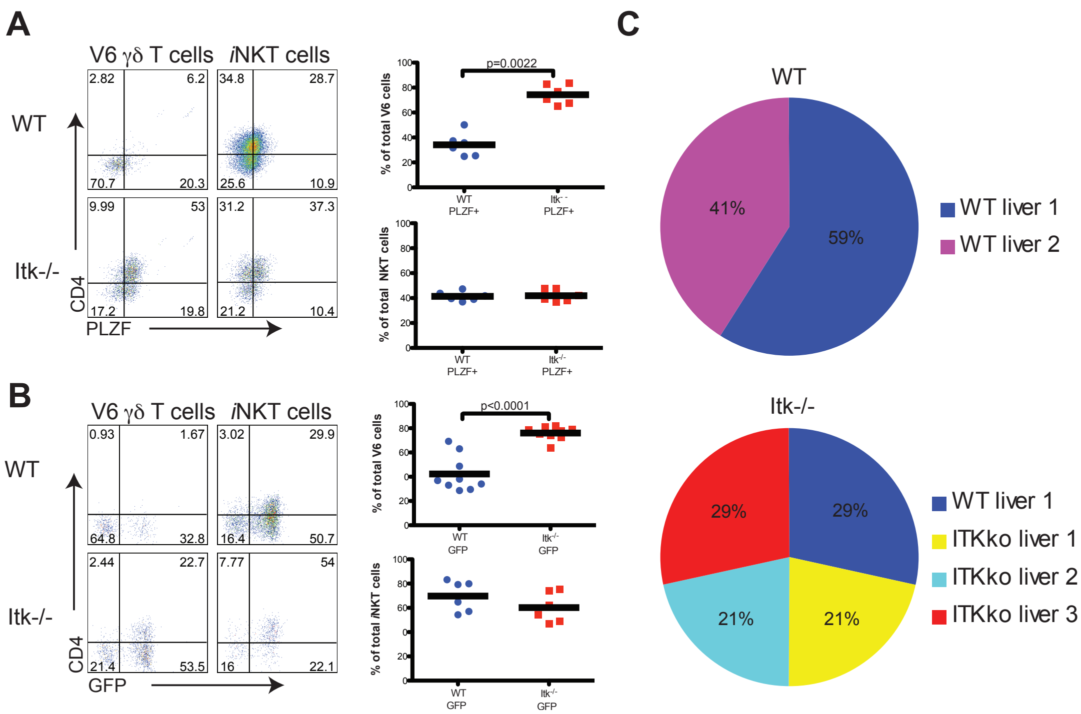
drastically altered phenotype when compared to wild type controls. While the majority of *Itk*$^{-/-}$ V6 cells express CD44 and CD122, like wild type cells, they retain expression of PLZF and CD4, suggesting that *Itk*$^{-/-}$ V6 cells are similar to the *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells. We then wondered whether this altered phenotype correlated with altered functionality as previous studies have demonstrated that as $\alpha\beta$ *i*NKT cells mature IL-4 production decreases (156). Using bicistronic IL-4/enhanced GFP reporter (4Get) mice (197) we were able to compare the proportion of HSA^{-} $\alpha\beta$ *i*NKT and V6 cells expressing IL-4 in the presence or absence of Itk. As shown in Figure 3.1C, the vast majority of V6 cells from *Itk*$^{-/-}$ mice were GFP positive compared to V6 cells from wild type controls similar to what is seen for wild type and *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells. In both $\alpha\beta$ *i*NKT and V6 cells from wild type and *Itk*$^{-/-}$ 4Get mice, cells were CD44^{+} and GFP^{+} yet upon examination of CD122, while many of the wild type 4Get $\alpha\beta$ *i*NKT and V6 cells were CD122^{+} and very few GFP single positive, $\alpha\beta$ *i*NKT and V6 cells from *Itk*$^{-/-}$ mice were still GFP single positive. This is consistent with the possibility that *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT and V6 cells are leaving the thymus at an immature stage. Interestingly, the cell surface phenotype of V6 cells from 4Get mice matched the cell surface phenotype of PLZF expressing V6 cells (Figures 3.1B and C), a trend that remained true in *Itk*$^{-/-}$ mice as well. Finally, Figure 3.1D shows that the total number and proportion of GFP positive cells is significantly increased in *Itk*$^{-/-}$ mice compared to WT counterparts. This observation is also true when looking at PLZF positive cells, indicating a correlation between PLZF expression and IL-4 transcription. Taken together, these data indicate that the majority of V6 cells from the spleens of *Itk*$^{-/-}$ mice display an immature phenotype.

Itk$^{-/-}$ *V6 cells do not mature in the periphery*

It remained to be seen, however if the V6 cells from *Itk*$^{-/-}$ mice displayed this immature phenotype throughout the periphery or only in the spleen. Since it is well documented that αβ *i*NKT cells continue to mature in the periphery (98, 100) we next examined these populations in the liver where αβ *i*NKT and V6 cells preferentially home (68, 99) to examine whether or not αβ *i*NKT and V6 cells continued to express elevated levels of PLZF and GFP in the liver. Indeed, the percentage of both αβ *i*NKT cells and V6 cells expressing low levels of PLZF from wild type mice are increased in the liver compared to the splenic compartment (Figure 3.2A). Furthermore, the majority of V6 cells from *Itk*$^{-/-}$ mice continue to display an immature phenotype, expressing both CD4 and PLZF (Figure 3.2A). Consistent with data from the spleen, V6 cells from the livers of *Itk*$^{-/-}$ 4Get mice remain positive for GFP compared to wild type counterparts (Figure 3.2B). One of the hallmarks of innate T lymphocytes is a restricted TCR repertoire (11). Therefore we wanted to examine the TCR delta usage of V6 cells from the livers of wild type and *Itk*$^{-/-}$ mice. Sequencing of the TCR also allows for closer examination of whether or not V6 cells were generated from fetal precursors or adult precursors based upon N nucleotide additions (76). As expected V6 cells from wild type mice exhibit limited diversity in their TCR delta

Figure 3.2: Liver V6 cells in *Itk*$^{-/-}$ mice display an immature phenotype.
Cells were prepared from livers of wild type and *Itk*$^{-/-}$ mice or 4Get and *Itk*-4Get mice and analyzed by flow cytometry. (A) Wild type and *Itk*$^{-/-}$ HSA^{-} V6 $\gamma\delta$ T cells and *i*NKT cells were analyzed for expression of CD4 and PLZF. Percentage of HSA^{-} V6 $\gamma\delta$ T cells and *i*NKT PLZF^{+} cells are shown. (B) 4Get and *Itk*-4Get HSA^{-} V6 $\gamma\delta$ T cells and *i*NKT cells were analyzed for expression of CD4 and PLZF. Percentage of HSA^{-} V6 $\gamma\delta$ T cells and *i*NKT PLZF^{+} cells are shown. Each data point represents one mouse with the black bar indicating the mean. (C) Chart represents sequences and classification of sequences of Vδ6 and Jδ1 junctions from sorted V6 $\gamma\delta$ T cells from wild type and *Itk*$^{-/-}$ livers.

Figure 3.2


A
V6 γδ T cells
iNKT cells
WT
Itk-/-
CD4
PLZF
2.82
6.2
70.7
20.3
34.8
28.7
25.6
10.9
9.99
53
17.2
19.8
31.2
37.3
21.2
10.4
p=0.0022
% of total V6 cells
% of total NKT cells
WT PLZF+
Itk-/- PLZF+
B
V6 γδ T cells
iNKT cells
WT
Itk-/-
CD4
GFP
0.93
1.67
64.8
32.8
3.02
29.9
16.4
50.7
2.44
22.7
21.4
53.5
7.77
54
16
22.1
p<0.0001
% of total V6 cells
% of total iNKT cells
WT GFP
Itk-/- GFP
C
WT
41%
59%
WT liver 1
WT liver 2
Itk-/-
29%
29%
21%
21%
WT liver 1
ITKko liver 1
ITKko liver 2
ITKko liver 3

usage, using only two unique sequences. These sequences very few N nucleotide additions suggesting that rearrangement occurred prior to TdT expression. In addition, all the sequences lack the Dδ1 gene segment and use of the canonical Dδ2-Jδ1 junction (Figure 3.2C and Figure 3.3). This suggests that wild type V6 livers cells in the liver develop from fetal or postnatal precursors. In contrast, V6 cells from *Itk*$^{-/-}$ mice use four unique sequences, three of which are not even found in the livers of wild type mice (Figure 3.2C and Figure 3.3). Interestingly, only one of the V6 sequences found in *Itk*$^{-/-}$ mice contains N nucleotide additions suggesting that this particular sequence was generated from an adult precursor. The inability of V6 cells from *Itk*$^{-/-}$ mice to restrict their TCR usage suggest that the majority of V6 cells in periphery of *Itk*$^{-/-}$ mice are immature.

Developmental progression of V6 cells is impaired in Itk$^{-/-}$ *mice*

The data thus far indicate that Itk is important in mediating the maturation of V6 cells and that in its absence V6 cells are inefficiently transitioning through the maturation stages similar to Itk$^{-/-}$ αβ *i*NKT cells. We therefore decided to inspect the thymi of wild type and *Itk*$^{-/-}$ mice by examining the expression of CD4, CD122, CD44 and PLZF expression on αβ *i*NKT and V6 cells to determine if and where the defect occurs. As observed previously, the percentage of *i*NKT cells in *Itk*$^{-/-}$ mice is decreased by approximately half compared to wild type mice (Figure 3.4A) (155, 157, 158). On the other hand, a substantial increase in the

Figure 3.3: Liver sequence analysis of Vδ6-Jδ1 junctions

Shown sequences are the junctional regions of Vδ6 (variable region), Dδ1 and 2 (diversity region) and Jδ1 (joining region) rearrangements in 17 wild type- and 14 *Itk*$^{-/-}$-V6 clones, respectively. Palindromic (P) and N nucleotides are marked in red color. Polymorphic sequences are compared to the germline sequence. The frequency (left) implies the clone numbers for the sequence and simple note (right) marks brief classification of each sequence. After sequencing, off-frame sequences were not considered.

Figure 3.3

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
	GCT CTC TGG GAG CTG G		ATGGCATAT		ATCGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E L V				I G G I R A		T D K	

WT liver sorted by V6

(Frequency)

Fetal precursor

(9)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 1
	A L W E H				I G G I R A		T D K	
(1)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACGAG		CT ACC GAC	WT liver 1'
	A L W E H				I G G I R A		T D	
(6)	GCT CTC TGG GAG CT	T			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 2
	A L W E L				I G G I R A		T D K	
(1)	GCT CTC TGG GAG CT	T			ATCGGAGGGATACGAG		CT	WT liver 2'
	A L W E L				I G G I R A			

ITKko liver sorted by V6

(Frequency)

Fetal precursor

(4)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 1
	A L W E H				I G G I R A		T D K	
(3)	GCT CTC TGG GAG CTG G				TCGGAGGGATACGAG		CT ACC GAC AAA	ITKko liver 1
	A L W E L V				G G I R A		T D K	
(3)	GCT CTC TGG GAG CTG G	C			CGGAGGGATACGAG		CT ACC GAC AAA	ITKko liver 2
	A L W E L A				G G I R A		T D	

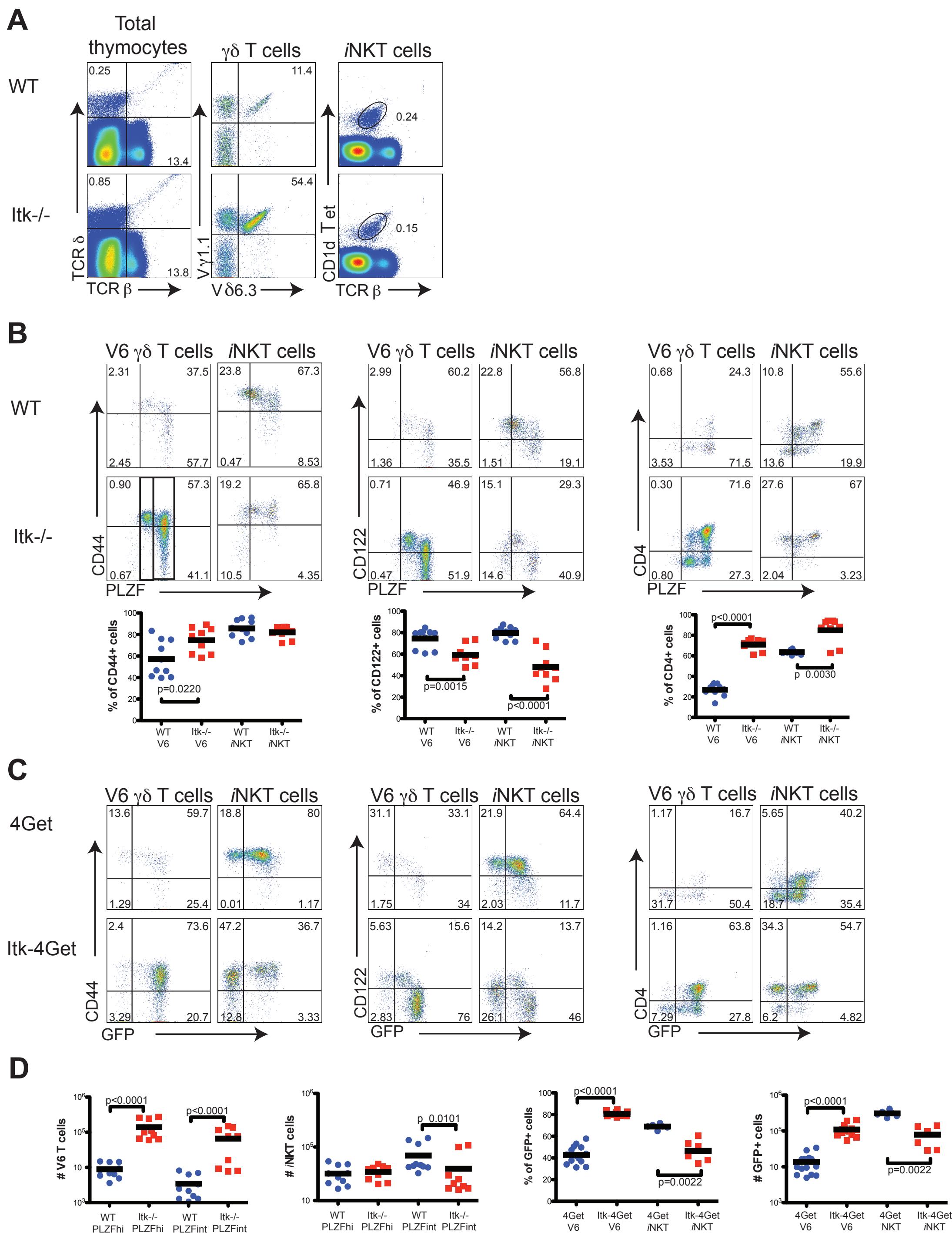
Adult precursor

(4)	GCT CTC TGG GAG CTG G	TCCCATAT	ATGGC	CCTTT	ATCGGAGGGATACG	GCA	ACC GAC AAA	ITKko liver 3
	A L W E L V	P Y	M A	L Y	R R D T	A	T D K	

Figure 3.4: Absence of Itk results in a partial block in $\gamma\delta$ NKT cell maturation.

Cells were prepared from thymuses of wild type and *Itk*$^{-/-}$ mice or 4Get and *Itk*-4Get mice and analyzed by flow cytometry. (A) Thymocytes were stained with anti-TCRδ for $\gamma\delta$ T cells. $\gamma\delta$ T cells were further stained with anti-Vγ1.1 and anti-Vδ6.3 to examine the V6 population. *i*NKT cells were also examined by staining with CD1d tetramer and anti-TCRβ. (B) HSA^{-} V6 $\gamma\delta$ T cells and *i*NKT cells were further analyzed for expression of CD44, CD122, CD4 and PLZF. Percentage of HSA^{-} V6 $\gamma\delta$ T cells and *i*NKT cells CD44^{+}, CD122^{+} and CD4^{+} are shown. Each data point represents one mouse with the black bar indicating the mean. (C) HSA^{-} V6 $\gamma\delta$ T cells and *i*NKT cells from 4Get and *Itk*-4Get mice were stained and analyzed for CD44 vs GFP, CD122 vs GFP and CD4 vs GFP. Data are representative of at least two independent experiments. (D) PLZF hi and intermediate V6 $\gamma\delta$ T cell and *i*NKT cell populations were gated as shown in B and absolute numbers are shown. Percent and absolute numbers of GFP^{+} from HSA^{-} V6 $\gamma\delta$ T cells and *i*NKT cells are shown.

Figure 3.4


A
Total thymocytes
γδ T cells
iNKT cells
WT
Itk-/-
TCR δ
TCR β
Vγ1.1
Vδ6.3
CD1d Tet
B
V6 γδ T cells
iNKT cells
CD44
CD122
CD4
PLZF
C
4Get
Itk-4Get
GFP
D
V6 T cells
iNKT cells
% of GFP+ cells
# GFP+ cells

proportion of $\gamma\delta$ T cells can be attributed to an expansion in the V6 cell population of *Itk*$^{-/-}$ mice (Figure 3.4A).

Several stages of $\alpha\beta$ *i*NKT cell differentiation have been identified previously using a variety of cell surface markers, cytokines, and transcription factors. For example, up-regulation of CD44 and CD122 demarks a transition from stage 2 to stage 3 (93, 99). Similar to $\alpha\beta$ *i*NKT cells, HSA^{-} V6 cells up-regulate CD44 while concurrently up-regulating CD122. A population of *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells remain CD122^{-}, in contrast the majority of wild type $\alpha\beta$ *i*NKT cells become CD122^{+} as has been previously shown (Figure 3.4B) (156-158). Compared to wild type mice, *Itk*$^{-/-}$ mice contain an increased percentage of CD44^{+} cells. Interestingly a proportion of the V6 cells from these mice remain CD122^{-} suggesting a defect during the stage 2 to stage 3 transition of the maturation process (Figure 3.4B). One of the few differences between $\alpha\beta$ *i*NKT cells and V6 cells seems to be the expression of CD4, with $\alpha\beta$ *i*NKT cells largely remaining CD4^{+} and V6 cells being CD4^{-}. Comparing the V6 cells in the periphery to those in the thymus, it appears that CD4 becomes down-regulated during development. However, a significant proportion of V6 thymocytes from *Itk*$^{-/-}$ mice remain positive for this marker (Figure 3.4B). One of the major transcription factors shown to be important in the development of $\alpha\beta$ *i*NKT cells is PLZF (81, 82). As $\alpha\beta$ *i*NKT cells progress through the maturation stages, they begin to down-regulate PLZF (81, 82). Given the similarities that have been seen in the spleen and liver between $\alpha\beta$ *i*NKT cells and V6 cells we examined

PLZF expression in the thymus. As both wild type and *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells and V6 cells mature, a progressive down-regulation of PLZF occurs such that two distinct populations arise: PLZF^{hi} and PLZF^{int} (Figure 3.4B). These populations are not seen in wild type $\alpha\beta$ *i*NKT and V6 cells found in the spleen and liver (Figure 3.1B and 3.2A) where there only seems to be a corresponding population of PLZF$^{lo/int}$ population which is in agreement with studies that show that PLZF down regulates as $\alpha\beta$ *i*NKT cells migrate out into the periphery (81, 82). While there is no difference in the ratio of PLZF^{hi} to PLZF^{int} cells between wild type and *Itk*$^{-/-}$ mice (data not shown), there is an overall increase in the total number of both the PLZF^{hi} and PLZF^{int} populations in *Itk*$^{-/-}$ mice (Figure 3.4D, far left panel).

It has been well documented that $\alpha\beta$ *i*NKT cell development also correlates with IL-4 cytokine mRNA which results in decreased IL-4 protein levels as they mature (101, 156). Therefore we wanted to examine the ability of V6 cells to produce IL-4 in the presence or absence of Itk. Using 4Get reporter mice crossed to *Itk*$^{-/-}$ mice, we were able to examine the various stages of both $\alpha\beta$ *i*NKT and V6 cell development using CD44, CD122 and CD4 surface staining in the thymus in conjunction with IL-4 transcription. Consistent with previous studies (101, 157) and in agreement with the PLZF data, wild type $\alpha\beta$ *i*NKT cells have very few GFP single positive cells classified as stage 2 cells (Figure 3.4C). While *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells have increased percent of GFP single positive cells, overall expression of GFP is decreased in *Itk*$^{-/-}$ compared to wild type $\alpha\beta$ *i*NKT cells (Figure 3.4D). Closer examination of V6 cells from wild type mice show three distinct populations in each of the three cell surface markers examined, with GFP expression correlating with each stage of development similar to $\alpha\beta$ *i*NKT cells. For example, as CD44 and CD122 expression is up-regulated, IL-4 transcription decreases representing a transition from stage 3 to sage 4 with GFP single positive cells representing stage 2. In *Itk*$^{-/-}$ mice however, an altered proportion of V6 cells in each stage of development is present. Consistent with PLZF staining, it appears that the majority of V6 cells in *Itk*$^{-/-}$ mice are inefficient at transitioning from the $GFP^{+}CD122^{-}$ to the $GFP^{+}CD122^{+}$ stage where there are 76% compared to 34% of V6 $GFP^{+}CD122^{-}$ cells in wild type mice (Figure 3.4C). A defect in the progression of V6 cells at these early maturation points could indicate an accumulation in cells that expressed PLZF as well as IL-4 transcription similar to what is observed for *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells. Indeed, when the total number of V6 cells expressing PLZF or GFP is quantified, there is a significant increase in *Itk*$^{-/-}$ mice compared to wild type (Figure 3.4D).

Altered Cytokine Profile in Itk$^{-/-}$ *V6 cells*

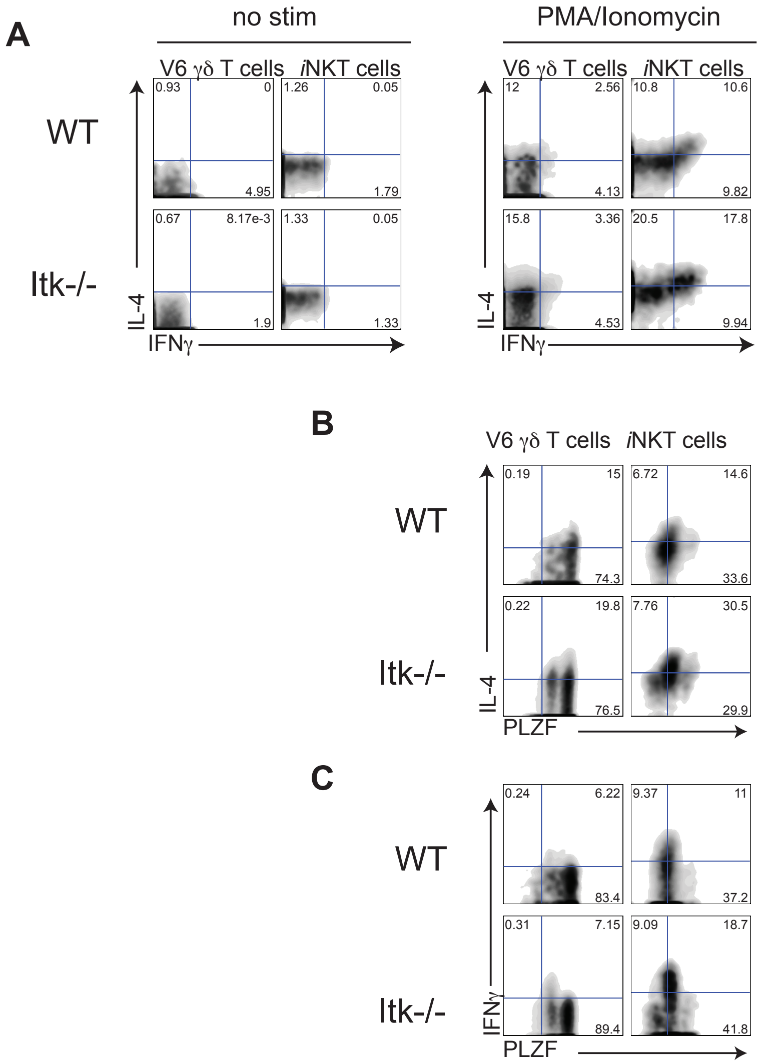
Although the data presented above suggests that both wild type and *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells constitutively express IL-4 mRNA which is thought to lead to rapid secretion of IL-4 it was unexpected that *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells are defective in IL-4 secretion upon stimulation with αGalCer (157, 158). In addition, given that the defect in maturation of *Itk*$^{-/-}$ V6 cells was similar to the defect seen in *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells, it was surprising that these cells could be responsible for the hyper-

IgE syndrome in *Itk*$^{-/-}$ mice. We have previously shown that splenic $\gamma\delta$ T cells in *Itk*$^{-/-}$ secrete high levels of IL-4 upon activation *ex vivo* (155). Together this suggested that there might be an altered cytokine profile associated with the V6 cell population. Furthermore, we wanted to know if there was a correlation between the amount of PLZF expressed and the cytokines produced. To this end, we stimulated wild type and *Itk*$^{-/-}$ thymocytes with PMA and ionomycin, which bypass the TCR, and examined IFNγ and IL-4 production in $\alpha\beta$ *i*NKT and V6 cells. Similar to wild type and *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells, V6 cells are capable of producing both cytokines upon stimulation (Figure 3.5A). However, the proportion of wild type and *Itk*$^{-/-}$ V6 cells producing IFNγ and IL-4 simultaneously is far less than $\alpha\beta$ *i*NKT cells. Surprisingly, there was very little difference in the frequency of total *Itk*$^{-/-}$ V6 cells making IFNγ and/or IL-4 compared to wild type V6 cells (Figure 3.5A). Because cytokine production in $\alpha\beta$ *i*NKT cells is mediated by PLZF and loss of PLZF in both $\alpha\beta$ *i*NKT and V6 cells results in a loss of IL-4 and IFNγ double secretors (81-83, 97), we decided to examine PLZF-expressing V6 cells to determine if there was a difference in whether or not PLZF^{hi} and PLZF^{int} preferentially secreted IL-4 or IFNγ. Following PMA and ionomycin stimulation, a greater proportion of *Itk*$^{-/-}$ V6 cells are making IL-4 than V6 cells from wild type mice (Figure 3.5B). In contrast, there is no difference in the frequency of IFNγ producing cells between wild type and *Itk*$^{-/-}$ V6 cells (Figure 3.5C). As expected, the majority of IL-4 production appears to come from V6 cells that are PLZF^{hi}

Figure 3.5: Level of PLZF expression correlates with IL-4 and IFNγ production.

Intracellular staining for IL-4, IFNγ and PLZF for wild type and *Itk*$^{-/-}$HSA^{-} V6 thymocytes were analyzed by flow cytometry. (A) Non stimulated (left) and stimulated (right) HSA^{-} V6 $\gamma\delta$ T cells and *i*NKT cells from the thymus were analyzed for IL-4 and IFNγ along PLZF expression (B and C). Wild type and *Itk*$^{-/-}$ thymocytes stimulated with 10ng/ml PMA and 2 μg/ml Ionomycin for 5 hours.

Figure 3.5


A
no stim
PMA/Ionomycin
V6 γδ T cells
iNKT cells
WT
Itk-/-
IL-4
IFNγ
0.93
0
4.95
1.26
0.05
1.79
0.67
8.17e-3
1.9
1.33
0.05
1.33
12
2.56
4.13
10.8
10.6
9.82
15.8
3.36
4.53
20.5
17.8
9.94
B
V6 γδ T cells
iNKT cells
WT
Itk-/-
IL-4
PLZF
0.19
15
74.3
6.72
14.6
33.6
0.22
19.8
76.5
7.76
30.5
29.9
C
WT
Itk-/-
IFNγ
PLZF
0.24
6.22
83.4
9.37
11
37.2
0.31
7.15
89.4
9.09
18.7
41.8

while IFNγ is primarily produced by PLZF^{lo} cells. Together, these data suggest that as cells down-regulate PLZF expression the cytokine profile changes from primarily IL-4 production to IFNγ, IL-4 co-producers. Further, V6 cells from *Itk*$^{-/-}$ mice are defective in this cytokine transition indicating a problem with maturation.

Gene signature of Itk$^{-/-}$ *V6 cells*

Thus far, the data suggests that wild type $\alpha\beta$ *i*NKT and V6 cells develop along a similar pathway and a defect exists in the developmental progression of V6 cells when Itk is not present. In order to better understand some of the molecular pathways involved in this process, we sorted V6 cells from the thymi of *Itk*$^{-/-}$ mice and compared them in a microarray to T cell populations found in wild type mice to determine the degree of similarity between $\alpha\beta$ *i*NKT and V6 cells from wild type and *Itk*$^{-/-}$ mice. As expected, the expression of a variety of genes is altered in *Itk*$^{-/-}$ V6 cells when compared with the most mature population of V6 cells in wild type mice (Figure 3.6A, Table 3.1 and 3.2). Using the Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis program, we were able to identify numerous KEGG pathways that appeared to be disregulated in *Itk*$^{-/-}$ V6 cells (Figure 3.6B). These pathways were involved in cytokine signaling, some of which are required for the development of T cells, as well as effector function, which included receptors frequently associated with $\alpha\beta$ *i*NKT and NK cells. As a case in point, we examined the expression of several NK cell receptors that can mediate effector function in $\alpha\beta$ *i*NKT cells that were

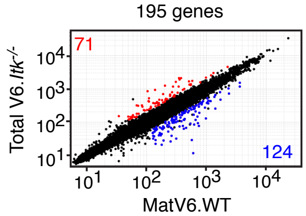
Figure 3.6: Gene expression analysis of V6 cells.

(A) Scatter plot showing expression of consolidated probe sets by populations of thymocytes from adult wild type and *Itk*$^{-/-}$ mice. Each dot represents one gene (mean of all probe sets and mean of replicates); red and blue indicate genes with expression increased or decreased, respectively, by more than twofold ($P < 0.05$ (Student's *t*-test); coefficient of variation < 0.5; mean expression value (MEV) > 120 in one subset); numbers in parentheses above plot indicates total number of these genes. The numbers within the plot indicate the number of up-regulated (red) or down-regulated (blue) genes in the comparison. Data are from three independent experiments with 4–30 mice each. (B) The 124 genes increased in TotalV6. *Itk*$^{-/-}$ versus MatV6.WT cells were classified into functional pathways using the KEGG analysis program (DAVID). The top five pathways enriched in the dataset are shown along with the number of genes, the gene names, the *P*-value, and the Benjamini *P*-value for each pathway. (C) Venn diagram comparing the list of genes that were increased or decreased in NKT (NKT.44^{+}NK1.1^{+}) cells versus MatCD4 and MatCD8 single positive thymoctyes that were identified previously (49) with the list of 124 genes down-regulated in TotalV6. *Itk*$^{-/-}$ versus MatV6.WT cells. Roughly 40% of the genes decreased in TotalV6. *Itk*$^{-/-}$ versus MatV6.WT cells are normally increased in NKT.44^{+}NK1.1^{+} cells versus mature single-positives. (D) Heat map showing relative expression of the 51 genes identified in 3.6C that were decreased in TotalV6.*Itk*$^{-/-}$ versus MatV6.WT cells and increased in NKT.44^{+}NK1.1^{+} cells versus MatCD4 and

MatCD8 single positive thymoctyes in populations of NKT and MatCD4 cells from WT mice and V6 cells from WT or *Itk*$^{-/-}$ mice. Data were log transformed, centered by gene row and hierarchically clustered by gene and subset. The clustering dendrogram for samples is shown.

Figure 3.6

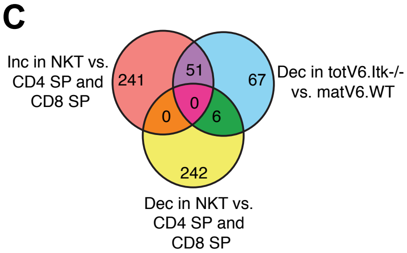
A



B

KEGG Pathway	# of Genes in Pathway	Genes	P-value	Benjamini
Natural killer cell mediated cytotoxicity	14	Cd244, Fasl, Fcer1g, Gzmb, Ifng, Ifngr1, Klrc1, Klrc2, Klrk1, Klrd1, Ncr1, Prkcb, Klra9, Tnfsf10	3.1×10^{-11}	2.3×10^{-9}
Cytokine-cytokine receptor interaction	13	Fasl, Xcl1, Ccl5, Ccr10, Ccr8, Ifng, Ifngr1, Il10ra, Il18rap, Il2rb, Cx3cr1, Il21, Tnfsf10	1.3×10^{-6}	4.7×10^{-5}
Graft-versus-host disease	7	Fasl, Gzmb, H2-Q6, Ifng, Klrc1, Klrd1, Klra9	7.7×10^{6}	1.9 x10
Chemokine signaling pathway	8	Lyn, Xcl1, Ccl5, Ccr10, Ccr8, Prex1, Prkcb, Cx3cr1	1.0×10^{-3}	1.9×10^{-2}
Jak-STAT signaling pathway	7	Ifng, Ifngr1, Il10ra, Il2rb, Stat4, Il21, Spry2	2.4×10^{3}	3.5×10^{2}

C



D

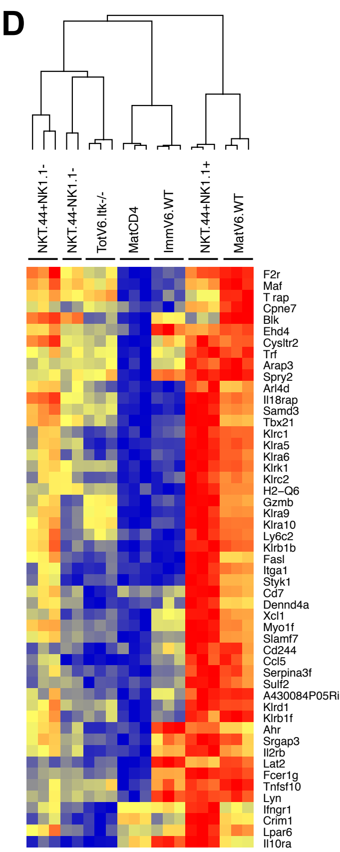


Table 3.1: Genes that are down-regulated between MatV6.WT and Total V6.*Itk*$^{-/-}$ cells.

List of the 124 genes that were decreased in Total V6.*Itk*$^{-/-}$ cells compared to MatV6.WT cells. Genes were classified as being decreased if the mean expression value (MEV) was changed by 2-fold or more in pair wise comparisons with a coefficient of variation among replicates <0.5, *t*-test P-value<0.05, and MEV>120 in at least one population.

Table 3.1: Genes that are down-regulated in Total V6.*Itk*$^{-/-}$ cells.

2010002N04Rik	*Ikzf2*	*Rnu3a*
5830411N06Rik	*Ikzf3*	*Rnu3b1*
A430084P05Rik	*Il10ra*	*Rorc*
A430093F15Rik	*Il18rap*	*Rxra*
Adamts10	*Il21*	*Samd3*
Ahr	*Il2rb*	*Sccpdh*
Angptl2	*Itga1*	*Scpep1*
Anxa6	*Itga4*	*Serpina3f*
Arap3	*Itgae*	*Sidt1*
Arl4d	*Jun*	*Slamf7*
Atp5d	*Klf2*	*Slc15a2*
Blk	*Klra10*	*Slc25a38*
Ccl5	*Klra5*	*Slc9a7*
Ccr10	*Klra6*	*Snord118*
Ccr8	*Klra9*	*Sox4*
Cd1d1	*Klrb1a*	*Spock2*
Cd244	*Klrb1b*	*Spp1*
Cd7	*Klrb1f*	*Spry2*
Cmah	*Klrc1*	*Srgap3*
Cpne7	*Klrc2*	*Stat4*
Crim1	*Klrd1*	*Styk1*
Cx3cr1	*Klrk1*	*Sulf2*
Cysltr2	*Lag3*	*Tagap*
Dad1	*Lass6*	*Tbx21*
Dennd4a	*Lat2*	*Thbs1*
Ehd4	*Lgals1*	*Tirap*
Emp1	*Lpar6*	*Tm6sf1*
Emp3	*Lrrc6*	*Tmem176b*
Epha3	*Ly6c2*	*Tnfsf10*
F2r	*Lyn*	*Tns4*
Fam20a	*Maf*	*Trf*
Fasl	*Maged1*	*Trps1*
Fcer1g	*Ms4a4b*	*Ubash3b*
Fosb	*Myo1f*	*Vdr*
Gpr114	*Ncr1*	*Xcl1*
Gpr177	*Neurl3*	*Xdh*
Gpr52	*Nkg7*	
Gzmb	*Nr4a1*	
H19	*Nrp1*	
H2-Q6	*Otud1*	
Hist1h2bg	*Plscr1*	
I730030J21Rik	*Prex1*	
Ifng	*Prkcb*	
Ifngr1	*Rnu1b1*	

Table 3.2: Genes that are up-regulated in Total V6.*Itk*$^{-/-}$ cells relative to MatV6.WT cells.

List of the 71 genes that were increased in Total V6.*Itk*$^{-/-}$ cells compared to MatV6.WT cells. Genes were classified as being increased if the mean expression value (MEV) was changed by 2-fold or more in pair wise comparisons with a coefficient of variation among replicates <0.5, *t*-test P-value<0.05, and MEV>120 in at least one population.

Table 3.2: Genes that are up-regulated in Total V6.*Itk*$^{-/-}$ cells relative to MatV6.WT cells.

1110002B05Rik	*Fam46c*	*Nrn1*
1810011H11Rik	*Fam84a*	*Pde7b*
2010016I18Rik	*Gadl1*	*Plce1*
Adh1	*Gm10673*	*Pttg1*
AI747699	*Gm10785*	*Rnf7*
Akap6	*Gm12643*	*Robo1*
Aldh2	*Gm13194*	*Rpl22l1*
Aqp9	*Gm6134*	*Rpl35a*
Art2b	*Gm6983*	*Rps19*
Asns	*Gm7239*	*Rps3a*
Ass1	*Gm7693*	*Rps6ka2*
Atp6v0d2	*Gm8936*	*Sgk3*
Atp8a2	*Gm9197*	*Slc15a1*
B930036N10Rik	*Gpnmb*	*Smpdl3b*
BC002163	*Grm4*	*Spc25*
BC005685	*Hc*	*St8sia1*
Ccr2	*Hectd2*	*Tdgf1*
Ccr9	*Hist1h1b*	*Tex2*
Cd4	*Impdh2*	*Themis*
Cdkn1b	*LOC625360*	*Tmie*
Cox6a1	*LOC641050*	*Trim36*
Ctla2a	*Lrig1*	*Utp3*
D10Ertd322e	*Lztfl1*	*Vegfa*
Fam169b	*Mpp6*	

shown to be down-regulated in *Itk*$^{-/-}$ V6 cells compared to wild type (Figure 3.7). Many of the receptors that were identified to be down-regulated in the microarray were confirmed by flow cytometry to be decreased or absent in V6 cells from *Itk*$^{-/-}$ mice. Acquisition of NK cell receptors is one of the final stages of αβ *i*NKT cell maturation (156, 195, 198) and appears to be a similar trait in V6 cells as well.

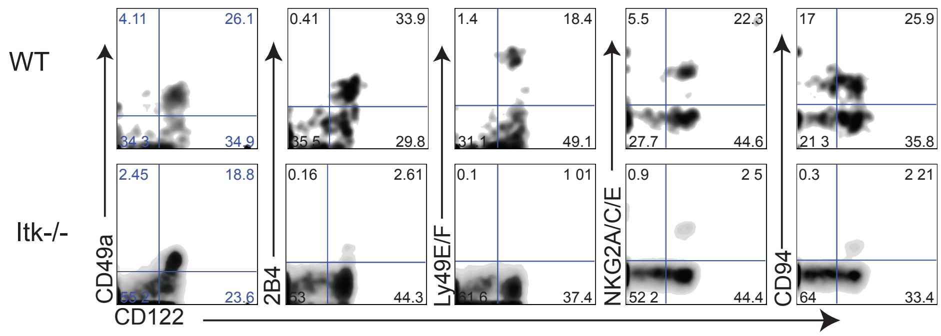
Given the similarity between αβ *i*NKT cells and V6 cells, we next used the αβ *i*NKT gene signature list generated previously by Narayan and colleagues (49). Figure 3.6C is a Venn diagram comparing wild type αβ *i*NKT cell signature genes previously described (49) that were found to be increased (292 genes) and decreased (248 genes) in αβ iNKT cells to genes that were found to be down-regulated (124 genes) in *Itk*$^{-/-}$ V6 cells versus wild type mature V6 cells. About 40% of genes that (51 genes) are normally up-regulated in mature αβ *i*NKT cells were found to be down-regulated in *Itk*$^{-/-}$ V6 cells (Figure 3.6C). This data suggests that not only are the wild type V6 cells similar to wild type αβ *i*NKT cells but that the *Itk*$^{-/-}$ V6 cells have a more immature gene profile. Using cluster analysis and comparing the 51 genes that wild type αβ *i*NKT and *Itk*$^{-/-}$ V6 cells share in Figure 3.6C, the heat map shows that wild type HSA^{-} V6 cells are similar to mature wild type αβ *i*NKT cells while the *Itk*$^{-/-}$ V6 cells are most similar to immature wild type αβ *i*NKT cells (Figure 3.6D).

Thymic environment influences V6 development

Figure 3.7: Decreased surface expression of NK cell markers in *Itk*$^{-/-}$ V6 cells.

HSA^{-} V6 thymocytes from wild type and *Itk*$^{-/-}$ mice were stained for expression of CD49a, 2B4, Ly49E/F, NKG2A/C/E, CD94 and CD122. Results are representative of 2 experiments with at least 3 mice per group.

Figure 3.7


WT
Itk-/-
CD49a
2B4
Ly49E/F
NKG2A/C/E
CD94
CD122
4.11
26.1
34.3
34.9
0.41
33.9
35.5
29.8
1.4
18.4
31.1
49.1
5.5
22.3
27.7
44.6
17
25.9
21 3
35.8
2.45
18.8
23.6
0.16
2.61
53
44.3
0.1
1 01
37.4
0.9
2 5
52 2
44.4
0.3
2 21
64
33.4

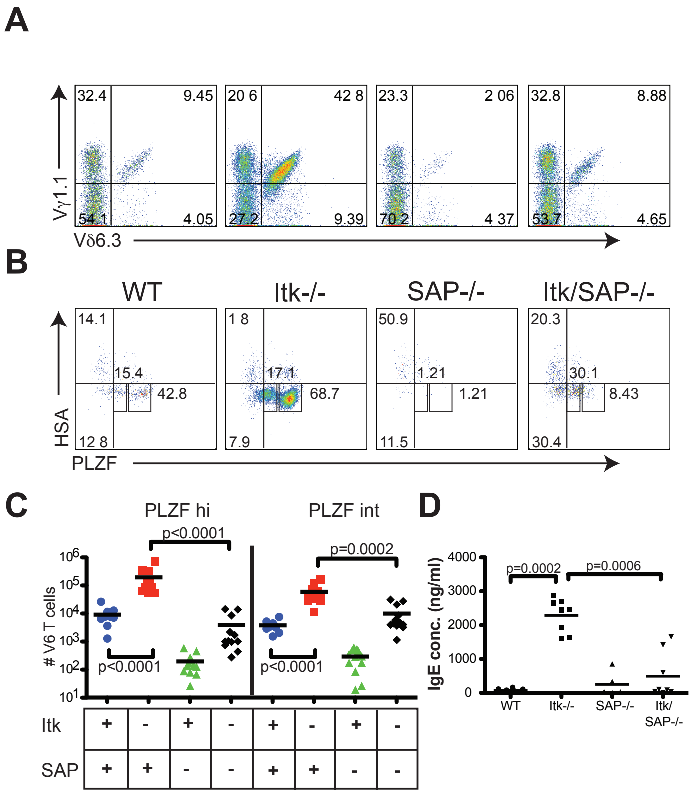
Considering the signals important for proper V6 cell development, we began to wonder what role other molecules played with respect to Itk. The SAP/SLAM signaling pathway (199, 200) have been shown to be important for the development of *i*NKT cells. In addition, the SAP/SLAM pathway has also been suggested to contribute to the development of V6 cells, as previous studies have shown lack of SAP results in a decrease in V6 cells (80, 83, 191). We crossed *Itk*$^{-/-}$ mice to *SAP*$^{-/-}$ animals generating a double knockout mice and examined the thymocytes to determine the developmental progression of the resulting V6 cells.

In the absence of Itk, the overall frequency of total V6 cells along with PLZF-expressing V6 cells from *SAP*$^{-/-}$ mice is diminished when compared to *Itk*$^{-/-}$ mice (Figure 3.8A-C). Closer inspection of the V6 cells in *Itk/SAP*$^{-/-}$ reveals that this difference is likely due to a severe reduction in the percentage of PLZF^{hi} V6 cells. Because the PLZF^{hi} V6 cells are responsible for the bulk of the IL-4 production, it is likely that the levels of serum IgE return to normal (Figure 3.8D) due to the decrease in the PLZF^{hi} V6 cell population and not because of an overall lack of total numbers, as their numbers of PLZF expressing cells are similar to wild type V6 cells. Interestingly, a large proportion of PLZF^{-} cells remain in the *Itk/SAP*$^{-/-}$ mice, a population that is not prevalent in both wild type and *Itk*$^{-/-}$ mice. Of even greater interest is the presence of PLZF^{-} HSA^{+} V6 cells in *SAP*$^{-/-}$ mice. These data suggest that SAP is required for certain developmental stages of V6 cells.

Figure 3.8: *Itk$^{-/-}$* V6 cells require SAP for hyper IgE phenotype

Thymocytes from wild type, *Itk$^{-/-}$, SAP$^{-/-}$* and *Itk/SAP$^{-/-}$* mice were analyzed by flow cytometry. (A) Thymocytes were stained for $\gamma\delta$ T cells using anti-TCRδ and further stained with anti-Vγ1.1 and anti-Vδ6.3 to examine the V6 population. (B) HSA^{-} V6 cells were analyzed for PLZF hi and intermediate expression for each genotype. (C) Absolute numbers of HSA^{-} V6 cells expressing PLZF hi and intermediate were compiled. (D) Serum obtained for each genotype were analyzed for IgE by ELISA.

Figure 3.8


A
32.4
9.45
20 6
42 8
23.3
2 06
32.8
8.88
Vγ1.1
54.1
4.05
27.2
9.39
70 2
4 37
53.7
4.65
Vδ6.3
B
WT
Itk-/-
SAP-/-
Itk/SAP-/-
14.1
1 8
50.9
20.3
15.4
42.8
17.1
68.7
1.21
1.21
30.1
8.43
HSA
12 8
7.9
11.5
30.4
PLZF
C
PLZF hi
PLZF int
p<0.0001
p=0.0002
# V6 T cells
p<0.0001
p<0.0001
Itk
SAP
D
IgE conc. (ng/ml)
p=0.0002
p=0.0006
WT
Itk-/-
SAP-/-
Itk/
SAP-/-

Vδ sequence of adult wild type V6 cell thymocytes

As *Itk*$^{-/-}$ V6 cells mature we wanted to examine if there was a restriction of the repertoire as they go through the different maturation stages given that the liver V6 cells surface phenotype and sequences from *Itk*$^{-/-}$ V6 cells suggested that they are leaving the thymus in an immature state. To determine if there was a restriction of the V6 TCR repertoire cells from wild type 4Get and *Itk*$^{-/-}$ 4Get mice were sorted and the Vδ6-(D)-Jδ1 junction was sequenced. Sequence analysis of maturation stages 2 (CD122^{-}GFP^{+}), 3 (CD122^{+}GFP^{+}) and 4 (CD122^{+}GFP^{-}) of V6 cells based upon CD122 and GFP expression from 4Get and *Itk*$^{-/-}$ 4Get thymocytes (Figure 3.9A and B respectively) indicated that as V6 cells mature there does not seem to be a restriction of the repertoire as the cells progress through the maturation stages. Instead a large proportion of the sequences (Figure 3.10) from both 4Get and *Itk*$^{-/-}$ 4Get thymocytes contain varying sequences of Vγ6.3 which is in contrast to the sequences found in wild type and *Itk*$^{-/-}$ liver (Figure 3.2C). Given that the sequences analyzed from wild type V6 liver cells do not contain N nucleotide additions this suggests that wild type liver V6 cells do not originate from adult thymus and that there are *Itk*$^{-/-}$ V6 cells that are derived from adult precursors migrating to the liver. Although there were many different sequences of V6 cells found in the both wild type and *Itk*$^{-/-}$ mice there are also a mixture of sequences containing both N nucleotides and contain the Dδ1 gene segment indicating these V6 cells were generated from adult precursors. On the other hand, there are also sequences containing only

Figure 3.9: V6 cells in the thymus develop from fetal and adult precursors

Chart represents frequency and classification of sequences of Vδ6 and Jδ1 junctions from CD122$^-$GFP$^+$ (Stage 2), CD122$^+$GFP$^+$ (Stage 3), CD122$^+$GFP$^-$ (Stage 4) V6 thymocytes sorted from 4Get (A) and *Itk*-4Get (B) mice.

Figure 3.9

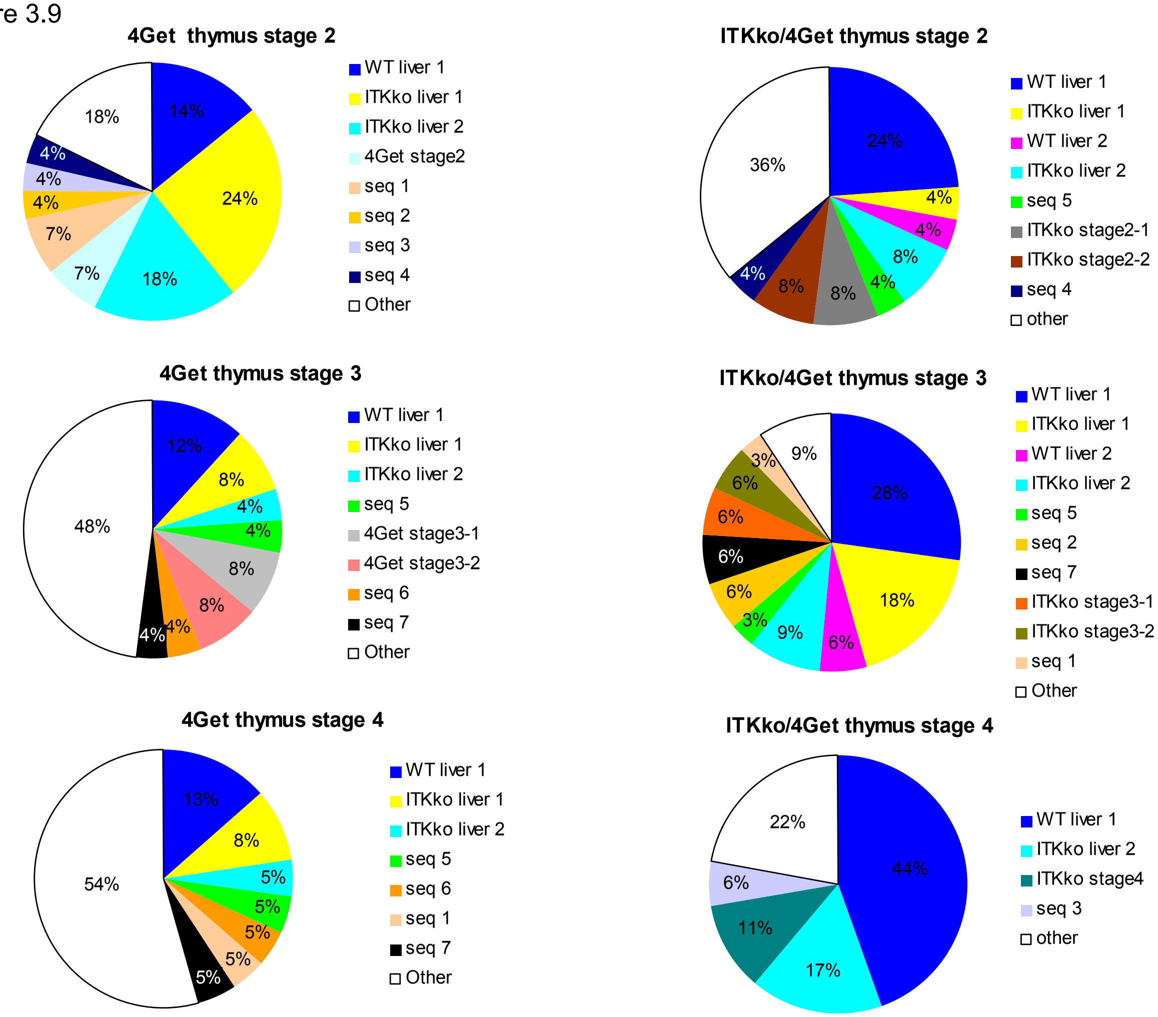

4Get thymus stage 2
14%
24%
18%
7%
7%
4%
4%
4%
18%
WT liver 1
ITKko liver 1
ITKko liver 2
4Get stage2
seq 1
seq 2
seq 3
seq 4
Other
ITKko/4Get thymus stage 2
24%
4%
4%
8%
4%
8%
8%
4%
36%
WT liver 1
ITKko liver 1
WT liver 2
ITKko liver 2
seq 5
ITKko stage2-1
ITKko stage2-2
seq 4
other
4Get thymus stage 3
12%
8%
4%
4%
8%
8%
4%
4%
48%
WT liver 1
ITKko liver 1
ITKko liver 2
seq 5
4Get stage3-1
4Get stage3-2
seq 6
seq 7
Other
ITKko/4Get thymus stage 3
28%
18%
6%
9%
3%
6%
6%
6%
6%
3%
9%
WT liver 1
ITKko liver 1
WT liver 2
ITKko liver 2
seq 5
seq 2
seq 7
ITKko stage3-1
ITKko stage3-2
seq 1
Other
4Get thymus stage 4
13%
8%
5%
5%
5%
5%
5%
54%
WT liver 1
ITKko liver 1
ITKko liver 2
seq 5
seq 6
seq 1
seq 7
Other
ITKko/4Get thymus stage 4
44%
17%
11%
6%
22%
WT liver 1
ITKko liver 2
ITKko stage4
seq 3
other

Figure 3.10: Thymus sequence analysis of Vδ6-Jδ1 junctions

Sequences from the junctional regions of Vδ6 (variable region), Dδ1 and 2 (diversity region) and Jδ1 (joining region) rearrangements in 28 (Stage 2), 25 (Stage 3), 22 (Stage 4) 4Get V6 clones- and 25 (Stage 2), 33 (Stage 3), 18 (Stage 4) *Itk*-4Get V6 clones, respectively are shown. P/N addition regions are shown in red and the sequence-polymorphisms are aligned to the germline sequence. The frequency (left) implies the clone numbers for the sequence and simple note (right) marks brief classification of each sequence. After sequencing, only in-frame sequences are determined.

Figure 3.10

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
	GCT CTC TGG GAG CTG G		ATGGCATAT		ATCGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E L V				I G G I R A		T D K	

4Get thymocyte stage 2 (CD122$^-$GFP$^+$)

(Frequency)

Fetal precursor

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
(4)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 1
	A L W E H				I G G I R A		T D K	
(5)	GCT CTC TGG GAG CTG G				TCGGAGGGATACGAG		CT ACC GAC AAA	ITKko liver 1
	A L W E L V				G G I R A		T D K	
(2)	GCT CTC TGG GAG CTG G				TCGGAGGGATACGAG		CT	ITKko liver 1'
	A L W E L V				G G I R A			
(5)	GCT CTC TGG GAG CTG G	C			CGGAGGGATACGAG		CT ACC GAC AAA	ITKko liver 2
	A L W E L A				G G I R A		T D K	

Adult precursor

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
(2)	GCT CTC TGG G	GCCT	A		ATCGGAGG	AATAT	CT ACC GAC AAA	4Get stage2
	A L W G	L			I G G	I S	T D K	
(1)	GCT CTC TGG GAG CTG G	T	TGGC	TT	GGAGGG	TCCAG	CT ACC GAC AAA	
	A L W E L V		G	L	E G	P A	T D K	
(1)	GCT CTC TGG GAG CTG G	CCT			CGGAGGG	CCCA	ACC GAC AAA	
	A L W E L A	P			E G	P	T D K	
(1)	GCT CTC TGG GAG CTG G	AG	GG	GGAT	ATCGGAGGGATAC	CTG	CT ACC GAC AAA	seq 1
	A L W E L E		G	D	I G G I P	A	T D K	
(1)	GCT CTC TGG GAG CTG	AAG	GG	AGAT	ATCGGAGGGAT	TCCCG	CT ACC GAC AAA	seq 1'
	A L W E L	K	G	D	I G G I	P A	T D K	
(1)	GCT CTC TGG GAG CT	C	CAT		ATCGGAGGGATACGAG	CTT	CC GAC AAA	seq 2
	A L W E L		H		I G G I R A	S	D K	
(1)	GCT CTC TGG GAG C	CAG	ATAT	C	GGG	G	CT ACC GAC AAA	seq 3
	A L W E P	D	I		G	A	T D K	
(1)	GCT CTC TGG GAG C	CCT	AT		ATCGGAGGGATA	GTCTTCT	CT ACC GAC AAA	seq 4
	A L W E P	Y			I G G I	V F S	T D K	
(1)	GCT CTC TGG GAG		TGGC		CGGAGGGATACGAG	CTTAGG	ACC GAC AAA	
	A L W E		W P		E G Y E	L R	T D K	
(1)	GCT CTC TGG GAG CT	TGAT			ATCGGAGGGATACG	TCCCG	CT ACC GAC AAA	
	A L W E L	D			I G G I R	P A	T D K	
(1)	GCT CTC TGG GAG CT	T			ATCGGAGGGATACG	GGGTTCCG	CT ACC GAC AAA	
	A L W E L				I G G I R	G S A	T D K	

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
	GCT CTC TGG GAG CTG G		ATGGCATAT		ATCGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E L V				I G G I R A		T D K	

4Get thymocyte stage 3 (CD122+GFP+)

(Frequency)

Fetal precursor

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
(1)	GCT CTC TGG GAG C	GT			ATCGGAGGGATACGAG		CT	WT liver 1'
	A L W E R				I G G I R A			
(1)	GCT CTC TGG GAG C	AG			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 1'
	A L W E Q				I G G I R A		T D K	
(2)	GCT CTC TGG GAG CTG G				TCGGAGGGATACGAG		CT ACC GAC AAA	ITKko liver 1
	A L W E L V				G G I R A		T D K	

Adult precursor

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
(1)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACG	GG	CT ACC GAC AAA	
	A L W E H				I G G I R	A	T D K	
(1)	GCT CTC TGG GAG CTG G	C			CGGAGGGATA	ACGG	CT ACC GAC AAA	ITKko liver 2'
	A L W E L A				G G I	T A	T D K	
(1)	GCT CTC TGG GAG	TTCG			AGGGATACGAG	CTTG	CC GAC AAA	
	A L W E	F E			G Y E	L A	D K	
(1)	GCT CTC TGG GAG C	AGT	ATGGCA	GAAT	ATCGGAGGGAT		CC GAC AAA	
	A L W E Q	Y	G R	I	S E G S		D K	
(1)	GCT CTC TGG GAG C		ATGGCATAT	CGTCT	ATCGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E H		G I S	S	I G G I R A		T D K	
(1)	GCT CTC TGG GAG C	ACAT	ATGGCAT		ATCGGAGGGATACGA		ACC GAC AAA	
	A L W E H	I	W H		I G G I R		T D K	
(1)	GCT CTC TGG GAG C			ACCTT	ATCGGAGGGATACGAG	CCCCGTA	T ACC GAC AAA	
	A L W E H			L	I G G I R A	P Y	T D K	
(1)	GCT CTC TGG GAG C	AT	ATGGCATAT	GA	CGGAGGGATACGAG		CC GAC AAA	
	A L W E H		M A Y	D	G G I R A		D K	
(1)	GCT CTC TGG GAG CTG G	ACGGA	ATGGCATAT	T	GGATACGAG		CT ACC GAC AAA	
	A L W E L D	G	M A Y	W	I R A		T D K	
(1)	GCT CTC TGG GAG CTG G	CGAGAGGCAT			ATCGGAGGGATACG	GGGGAT	CC GAC AAA	
	A L W E L A	R G I			S E G Y G	G S	D K	
(1)	GCT CTC TGG GAG CTG G			AGGGA	CGGAGGGATACG	C	CC GAC AAA	
	A L W E L E			G	R R D T	P	D K	
(1)	GCT CTC TGG GAG C	CTCAT			ATCGGAGGGATACGAG	CTC	CC GAC AAA	seq 5
	A L W E P	H			I G G I R A	P	D K	
(1)	GCT CTC TGG GAG C	CTCAT			ATCGGAGGGATAC	CCTCCC	CC GAC AAA	
	A L W E P	H			I G G I P	S P	D K	
(2)	GCT CTC TGG GAG CTG G	GGGGA	GG	T	ATCGGAG	CC	ACC GAC AAA	4Get stage3-1
	A L W E L G	G	G		I G A		T D K	
(2)	GCT CTC TGG GAG CTG G			GGG	CGGAGGGATACG	GGG	CT ACC GAC AAA	4Get stage3-2
	A L W E L G			A	E G Y G	A	T D K	
(1)	GCT CTC TGG GAG CTG G	CGGCCCT			ATA	TGGCCCTC	CT ACC GAC AAA	
	A L W E L A	A L			Y	G P P	T D K	
(1)	GCT CTC TGG GAG CTG G	GG			GGAGGGATACGAG	CCC	CT ACC GAC AAA	seq 6
	A L W E L G				G G I R A	P	T D K	
(1)	GCT CTC TGG GAG CT	AGGG	GGC	TTCTT	ATCGGAGGGATAC	C	CC GAC AAA	
	A L W E L	G	G	F L	S E G Y	P	D K	
(1)	GCT CTC TGG GAG CT	C	TAT		ATCGGAGGGATACGAG	CTC	CC GAC AAA	seq 7'
	A L W E L		Y		I G G I R A	P	D K	

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
	GCT CTC TGG GAG CTG G		ATGGCATAT		ATCGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E L V				I G G I R A		T D K	
4Get thymocyte stage 4 (CD122⁺GFP⁻)								
(Frequency)								
Fetal precursor								
(3)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 1
	A L W E H				I G G I R A		T D K	
(2)	GCT CTC TGG GAG CTG G				TCGGAGGGATACGAG		CT ACC GAC AAA	ITKko liver 1
	A L W E L V				G G I R A		T D K	
Adult precursor								
(1)	GCT CTC TGG GAG CTG G	C			CGGAGGGATACGA	A	CT ACC GAC AAA	ITKko liver 2'
	A L W E L A				G G I R	T	T D K	
(1)	GCT CTC TGG GAG CTG G				GGGATA		CT ACC GAC AAA	
	A L W E L G				D T		T D K	
(1)	GCT CTC TGG GAG CTG	AC			CGGAGGG	GTACGGT	CT	
	A L W E L	T			G G	V R S		
(1)	GCT CTC TGG GAG	T			TCGG		T ACC GAC AAA	
	A L W E				G		T D K	
(1)	GCT CTC TGG GAG C	CTCAT			ATCGGAGGGATACGAG	CTC	CC GAC AAA	seq 5
	A L W E P	H			I G G I R A	P	D K	
(1)	GCT CTC TGG GAG C	C	ATGGCA	ACTCC	GAGGGATACGAG		CT ACC GAC AAA	
	A L W E P		W Q	L R	G I R A		T D K	
(1)	GCT CTC TGG GAG CTG G	GGTTGGG	TGG	CCCCCAT	ATCGGAGGG	GT	C GAC AAA	
	A L W E L G	L G	G	P H	I G G	V	D K	
(1)	GCT CTC TGG GAG CTG G	AG	GG	GGAT	ATCGGAGGGATAC	CTG	CT ACC GAC AAA	seq 1
	A L W E L E		G	D	I G G I P	A	T D K	
(1)	GCT CTC TGG GAG		TGG		CGGAGGGATAC	CCCTC	CT ACC GAC AAA	
	A L W E		W		R R D T	P P	T D K	
(1)	GCT CTC TGG GA	CAAT			ATCGGAGGGATACG	GG	CT ACC GA	
	A L W D	N			I G G I R	A	T	
(1)	GCT CTC TGG GA	CCAGGT	ATGGC	CCCCACC	CGGAGGGATACGAG		CT ACC GAC AAA	
	A L W D	Q V	W P	P P	G G I R A		T D K	
(1)	GCT CTC TGG GAG CT	T			ATCGGAGGGATACGAG	CTCCCC	CT ACC GAC AAA	
	A L W E L				I G G I R A	P P	T D K	
(1)	GCT CTC TGG GAG CTG G	GG			GGAGGGATACGAG	CCC	CT ACC GAC AAA	seq 6
	A L W E L G				G G I R A	P	T D K	
(1)	GCT CTC TGG GAG CTG G	CGGGG			CGGAGGGATA	CCCCCG	CT ACC GAC AAA	
	A L W E L A	G			R R D T	P A	T D K	
(1)	GCT CTC TGG GAG CTG		TAT		ATCGGAGGG	GTTG	CT ACC GAC AAA	seq 7'
	A L W E L		Y		I G G	V A	T D K	
(1)	GCT CTC TGG GAG CTG		TAT		ATCGGAGGGATACGAG	CTA	CT ACC GAC AAA	
	A L W E L		Y		I G G I R A	T	T D K	
(1)	GCT CTC TGG GAG CT	CCG			CGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E L	R			G G I R A		T D K	

(Frequency)	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
	GCT CTC TGG GAG CTG G		ATGGCATAT		ATCGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E L V				I G G I R A		T D K	

Itk$^{-/-}$-4Get thymocyte stage 2 (CD122^{-}GFP^{+})

(Frequency)	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
Fetal precursor								
(4)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 1
	A L W E H				I G G I R A		T D K	
(1)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACGAG		CT ACC	WT liver 1'
	A L W E H				I G G I R A		T	
(1)	GCT CTC TGG GAG C	GT			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 1'
	A L W E R				I G G I R A		T D K	
(1)	GCT CTC TGG GAG CTG G				TCGGAGGGATACGAG		CT ACC GAC AAA	ITKko liver 1
	A L W E L V				G G I R A		T D K	
(1)	GCT CTC TGG GAG CTG				ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 2
	A L W E L				I G G I R A		T D K	
(2)	GCT CTC TGG GAG CTG G	C			CGGAGGGATACGAG		CT	ITKko liver 2'
	A L W E L A				G G I R A			
Adult precursor								
(1)	GCT CTC TGG GAG CT	TGAT			ATCGGAGGGATACGAG		CT	seq 5'
	A L W E L	D			I G G I R A			
(2)	GCT CTC TGG GAG CTG G				GGGATACG	G	CT ACC GAC AAA	ITKko stage2-1
	A L W E L G				D T	A	T D K	
(1)	GCT CTC TGG GAG CTG G	T			GGG	TC	CT ACC GAC AAA	
	A L W E L V				G	P	T D K	
(1)	GCT CTC TGG GAG CT	TCATCG			AGGGATACGAG		CT ACC GAC AAA	
	A L W E L	H R			G I R A		T D K	
(1)	GCT CTC TGG GAG CTG G	TCCTCTA			CGGAG		CT ACC GAC AAA	
	A L W E L V	L Y			G A		T D K	
(1)	GCT CTC TGG GAG CTG G		CATAT		ATCGGAGGGATAC		CC GAC AAA	
	A L W E L A		Y		I G G I P		D K	
(1)	GCT CTC TGG GAG CTG G		ATGGC	CCTT	GGATCGGGGC	A	ACC GAC AAA	
	A L W E L D		G	P W	I G A		T D K	
(1)	GCT CTC TGG GAG CTC G	AGCCGG	TGGCATAT	ATGTGG	CGGAGGGATACGAG	A	ACC GAC AAA	
	A L W E L E	P V	A Y	M W	R R D T R		T D K	
(2)	GCT CTC TGG GAG	TTCAAT			ATCGGAGGGATACGAG		CT ACC GAC AAA	ITKko stage2-2
	A L W E	F N			I G G I R A		T D K	
(1)	GCT CTC TGG GAG	GAAT			ATCGGAGGGGT	G	CT ACC GAC AAA	
	A L W E	E Y			R R G	A	T D K	
(1)	GCT CTC TGG GAG C	CCTAT			ATCGGAGGGATA	GTCTTCT	CT ACC GAC AAA	seq 4
	A L W E P	Y			I G G I	V F S	T D K	
(1)	GCT CTC TGG GAG CTG	TCAT	ATGGC		ATAGGGGGGATACGAG		CT ACC GAC AAA	
	A L W E L	S Y	G		I G G I R A		T D K	
(1)	GCT CTC TGG GAG CT		ATGGCAT		ATCGGAGGGATACGAG	AAC	CC GAC AAA	
	A L W E L		W H		I G G I R E	P	D K	

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
	GCT CTC TGG GAG CTG G		ATGGCATAT		ATCGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E L V				I G G I R A		T D K	

Itk$^{-/-}$-4Get thymocyte stage 3 (CD122^{+}GFP^{+})

(Frequency)	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
Fetal precursor								
(9)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 1
	A L W E H				I G G I R A		T D K	
(6)	GCT CTC TGG GAG CTG G				TCGGAGGGATACGAG		CT ACC GAC AAA	ITKko liver 1
	A L W E L V				G G I R A		T D K	
(1)	GCT CTC TGG GAG CT	T			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 2
	A L W E L				I G G I R A		T D K	
Adult precursor								
(1)	GCT CTC TGG GAG CTG				ATCGGAGGGATA	TATG	CT ACC GAC AAA	WT liver 2'
	A L W E L				I G G I Y A		T D K	
(1)	GCT CTC TGG GAG CTG G	C			CGGAGGGATAC	CCG	CT ACC GAC AAA	ITKko liver 2
	A L W E L A				G G I P	A	T D K	
(2)	GCT CTC TGG GAG CT	CGC			CGGAGGGATACG	GG	CT ACC GAC AAA	ITKko liver 2'
	A L W E L	A			G G I R	A	T D K	
(1)	GCT CTC TGG GAG C	CGGAT			ATCGGAGGGATACGAG		CT	seq 5'
	A L W E P D				I G G I R A			
(2)	GCT CTC TGG GAG CTG G	AT			ATCGGAGGG	T	CT ACC GAC AAA	ITKko stage3-1
	A L W E L D				I G G	S	T D K	
(1)	GCT CTC TGG GAG CTG G				AAA GAGGGATACGAG		CT ACC GAC AAA	
	A L W E L E				R G I R A		T D K	
(1)	GCT CTC TGG GAG CTG G		AGGGGG AT		ATCGGAGGGATACCTG		CT ACC GAC AAA	seq 1
	A L W E L E		G D		I G G I P A		T D K	
(2)	GCT CTC TGG GAG C		TC CAT		ATCGGAGGGATACGAG		CT GCC GAC AAA	seq 2'
	A L W E L		H		I G G I R A		A D K	
(2)	GCT CTC TGG GAG C		TG TAT		ATCGGAGGGATACGAG		CT ACC GAC AAA	seq 7
	A L W E L		Y		I G G I R A		T D K	
(1)	GCT CTC TGG GA	CCAG	ATAT	GGCCCCCACC	CGGAGGGATACGAG		CT ACC GAC AAA	
	A L W D	Q	I W	P P P	G G I R A		T D K	
(2)	GCT CTC TGG GAG CTG G				TCCCAGCC GAG	CTCG	CC GAC AAA	ITKko stage3-2
	A L W E L V				P A E	L A	D K	
(1)	GCT CTC TGG GAG CT				CGGAGGGATACCCC	CT G	CT ACC GCC AGA	
	A L W E L				G G I P P	A	T A R	

	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
	GCT CTC TGG GAG CTG G		ATGGCATAT		ATCGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E L V				I G G I R A		T D K	

Itk$^{-/-}$-4Get thymocyte stage 4 (CD122^{+}GFP^{-})

(Frequency)	Vδ6.3	P/N	Dδ1	P/N	Dδ2	P/N	Jδ1	
Fetal precursor								
(8)	GCT CTC TGG GAG C	AT			ATCGGAGGGATACGAG		CT ACC GAC AAA	WT liver 1
	A L W E H				I G G I R A		T D K	
(3)	GCT CTC TGG GAG CTG G	C			CGGAGGGATACGAG		CT ACC GAC AAA	ITKko liver 2
	A L W E L A				G G I R A		T D K	
Adult precursor								
(1)	GCT CTC TGG GAG	T			GGAGGGATACGAG	CC	CC GAC AAA	
	A L W E	W			R D T S	P	D K	
(1)	GCT C C	AT			ATCGGAGGGATACG	GAA		
	A L	W			E L P Y	R		
(2)	GCT CTC TGG GAG CT			CCCAT	ATCGGAGGGATACG	GAA		ITKko stage4
	A L W E L			P Y	R R D T	E		
(1)	GCT CTC TGG GAG CT	C	T ATAT	GGCCCCT	ATCGGAGGGATACGAG		CT ACC GAC AAA	
	A L W E L		Y M	A P	I G G I R A		T D K	
(1)	GCT CTC TGG GAG C	CAG	ATAT	C	GGG	G	CT ACC GAC AAA	seq 3
	A L W E P	D	I		G	A	T D K	
(1)	GCT CTC TGG GAG C			CTTCT	ATCGGAGGGATACGA	AT	C GAC AAA	
	A L W E P			S	I G G I R	I	D K	

one to two nucleotide additions and use of the Dδ2-Jδ1 junction indicating they were generated from fetal precursors (Figure 3.10). Sequences that were determined to be from fetal precursors were based upon sequences that have been previously published (76) Examination of the frequency of sequences from adult precursors from wild type 4Get and *Itk*$^{-/-}$ 4Get V6 thymocytes from each maturation stage indicate that there are fewer sequences from *Itk*$^{-/-}$ V6 thymocytes being generated from adult precursors (Wild type 4Get: 43%, 84%, 77%, *Itk*$^{-/-}$ 4Get: 60%, 52%, 39% of V6 cells generated from adult precursors from Stage 2, 3, and 4 respectively)(Figure 3.10). This data also demonstrates that there are more V6 cells being generated from fetal precursors in the thymus of *Itk*$^{-/-}$ 4Get mice.

Wild type liver sequence 1 was represented in both wild type and *Itk*$^{-/-}$ livers (Figure 3.2C). Upon closer examination of the sequences found in wild type 4Get and *Itk*$^{-/-}$ 4Get thymocytes, wild type liver sequence 1 was found in all three V6 stages from wild type 4Get and *Itk*$^{-/-}$ 4Get mice. Interestingly, there was a higher proportion of wild type liver sequence 1 found in all three V6 stages of *Itk*$^{-/-}$ 4Get thymocytes suggesting that these V6 cells are accumulating in the thymus while at the same time other V6 cells with different sequences are migrating into the periphery. In addition, the three thymic V6 cell subsets analyzed in wild type mice suggest that they are related given many of the shared sequences represented in each of the different maturation subsets are found in both wild type 4Get or *Itk*$^{-/-}$ 4Get mice. Alternatively, it is also possible that the similar sequences found in each subset maybe accumulating in each maturation stage.

Discussion

The role of Itk in conventional $\alpha\beta$ T cell development, differentiation and function has been well documented (146, 159, 183, 201). Although it has been previously shown that Itk is dispensable for the development of $CD8^+$ innate T cells (187-189) previous reports have demonstrated that Itk is required for the development and maturation of $\alpha\beta$ *i*NKT cells (156-158). One report showed that along with a decrease in number of *i*NKT cells in the thymus and periphery of $Itk^{-/-}$ mice, $Itk^{-/-}$ $\alpha\beta$ *i*NKT cells also had defects in the final maturation stage (156-158).

Although Itk seems to play different roles between $CD8^+$ T cells and $\alpha\beta$ *i*NKT cells there are general similarities shared between nonconventional T cell lineages, some lineages appear to be more related to each other than others. V6 cells were previously reported to share phenotypic and functional characteristics with $\alpha\beta$ *i*NKT cells in terms of their surface phenotype, tissue distribution, cytokine response and transcription factor expression (66-69, 83, 155, 191). Even though there are many similarities between V6 and $\alpha\beta$ *i*NKT cells, studies have not been performed to determine if V6 cells develop in a similar manner to $\alpha\beta$ *i*NKT cell. In the absence of Itk we have previously shown that there is an expansion of $CD4^+$ $\gamma\delta$ T cells along with an enriched V6 population. The V6 cells have the ability to rapidly produce large quantities of IL-4 and IL-13 in addition to the ability to secrete IFNγ. These cells also express the transcription factor PLZF. In addition to the expansion of V6 cells in *Itk*$^{-/-}$ mice, *TCR*$\alpha^{-/-}$ (202), *KLF2*$^{-/-}$ (203), and *Id3*$^{-/-}$ mice (86, 87) also have an expansion in V6 cells. Given that $\alpha\beta$ *i*NKT cells and V6 cells diverge early during thymic development, it is interesting that they can acquire the expression of the same transcription factor, PLZF, suggesting that these two populations of cells may undergo nearly identical differentiation programs (80-83, 191). In this study, we compare the maturation of V6 cells to $\alpha\beta$ *i*NKT cells and show that *Itk*$^{-/-}$V6 cells have a partial block in maturation.

$\alpha\beta$ *i*NKT cell developmental stages are associated with defined changes in surface marker expression and functional changes. In addition to confirming the role of Itk in $\alpha\beta$ iNKT maturation in this study, our data suggests that adult wild type $\alpha\beta$ *i*NKT and V6 cells mature in a similar manner. We find that wild type V6 cells, similar to wild type $\alpha\beta$ *i*NKT cells, express two distinct populations of cells positive for PLZF, along with CD4 and contain IL-4 mRNA transcripts. In addition, as wild type V6 cells mature they begin to down-regulate PLZF, CD4 and IL-4 transcription. At the same time, wild type V6 cells, similar to wild type $\alpha\beta$ *i*NKT cells begin to up-regulate CD44, CD122 and NK cell surface markers (Figure 3.4 and 3.7). The NK cell surface markers were mainly found on PLZF$^{-/lo}$ V6 cells suggesting that as V6 cells mature they up-regulate NK cell markers similar to $\alpha\beta$ *i*NKT cells (156, 195, 198). $\boldsymbol{\alpha\beta}$ *i*NKT cell development has also been shown to be affected by the SAP/SLAM signaling (199, 200). We find that while V6 development is influenced by lack of SAP, a population of V6 cells still persists unlike $\alpha\beta$ *i*NKT cells though many of the V6 cells still present are HSA^+ compared to wild type V6 cells (Figure 3.8A and B) indicating that SAP may have a role during stage 1 and 2 of maturation when HSA is down-regulated. In addition to $\alpha\beta$ *i*NKT cells and V6 cells requiring many of the same molecules for development, global gene-expression analysis of wild type $\alpha\beta$ *i*NKT cells compared to wild type V6 cells suggests that these two cell populations are similar. This data taken together indicates that despite $\alpha\beta$ *i*NKT cells and V6 cells diverging during development, they share a common maturation pathway. While our data suggests that $\alpha\beta$ *i*NKT cells and V6 cells mature through similar pathways, further experiments need to be performed on V6 cells in which each stage would need to be sorted and transferred back into mice to determine if they can continue to mature.

Upon closer examination of the maturation pathway of $Itk^{-/-}$ V6 cells we find that, similar to $Itk^{-/-}$ $\alpha\beta$ *i*NKT cells, $Itk^{-/-}$ V6 thymocytes are impaired in maturation since they do not down-regulate PLZF and IL-4 transcription and they have a defect in up-regulating CD122 (Figure 3.4). In addition, $Itk^{-/-}$ V6 cells are defective in up-regulating NK cell markers (Figure 3.7) further suggesting that $Itk^{-/-}$ V6 thymocytes have a defect in maturation similar to $Itk^{-/-}$ $\alpha\beta$ *i*NKT cells. Global gene-expression analysis of $Itk^{-/-}$ V6 thymocytes compared to mature and immature $\alpha\beta$ *i*NKT cells further supported these results as *Itk*$^{-/-}$ V6 thymocytes were hierarchically clustered with immature $\alpha\beta$ *i*NKT cells (Figure 3.6D). Further evidence supporting that *Itk*$^{-/-}$ V6 cells are more immature was demonstrated by examining PLZF expression in the spleen (Figure 3.1B). In wild type $\alpha\beta$ *i*NKT cells and V6 cells there is only a PLZF^{lo} population of cells. However, *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells and V6 cells contain a PLZF^{hi} along with a PLZF^{lo} population of cells in the spleen indicating that a proportion of the *Itk*$^{-/-}$ V6 cells are migrating out into the periphery with $\alpha\beta$ *i*NKT stage 2/3 characteristics. Due to limitations of staining of PLZF and IL-4 transcription levels together, intracellular staining on PMA/Ionomycin stimulated thymocytes was performed instead. Similar to what another group has shown, while both $\alpha\beta$ *i*NKT and V6 PLZF^{hi} and PLZF^{lo} cells can produce IL-4, $\alpha\beta$ *i*NKT and V6 PLZF^{lo} cells predominantly produce IFNγ (Figure 3.5B and C)(80, 204). Given that there is an accumulation of immature V6 PLZF^{hi} cells in the spleen that preferentially secrete IL-4 in the *Itk*$^{-/-}$ mice, it is likely that these cells are responsible for the hyper IgE syndrome in *Itk*$^{-/-}$ mice.

Interestingly, Itk seems to affect TCR signaling differently between V6 cell and $\alpha\beta$ *i*NKT cells. We and others show that while *Itk*$^{-/-}$ *i*NKT cells constitutively contain mRNA for IL-4 and are poised for cytokine synthesis (Figure 3.1C, 3.2B and 3.4C, (157)), stimulation through the TCR failed to produce effector cytokines. Similar to *Itk*$^{-/-}$ $\alpha\beta$ *i*NKT cells, *Itk*$^{-/-}$ V6 cells also constitutively contain mRNA for IL-4, but in contrast, *Itk*$^{-/-}$ V6 cells produce effector cytokine after stimulation through the TCR. These results suggest that while Itk-dependent signals are not required for activation of cytokine loci in either $\alpha\beta$ *i*NKT cells or V6 cells, Itk is required for regulating effector function and development of V6 cells.

It still remains unclear as to why there expansion of V6 cells in *Itk*$^{-/-}$ mice. Examination of cell cycle status determined by Ki-67 staining of *Itk*$^{-/-}$ V6 cells showed a decrease in Ki-67^{+} fraction compared to wild type V6 cells (unpublished observation). Proliferation rates determined by BrdU labeling also showed that *Itk*$^{-/-}$ V6 cells proliferated less then wild type V6 cells (unpublished observation). It is possible that by examining the V6 cell population as a whole we may not see any differences in Ki-67 staining. Similar to $\alpha\beta$ *i*NKT cells wihich are very heterogeneous in their function given the recent discovery of an IL-17-secreting $\alpha\beta$ *i*NKT cell population (205), V6 cells can also be divided into two distinct subsets based upon their expression of PLZF (204). Therefore it is possible that a subset of V6 cells may be expanding. A study has also suggested that $\alpha\beta$ *i*NKT cells compete for a thymic niche with V6 $\gamma\delta$ NKT cells, specifically the V6 cells that express NK1.1 (204). However, *β2m*$^{-/-}$ mice, which lack *i*NKT cells, do not have an increase in proportions of $\gamma\delta$ T cells (44) or $\gamma\delta$ NKT cells (unpublished observation) which makes it unlikely that this is the case. This data together suggests that *Itk*$^{-/-}$ V6 cells are not proliferating to fill a niche and that they are not dying. This may be due to lack of negative selection or down-regulation of anti-apoptotic molecules such as Fasl and Gzmb (Table 3.1).

A hallmark characteristic of innate T lymphocytes is the use of a restricted TCR repertoire as these cells mature (11). Analysis of wild type V6 liver Vδ6-Jδ1 sequences for N nucleotide additions demonstrated diversity in their TCR delta chain usage indicating that these cells do not restrict their repertoire as they go through the maturation stages (Figure 3.3 and 3.10). Instead studies have proposed that a large proportion of V6 cells in adult mice are the progeny of precursors that originate from fetal thymuses while a smaller proportion of V6 cells that develop in the adult thymus are from adult precursors (76). During early T cell ontogeny, T cell receptors contain limited diversity at the junction of the V, diversity (D) and joining (J) segments (206, 207) due to the absence of terminal deoxylnucleotidyl transferase (TdT). TdT is a polymerase that adds non-templated nucleotides that is absent in embryonic cells but begins to be expressed in developing thymocytes three to five days after birth resulting in TCR chains lacking N region additions during fetal and early post-natal life (71-75). V6 cells first arise in the thymus after birth and their numbers are thought to greatly expand during the first weeks of life (66). Interestingly, the gene arrangements associated with the V6 population are typically found in the fetal rather than the adult thymus. Characteristics of gene arrangements found in the fetal thymus include minimal to no N nucleotides at junctions, in addition to the presence of a single D element in the rearranged δ-chain gene (66, 67, 70). These characteristics are also associated with the V3 population of γδ T cells that migrate to the skin (208) which develop around embryonic day 14 (30, 34). Examination of the TCRδ chain of V6 cells in the liver of *Itk*$^{-/-}$ mice compared to wild type V6 liver cells (Figure 3.2C) revealed an increase in the number of TCRδ sequence usage. Interestingly, in wild type V6 liver cells, the two sequences present lacked N sequence additions whereas one of the most represented sequences in *Itk*$^{-/-}$ V6 liver cells contained N nucleotide additions (Figure 3.3). Lack of N nucleotide additions and predominant use of the Dδ2 gene segment in wild type V6 liver cells indicate that these cells develop from fetal precursors, where as in *Itk*$^{-/-}$ V6 cells, some of the V6 cells but not all the cells could have developed later in life. This data Indicates that perhaps lack of Itk may be affecting the selection of the V6 cells where cells that would normally be selected against are now being selected for in the thymus.

GFP^{-} V6 cells found in the thymus (Figure 3.4C) of wild type and *Itk*$^{-/-}$ mice consist of both invariant V6 receptors and receptors containing N nucleotide additions and use of Dδ1 segment. This indicates that the adult thymus contains V6 cells that developed from both fetal and adult precursors. Like $\alpha\beta$ *i*NKT cells, a small portion of V6 cells reside in the thymus after maturation (76, 209). Wild type V6 cells that developed from fetal precursors found in the thymus may have accumulated there after development similar to thymic resident $\alpha\beta$ *i*NKT cells. There seemed to be an overall increase in *Itk*$^{-/-}$ V6 cells in the thymus that developed from fetal precursors indicating Itk may be affecting the migration of V6 cells. Consistent with this idea, Itk has been shown to be involved in the migration of T cells and that lack of Itk results in defects in chemokine induced migration (210). Alternatively, as mentioned above these cells may normally be selected against, but in the absence of Itk they may be passing negative selection due to defective TCR signaling. Supporting this theory includes studies that have shown that $\gamma\delta$ T cells that recognize the ligands T10 and T22 have been found in the epithelial layer of the small intestine (IEL) in mice lacking the nonclassical MHC class I molecules T10 and T22 (211). Along with the development of T10/T22 specific $\gamma\delta$ cells in the absence of ligand, there is also an increase of T10/T22 specific cells in the IEL compartment indicating that there is some type of selection process occurring (211). While the ligand is not known for V6 cells, it is possible that something similar is occurring with *Itk*$^{-/-}$ V6 cells given that they have defective TCR signaling.

The sequencing data also clarifies some of the inconsistent data regarding the development of V6 cells in *Id3*$^{-/-}$ and *Itk*$^{-/-}$ mice. Id3 (inhibitor of DNA binding 3) belongs to the ERK-Egr-Id3 signaling pathway and is induced upon TCR signaling (85) therefore, Id3 is downstream of Itk. Previous reports demonstrated that similar to *Itk*$^{-/-}$ mice, *Id3*$^{-/-}$ mice had a similar increase in V6 cells (80, 86). We previously showed reconstitution of wild type mice with *Itk*$^{-/-}$ bone marrow resulted in reconstitution of the V6 population (155); whereas reconstitution of wild type mice with *Id3*$^{-/-}$ bone marrow failed to reconstitute the V6 population (191). Based upon our findings, we propose Id3 affects the development of the V6 cell population that develops during the late fetal or neonatal wave resulting in the lack of V6 cell reconstitution in bone marrow chimeras; while lack of Itk affects the development of V6 cells generated in the neonatal and adult thymus.

Further experiments should examine V6 cells from neonatal *Itk*$^{-/-}$ mice. All the experiments in this chapter have examined V6 cells in adult mice yet V6 cells are thought to expand during the first few weeks after birth (76). Therefore it would be interesting to examine the V6 cell population in *Itk*$^{-/-}$neonatal mice. In addition, it would also be of interest to sort for the different stages of V6 cells to examine the sequence variation to determine if there is a difference between wild type and *Itk*$^{-/-}$neonatal mice.

In this study, we elucidated the role of Itk in the maturation stages of V6 cells and that the lack of Itk results in V6 cells that migrate out into the periphery in an immature state, which can lead to hyper IgE. Additionally, in the absence of both αβ and γδ T cells with regulatory properties, V6 cells can cause uncontrolled inflammation due to recruitment of granulocytes and lymphocytes (83). While we do not see this in our model due to the presence of regulatory cells, our data indicates that Itk plays an important role in regulating the maturation and function of V6 cells.

Chapter IV: Discussion

Chapter IV Attributions and Copyright information

Some of the material in the discussion was derived from a manuscript that has been submitted by Malhotra et al. [figure 4.1] or has been published by Prince *et al.,* 2009 *Immunological Reviews* review [figures 4.2-4.4] (9). All of the material derived from papers was from sections originally written by me.

Malhotra, N., K. Narayan, O. Cho, K. Sylvia, C.C. Yin, H. Melichar, V. Lefebvre, L.J. Berg, J. Kang. A high mobility group box transcription factor network programs innate IL-17 production. *Manuscript submitted*

Prince, A. L., C. C. Yin, M. Enos, M. Felices, and L. J. Berg. 2009. The Tec kinases Itk and Rlk regulate conventional versus innate T-cell development. *Immunol. Rev.* 228: 115–131.

Synopsis of the material

The cell mediated immune response evolved over 450 million years ago when jawed vertebrates diverged from jawless fish, to play a crucial role to defend against numerous pathogens (212). While we have a greater understanding of B and $\alpha\beta$ T cells, much less is known about $\gamma\delta$ T cells and the role Itk plays in the development and function of these cells. Previous studies have shown Itk deficiency in mice resulted in the failure to mount an effective T_H2 response (213). Given the profound effects of Itk on T_H2 responses, it has become an attractive target for the development of drugs to treat asthma and other diseases of hypersensitivity. However, recent data has demonstrated aberrant Itk expression in humans result in fatal consequences. Therefore, a better understanding of the role of Itk in cells aside from T_H2 cells is required. The data presented in this thesis clearly demonstrates a role for Itk signaling in regulating $\gamma\delta$ T cell lineage development, maturation and effector function.

It has been long observed that naïve *Itk*$^{-/-}$ mice have spontaneously elevated serum IgE levels despite the observed defects in T_H2 cytokine secretion from conventional $CD4^+$ and *i*NKT cells. In Chapter II, we show in the absence of Itk, a specific subset of $\gamma\delta$ T cells is responsible for the spontaneously elevated serum IgE levels in *Itk*$^{-/-}$ mice as mice deficient in both Itk and TCRδ have normal levels of serum IgE. Upon stimulation through the TCR, *Itk*$^{-/-}$ $\gamma\delta$ T cells secrete low levels of IFNγ but high levels of T_H2 cytokines. In addition, we found an increase in $\gamma\delta$ T cell numbers in *Itk*$^{-/-}$ mice, most notably the V6 population that also has an increase in CD4 expression. The V6 population has previously been described to be the dominant population of $\gamma\delta$ NKT cells. Aside from *Itk*$^{-/-}$ $\gamma\delta$ T cells secreting large amounts of IL-4, these cells are also PLZF^{+}, which indicates that there is a common molecular program between $\alpha\beta$ *i*NKT and V6 cells.

Chapter III described an important role for Itk in the terminal maturation of the V6 $\gamma\delta$ NKT cells. The data from Chapter II, along with previous studies (66, 69, 76), and high transcriptome analysis of $\alpha\beta$ *i*NKT and V6 cells (49) indicate that there is a common molecular program between $\alpha\beta$ *i*NKT and V6 cells. Therefore, we further examined whether V6 cells demonstrate similar maturation properties to $\alpha\beta$ *i*NKT cells. We investigated V6 cell development in the presence and absence of Itk and compared these data to parallel analyses of $\alpha\beta$ *i*NKT cell maturation subsets in the thymus. In the presence of Itk, the subsets of V6 and $\alpha\beta$ *i*NKT cells show strong similarities in maturation based upon CD122, CD4, CD44, PLZF and IL-4 expression. These similarities suggest that these subsets undergo similar differentiation programs. The absence of Itk in V6 cells resulted in impaired maturation of these cells in the thymus, as illustrated by the inability of V6 cells to up-regulate NK cell markers and CD122. This impairment in maturation is similar to defects previously seen (156-158) in the terminal differentiation of Itk deficient $\alpha\beta$ *i*NKT cells. The hyper-IgE syndrome seen in *Itk*$^{-/-}$ mice can be attributed to the requirement for Itk in the terminal maturation of V6 cells, a process normally accompanied by down-regulation of PLZF and IL-4 production. Therefore, data in this chapter demonstrates that wild type V6 cells progress to a mature stage of development in a parallel manner to αβ *i*NKT cells and that Itk is required for proper development and function of both αβ *i*NKT and V6 cells.

Overall, my thesis extends the general knowledge of the development and function of V6 cells and the role Itk plays in this subset. Specifically, we demonstrate that Itk is required for terminal maturation and function of V6 cells. Our data also indicates each γδ T cell subset should be examined as an individual population since the absence of Itk affected some γδ T cell subsets (*i.e.* V6 cells), but not all. This can be due to not all γδ T cell populations being equally affected given that each subset has differing signal requirements. The following sections will attempt to reconcile the role of Itk in V6 cells and some of the questions that were raised based upon findings presented in the previous chapters.

Alternative signaling pathways in V6 cells

Three Tec family kinases are expressed in conventional αβ TCR T cells, Itk, Rlk and Tec with Itk being the predominant kinase in these cells. Upon stimulation of the αβ TCR, a cascade of signaling events is initiated where ZAP-70 and LAT are recruited and activate Itk to further propagate the signaling cascade. This results in the ability of αβ T cells to synthesize effector cytokines.

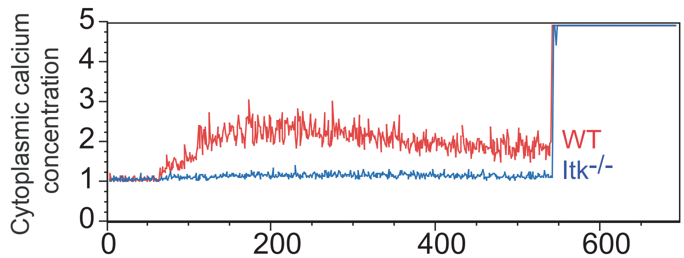
Therefore, it is not surprising that the lack Itk in $\alpha\beta$ T cells results in defects in Ca^{2+} mobilization and Erk activation that affect the synthesis of effector cytokines. In Chapter II, we find the absence of Itk in V6 cells resulted in an expansion of these cells. In addition, V6 cells have the ability to produce large amounts of IL-4 following stimulation through their TCR despite the inability of $\gamma\delta$ T cells lacking Itk to flux calcium (Figure 4.1). This finding raises an interesting issue regarding the development and signaling properties of $\gamma\delta$ T cells. Similar to $\alpha\beta$ T cells lacking Itk, $\gamma\delta$ T cells appear to have dampened TCR signals in the absence of Itk. However, in contrast to $\alpha\beta$ T cells lacking Itk, $\gamma\delta$ T cells still have the ability to produce cytokines. Therefore it is possible that the signaling pathways downstream of the $\gamma\delta$ TCR differ from $\alpha\beta$ TCR signaling.

While Itk has the predominant role in TCR-dependent thymic selection events and in TCR-dependent activation of mature T cells, Rlk's function is still uncertain. Combined deficiency of Itk and Rlk in conventional $\alpha\beta$ T cells results in a greater impaired signaling defect compared to conventional $\alpha\beta$ T cells lacking only Itk. Thus, this greater impaired signaling indicates a possible redundant role for Rlk in $\alpha\beta$ TCR signaling (121). Further evidence indicating Rlk can compensate for the loss of Itk includes a study by Sahu *et. al.* *In vivo* studies have demonstrated that lack of Itk in $CD4^+$ $\alpha\beta$ T cells results in aberrant T_H2 cell responses in an allergic model or upon challenge with eggs of *S. mansoni* (213). However, Sahu *et. al.,* demonstrated that the aberrant T_H2 cell response in *Itk*$^{-/-}$ mice can be rescued when an Rlk transgene is expressed (214).

Figure 4.1 *Itk*$^{-/-}$ γδ T cells do not flux Ca^{2+} upon TCR activation.

Intracellular Ca^{2+} concentration was determined by cross-linking wild type and *Itk*$^{-/-}$ thymocytes with anti-CD3ε biotin. Wild type and Itk$^{-/-}$ cells were stained with surface markers for TCRδ and then loaded with 16 μM of Fluo-3 (FL-1) and 16 μM Fura Red (FL-3). Baseline Ca^{2+} was taken for 2 min before stimulation via the TCR with 20 μg/ml streptavidin for 8 min. Intracellular Ca^{2+} concentration was determined by gating on γδ T cells and calculating the mean fluorescence ratio of FL1:FL3 using FlowJo. Results are representative of 2 independent experiments.

Figure 4.1


Cytoplasmic calcium concentration
5
4
3
2
1
0
0
200
400
600
WT
Itk-/-

Therefore, this data indicates that it is possible for Rlk to compensate for Itk in its absence.

Interestingly, Martin Felices examined the serum IgE levels in *Itk/Rlk*$^{-/-}$ mice and found IgE serum levels elevated about ~2 fold higher than in *Itk*$^{-/-}$ mice. Surprisingly, the absence of $\gamma\delta$ T cells does not rescue the elevated serum levels in *Itk/Rlk*$^{-/-}$ mice (data not shown). *Itk/Rlk*$^{-/-}$ mice have an increase in the proportion and number of $\gamma\delta$ T cells in the thymus but not in the spleen (Figure 4.2 A and B). Similar to *Itk*$^{-/-}$ mice, *Itk/Rlk*$^{-/-}$ mice also have elevated proportions of $CD4^+$ $\gamma\delta$ T cells in the thymus and spleen (Figure 4.3 A and B). Examination of $\gamma\delta$ T cell subsets in *Itk/Rlk*$^{-/-}$ mice showed an increase in proportion of V6 cells. *Itk/Rlk*$^{-/-}$ mice crossed to the IL-4 reporter mice (4Get) mice showed elevated levels of IL-4 message similar to 4Get mice lacking Itk (Figure 4.4 A) yet *ex vivo* stimulation of *Itk/Rlk*$^{-/-}$ $\gamma\delta$ T cells demonstrated that these cells were unable to secrete IL-4, IL-10, IL-13 and IFNγ (Figure 4.4 B). This could suggest that the TCR on *Itk/Rlk*$^{-/-}$ $\gamma\delta$ T cells is not functional and that the $\gamma\delta$ T cells may be spontaneously activated by other receptors such as Toll-like receptors (TLRs) (215) which has been shown to be the case for innate lymphoid cells. Innate lymphoid cells can be classified similar to T_H cells yet they do not have antigen – specific receptors but are able to secrete similar cytokine profiles to conventional $CD4^+$ $\alpha\beta$ T cells (216). Instead these cells have pattern recognition receptors (216, 217). In addition this data suggests that while Rlk is not required for activation of cytokine loci, it is required for optimal cytokine production upon TCR

Figure 4.2 $\gamma\delta$ T cells in *Itk/Rlk$^{-/-}$* mice have an increased proportion of $\gamma\delta$ T cells in the thymus

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

Figure 4.2

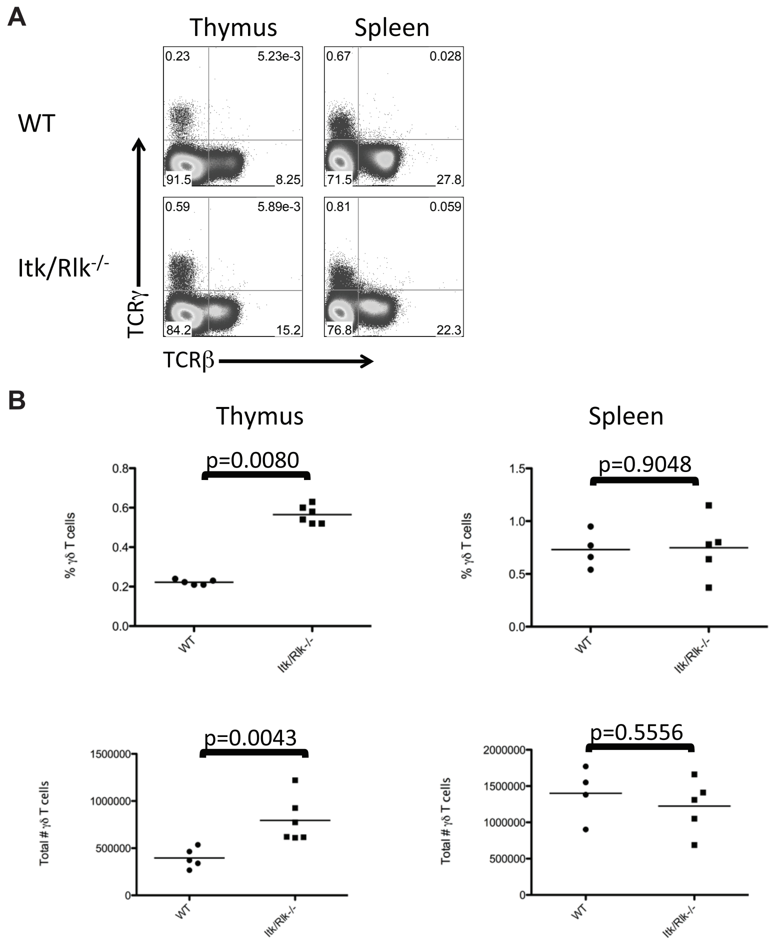

A
Thymus
Spleen
WT
Itk/Rlk-/-
0.23
5.23e-3
91.5
8.25
0.67
0.028
71.5
27.8
0.59
5.89e-3
84.2
15.2
0.81
0.059
76.8
22.3
TCRγ
TCRβ
B
Thymus
Spleen
p=0.0080
p=0.9048
% γδ T cells
WT
Itk/Rlk-/-
p=0.0043
p=0.5556
Total # γδ T cells

Figure 4.3 Altered $\gamma\delta$ T cell subsets in the thymus and spleen of *Itk/Rlk*$^{-/-}$ mice.

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

Figure 4.3

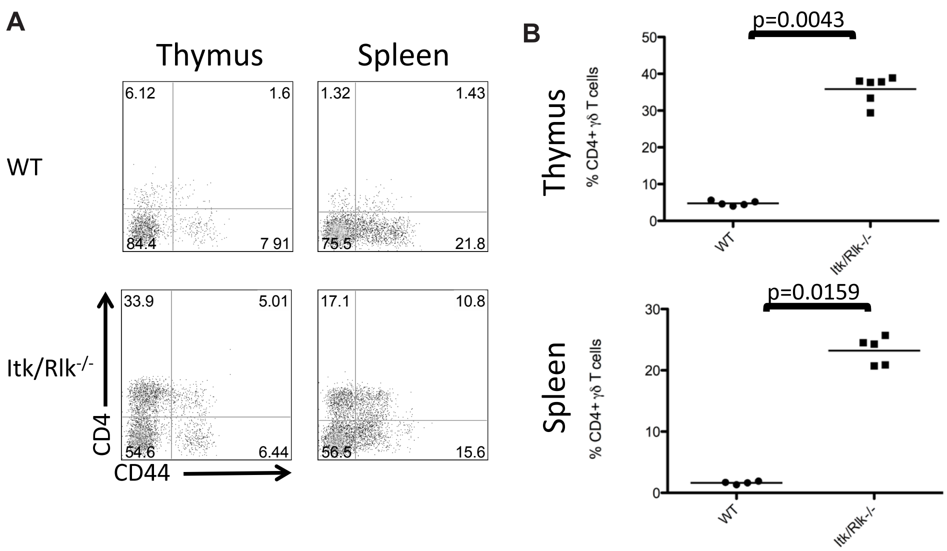
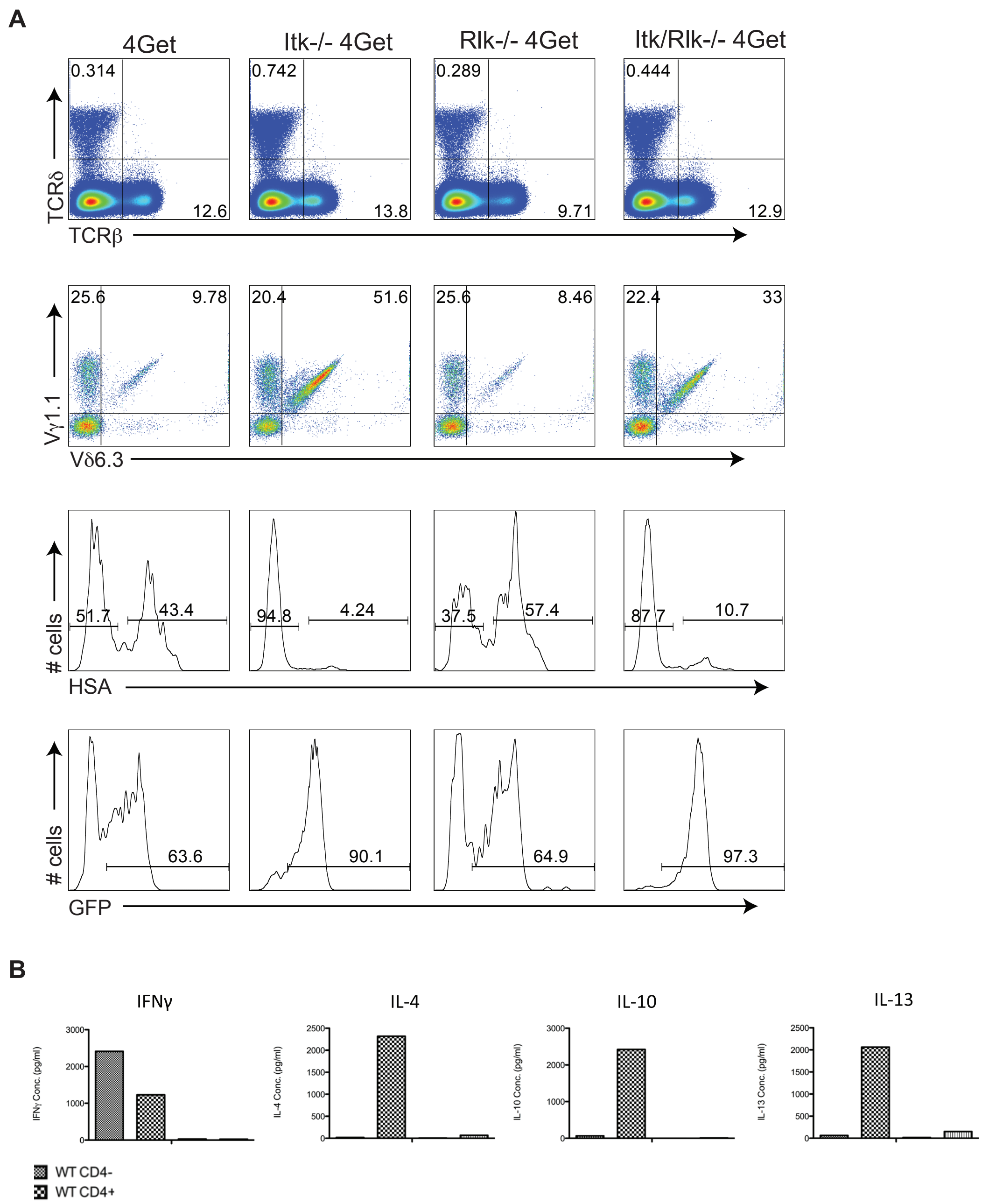

A
Thymus
Spleen
WT
6.12
1.6
84.4
7 91
1.32
1.43
75.5
21.8
Itk/Rlk-/-
33.9
5.01
54.6
6.44
17.1
10.8
56.5
15.6
CD4
CD44
B
Thymus
% CD4+ γδ T cells
p=0.0043
WT
Itk/Rlk-/-
Spleen
% CD4+ γδ T cells
p=0.0159
WT
Itk/Rlk-/-

Figure 4.4 *Itk/Rlk*$^{-/-}$ γδ T cells are impaired in the production of cytokines following *ex vivo* activation.

Cells were prepared from thymus of 4Get, *Itk*$^{-/-}$-4Get, *Rlk*$^{-/-}$-4Get and *Itk/Rlk*$^{-/-}$-4Get mice. (A) Cells from thymus were stained with anti-TCRγ, anti-TCRβ, anti-Vγ1.1, anti-Vδ6.3, and anti-CD24 antibodies and Vγ1.1^{+}Vδ6.3^{+} cells were further examined for CD24 expression by histogram. Histograms gated on CD24^{-} Vγ1.1^{+}Vδ6.3^{+} cells show GFP expression. (B) Lymph node and spleen cells from WT and *Rlk/Itk*$^{-/-}$ mice were pooled and TCRγ^{+} cells were isolated by cell sorting. 5× 10^4 cells were stimulated with 10 µg/ml of anti-TCRγ antibody for 72 h, and supernatants were analyzed for the presence of IL-4*,* IL-10, IL-13, and IFNγ by cytometric bead array (CBA).

Figure 4.4


A
4Get
Itk-/- 4Get
Rlk-/- 4Get
Itk/Rlk-/- 4Get
0.314
12.6
0.742
13.8
0.289
9.71
0.444
12.9
TCRδ
TCRβ
25.6
9.78
20.4
51.6
25.6
8.46
22.4
33
Vγ1.1
Vδ6.3
51.7
43.4
94.8
4.24
37.5
57.4
87.7
10.7
cells
HSA
63.6
90.1
64.9
97.3
cells
GFP
B
IFNγ
IL-4
IL-10
IL-13
WT CD4-
WT CD4+
DKO CD4

stimulation. These preliminary results further suggest that TCR signaling in *Itk*$^{-/-}$ γδ T cells may still function via compensation by Rlk. Further studies with *Rlk*$^{-/-}$ mice will be required to assess the function of Rlk in γδ T cell signaling.

Knowledge of TCR signal transduction has come from studies of αβ T cells given that γδ T cells are a rare population. Earlier studies identifying molecules involved in αβ TCR signaling were performed using genetic models and biochemical studies to examine the effects of the absence of tyrosine kinase molecules on CD4SP and CD8SP αβ T cells and total γδ T cells. Based upon these original studies, γδ T cell signal transduction is thought to be similar to αβ T cell signal transduction. However, data in this thesis suggests that different γδ T cell subsets may have different requirements for signaling as mentioned previously. Evidence suggests that αβ T and γδ T cell signaling share common features. This evidence includes mouse models lacking proximal signaling molecules, such as Lck, SLP76 and LAT, that impact both αβ and γδ T cell generation (80, 179, 218, 219). Interestingly, mutations of these molecules negatively affect the generation of αβ T cells, while the γδ T cell population is expanded. Furthermore, mutations in LAT, which is lacking a critical tyrosine in cytoplasmic tail, and in SLP76, where tyrosine residues were mutated to phenylalanine at position 145 or 112 and 128, showed elevated levels of serum IgE along with V6 cells producing elevated levels of IL-4 (80, 179). These data indicate that while $\alpha\beta$ and $\gamma\delta$ T cells may share similar signaling molecules there is still distinct biochemical requirements between $\alpha\beta$ and $\gamma\delta$ TCR signaling.

Further evidence indicating $\alpha\beta$ and $\gamma\delta$ TCR signaling have distinct requirements has been demonstrated recently in mice lacking RasGRP1. RasGRP1, a guanine nucleotide exchange factor for Ras, is a critical upstream activator of the Ras-Erk1/2 pathway in $\alpha\beta$ T cells following TCR engagement (220-222). As expected $\alpha\beta$ T cells in *RasGRP1*$^{-/-}$ mice were markedly decreased. In contrast, $\gamma\delta$ T cells did not require RasGRP1 for development. Closer examination of $\gamma\delta$ T cell subsets in RasGRP1 deficient mice further demonstrated that V6 cells were increased by the absence of RasGRP1. Although the development of $\gamma\delta$ T cells did not require RasGRP1, it was required for the generation and maintenance of an IL-17 producing subset of $\gamma\delta$ T cells ($CD27^{-}CD44^{+}$) and an IFNγ producing subset of $\gamma\delta$ T cells ($CD27^{+}CD44^{-}$) (223). Genetic studies with Lck, SLP-76, LAT, RasGRP1, and our studies with Itk indicate that these molecules are differentially required for $\alpha\beta$ versus $\gamma\delta$ T cell development. Activation of Ras-Erk1/2 pathway induces early growth response (Egr) 1-3 which in turn promotes the activation of the transcription factor inhibitor of differentiation 3 (Id3) which are both involved in V6 cell development. Thus the absence of RasGRP1 leads to decreased expression of Egr1/2 and Id3. Therefore, it is possible that these molecules are required for licensing or arming of γδ T cells during their development to ensure proper activation maturation, which is a process required for NK cell development (224, 225).

We have assumed that the effects mediated by Itk in γδ T cells are due to signals downstream of the TCR and this appears to be true. For example, in the absence of Itk, γδ T cells cannot flux calcium upon activation through the TCR (Figure 4.1). However, given the mutations in Itk and signaling molecules previously mentioned tend to differentially affect various γδ T cell subsets, therefore Itk may have cell type specific functions. Reinforcing this notion is that Itk is also downstream of FcεR1 in mast cells (143, 201). Hence, it is possible in γδ T cells Itk is potentially downstream of an unidentified signal.

A possible negative role for Itk in V6 cells

Based upon data derived from conventional T cell studies, Tec family kinases are typically thought to have a positive role in signaling (146, 201). In contrast to αβ T cells, Chapter II demonstrated that γδ T cells are not reduced in the absence of Itk and are actually expanded similar to innate $CD8^+$ T cells also found in the absence of Itk (187). In addition to the V6 cells expanding in the absence of Itk, they had increased cytokine secretion as shown by *ex vivo* experiments. Therefore, it is possible that Itk may be involved in a negative signaling pathway inhibiting the development or function of V6 cells.

There is evidence to support Itk having a negative role in signaling. Our lab and others have shown that Tec family kinases can have both a negative and positive role in mast cells. In contrast to T and B cells, which predominantly express Itk and Btk respectively, mast cells express Btk, Itk, Rlk and Tec downstream of the high affinity receptor for IgE (FcεR1). This receptor has a similar signaling pathway to the $\alpha\beta$ TCR (141-143, 201). Studies on the role of Btk in mast cells indicate that Btk has a positive role in mast cell signaling, and the absence Btk results in an effector function defect. Examination of Itk in mast cells by Forssell *et. al.,* indicated that Itk may also have a positive role in mast cell signaling (226). However, in contrast to Forssell *et. al*., work performed in our lab (Yoko Kosaka, Markus Falk and John Evans) and others found that Itk has a predominantly negative role in mast cell signaling (227). Unpublished work from our lab demonstrated increased PLCγ activation and cytokine secretion capabilities in mast cells lacking Itk. In addition, Markus Falk demonstrated Itk can associate with and phosphorylate SH2 domain-containing inositol 5'-phosphatase (SHIP)-1, Dok-1 and Dok-2, molecules that negatively regulate receptor signaling. He has also demonstrated that Itk can co-localize with a negative signaling complex upon antigen-mediated stimulation.

If Itk plays a negative role in the regulation of mast cells, then Itk may also play a negative role in V6 cell function. Evidence suggesting this negative role of Itk in V6 cells includes the abundant production of IL-4 and IL-13 in the absence of Itk. Given that Itk can phosphorylate SHIP-1, Dok-1 and Dok-2 in mast cells, these molecules may be involved in inhibiting the ability of V6 cells to secrete increased amounts of cytokine. Additionally, SHIP-1, Dok-1 and Dok-2 are engaged downstream of the SAP/SLAM signaling pathway (228) which as previously mentioned is important for the development of both V6 and $\alpha\beta$ *i*NKT cells. The SAP/SLAM signaling pathway modulates both positive and negative signaling. SAP/SLAM signaling can either positively regulate signals by activating NF-κB signaling through PKCθ, or reduce TCR signaling by dampening MAPK activation (93). SAP/SLAM pathway has been shown to be important for not only $\alpha\beta$ *i*NKT cell development (91, 92, 95, 199) but also V6 cell development (83, 191). SLAM family receptors along with SAP have also been shown to mediate IL-4 production by $CD4^+$ T cells (229, 230). Traditionally Dok-1 has been identified as an inhibitory molecule but Dok-1 has previously been reported as a positive regulator of IL-4 signaling and IgE response (231) though the exact pathway is unclear. In a study by Inoue *et. al.*, mice deficient in Dok-1 displayed impaired responses to IL-4 along with impaired production of IgE in mice immunized with a T cell-dependent antigen. Furthermore, overexpression of Dok-1 in a myeloid cell line was found to augment IL-4 induced proliferation. In accordance with this finding, a recent study of transgenic mice overexpressing Dok-1 under the control of the CD2 promoter led to the expansion of V6 cells (232). Similar to V6 cells lacking Itk, Dok-1 overexpressing V6 cells were mostly $PLZF^+$. Therefore it is possible that Dok-1 exerts a negative feedback regulation on Itk activity. Interestingly, this data would also suggest that Itk is negatively affecting the SAP/SLAM signaling pathway through Itk's interaction with Dok-1. In addition to the TCR signaling pathway thereby further indicating that the SAP/SLAM signaling pathway may directly interact with TCR signaling (Figure 4.5).

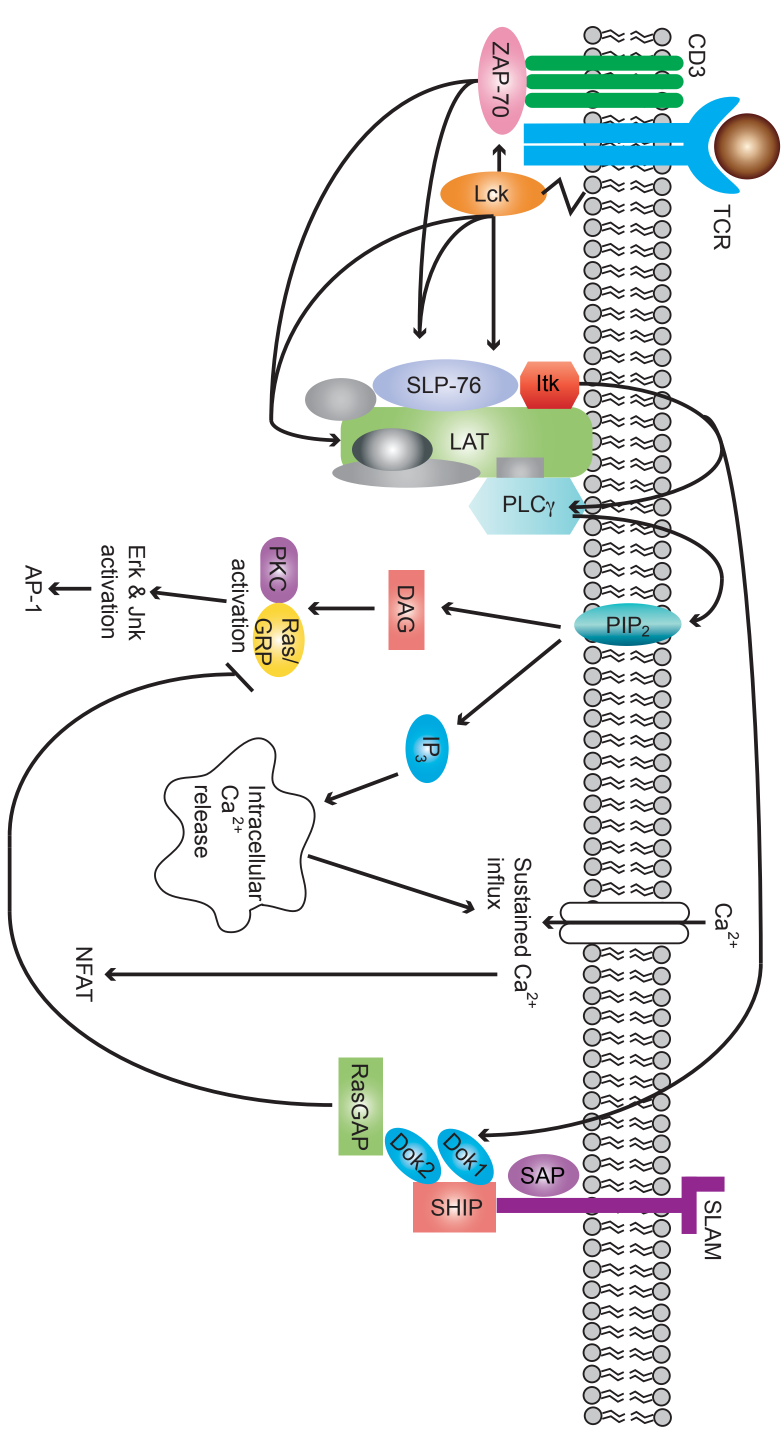
In addition, as discussed in Chapter III, wild type V6 cells mature similar to $\alpha\beta$ *i*NKT cells. For example, both cell types undergo changes in cytokine production, up-regulate maturation markers and inhibitory receptors such as CD94 and Ly49E. Functional studies on human $\gamma\delta$ T cells have demonstrated that activation of KIRs and CD94/NKG2 receptors leads to the inhibition of cytotoxicity and cytokine receptors (233). In mice, some $\gamma\delta$ T cells express NKG2A-F/CD94, Ly49C/I, Ly49A, Ly49E and Ly49G2 in the thymus and peripheral tissues (234-237). In $\alpha\beta$ T cells, expression of Ly49 inhibitory receptors has been shown to confer inhibition on T cell activation and effector functions (238-240). Contrastingly, *Itk*$^{-/-}$ V6 cells have decreased expression of Ly49 receptors and CD94. Therefore, it is possible that the increase in cytokine production in V6 cells is due to decreased expression of the inhibitory receptors. It is unclear if Itk has a direct role in regulating the expression of these inhibitory receptors or if it is due to Itk affecting the maturation of V6 cells.

Development of V6 cells from fetal precursors versus adult bone marrow

Figure 4.5 Model: Itk negatively regulates TCR pathway through interaction with the SAP/SLAM pathway

In wild type mice, the SAP/SLAM pathway is triggered in conjunction with the T cell receptor pathway and Itk negatively regulates the TCR signaling by acting upon the SHIP-Dok-1-Dok-2 complex in the SAP/SLAM pathway. Upon activation of the SHIP-Dok-1-Dok-2 complex RasGAP is recruited which goes on to inhibit Ras activation, culminating in inhibition of the MAPK signaling. Therefore, it is possible that lack of Itk results in constant activation of the TCR pathway.

Figure 4.5



CD3
TCR
ZAP-70
Lck
SLP-76
Itk
LAT
PLCγ
PIP2
DAG
PKC
Ras/GRP
activation
Erk & Jnk activation
AP-1
IP3
Intracellular Ca2+ release
Sustained Ca2+ influx
Ca2+
NFAT
RasGAP
Dok2
Dok1
SAP
SHIP
SLAM

Several studies have suggested that TCR signal strength determines T-cell lineage commitment where stronger signals favor γδ T-cell development and weaker signals favor αβ T cell development (25-27). Based upon this model one may expect, due to Itk possibly modulating TCR signal strength, T cells lacking Itk would receive reduced signal strength that would lead to reduced γδ T cell development. In contrast, we show that this is not the case. Instead there is an increase in the percentage and number of γδ T cells in the adult thymus and secondary lymphoid organs, and more specifically, an increase in the V6 cell population. Signal strength studies have treated γδ T cells as a single cell lineage but it has recently been shown that some γδ T cell subsets are as molecularly distinct from each other as they are from αβ T cells (49). In fact, closer examination of the gene profiles of immature γδ T cell subsets showed that they were already embedded with gene programs that were distinct for each subset. These gene programs direct the subset-specific effector function. This indicates that γδ function is molecularly programmed, and quite possibly, this programming is reinforced through the TCR (49). Additionally, it has been further suggested that the V6 cell population may receive stronger TCR signals compared to other γδ T cell subsets, which in wild type mice could lead to negative selection during development (83). Given that lack of Itk affects TCR signaling by dampening Ca^{2+} influx and activation of PLCγ, it was proposed that Itk deficiency may decrease signal strength. This decrease in signal strength would then allow V6 cells that would normally be negatively selected to survive. This escape of V6 cells from negative selection was thought to be the reason for their expansion in *Itk*$^{-/-}$ mice.

A recent study by Yuan *et. al.,* could provide an alternate explanation for the expansion of V6 cells in mice lacking Itk. V6 cells have been hypothesized to develop from late embryonic precursors and expand during neonatal life. Evidence arose for this model based upon the absence or minimal presence of N nucleotide addition at the junctions within the δ-chain due to lack of TdT expression in thymocytes until 3-5 days after birth. During hematopoiesis, the major site of thymocyte development occurs from the fetal liver to the fetal spleen and bone marrow during late embryogenesis. Fetal liver hematopoietic stem cells (HSCs) differ from adult bone HSCs both phenotypically and functionally and the developmental switch is thought to occur with in the first weeks after birth in mice (241, 242). It has previously been shown innate-like lymphocytes such as certain subsets of γδ T cells preferentially arise from fetal liver HSCs (243). Yuan *et. al.* further elucidated the molecular basis of innate like cells preference to develop from fetal or neonatal precursors. They reported Lin28b, which is known to specifically block the biogenesis of let-7 miRNA's post-transcriptionally, is expressed specifically in mouse fetal liver and thymus but is not sustained during ontogeny and ectopic expression of Lin28 in adult HSCs endows them with the ability to mediate multi-lineage reconstitution resembling fetal lymphopoiesis. Additionally, ectopic expression of Lin28 resulted in an increase in development of V6 cells thereby providing further evidence that these cells may develop from a yet unidentified fetal precursor (244). Since our data indicate that the absence of Itk allows for the development of V6 cells from adult HSCs, this suggests that Itk may negatively regulate Lin28b. Therefore, it is possible that as a result of Itk deficiency, Lin28b expression may still be aberrantly expressed in adult HSCs and would promote the increase of V6 cells.

Final thoughts

Further work needs to be undertaken to elucidate the effects of Itk on the fetal precursors of γδ T cells and determine if increased cytokine production is a cause or effect of aberrant maturation or function. Future studies should include identifying the fetal precursor of V6 cells in order to better understand the role Itk has in the development of these cells. In addition, sorting immature and mature subsets of neonatal γδ T cells for analysis of gene signatures will be useful identify the role Itk has to play in γδ T cell development. Moreover, additional studies are required to differentiate the role of Itk in development versus function.

Aside from the role Itk plays in the development of V6 cells, the functional relevance of the V6 cells have not been studied in detail. Previous studies in understanding the function of γδ T cells have focused on the population as a whole and have not examined the individual subsets of γδ T cells during an infectious response. Studies using infectious models have generalized that

$V\gamma1.1^+$ cells, which includes both $V\gamma1.1^+V\delta6.3^-$ and V6 cells, primarily act a immunoregulatory and anti-inflammatory cells (34, 168, 245). As shown by *Narayan* et. al., these are two distinct populations that could have differing functions during pathogen challenge (49). Therefore, further studies with infectious models should focus on V6 and V1 subsets independently and on how Itk affects the immune response of V6 cells.

The data represented in this thesis illustrate a role for Itk in the development and function of V6 cells. Itk is required for terminal maturation of these cells along with regulating their function. Efforts are underway to develop pharmacological inhibitors of Itk for use as a potential target for asthma and other diseases of hypersensitivity (246). A more thorough understanding of how Itk affects immune cells is required given recent findings of human patients with fatal Epstein-Barr Virus-associated lymphoproliferative disorder due to mutations in Itk. Furthermore, understanding how Itk affects V6 cells during allergic responses and other diseases of hypersensitivity may benefit new strategies to block other disease processes where $\gamma\delta$ T cells are involved.

Chapter V: Materials and methods

Mice:

$Itk^{-/-}$ mice (129) on the C57BL/10 strain were previously generated in our lab and were either backcrossed >13 times to C57BL/10 background or were backcrossed 6 times to C57BL/6 background. Wild type C57BL/10 or C57BL/6 were purchased from Jackson laboratories, Charles River or Taconic laboratories. *$Tcrd^{-/-}$* mice (167) on the B57BL/6 background (Jackson Laboratories) were crossed to *$Itk^{-/-}$* mice to obtain *$Itk^{-/-}Tcrd^{-/-}$* mice. Wild type mice were *$Itk^{+/-}Tcrd^{+/-}$* littermates or C57BL/10 mice (Jackson Laboratories). *$\beta 2m^{-/-}$*, *$MHC2^{-/-}$* and *$TCR\beta^{-/-}$* on the C57Bl/6 background (Jackson Laboratories) were crossed to *$Itk^{-/-}$* mice to obtain *$Itk/\beta 2m^{-/-}$*, *$Itk/MHC2^{-/-}$* and *$Itk/TCR\beta^{-/-}$* mice. *$SAP^{-/-}$*, *$Itk/SAP^{-/-}$* and *$Itk/Rlk^{-/-}$* were a gift from P. Schwartzberg (National Human Genome Research Institute, National Institutes of Health, Bethesda, MD). 4Get mice (197) were crossed to *$Itk^{-/-}$* , *$Rlk^{-/-}$* and *$Itk/Rlk^{-/-}$* mice to obtain *$Itk^{-/-}$*-4Get, *$Rlk^{-/-}$*-4Get, and *$Itk/Rlk^{-/-}$*-4Get mice. C57BL/6 or 4Get mice were used as controls. Mice were used between 2-3 months of age and were maintained at the University of Massachusetts Medical School under specific pathogen-free conditions after approval by the institutional animal care and use committee.

Cell preparations, antibodies and flow cytometry

Liver lymphocytes were isolated by collagenase digestion of minced liver followed by Ficoll gradient centrifugation; iIELs were isolated by incubation of cleaned intenstine followed by Ficoll gradient centrifugation. The following antibodies were purchased from BD Pharmingen: TCRδ(GL3)-FITC, Vγ2-FITC, Vγ3-FITC, Vδ6.2/6.3-PE, TCRβ-allophycocyanin, TCRβ-PE, TCRβ-biotin (bio), CD4-allophycocyanin, CD4-PE, CD8α-PerCP-Cy5.5, NK1.1-PE-Cy7, CD5-CyChrome, IL-4-PE, IFNγ-allophycocyanin, B220-allophycocyanin, streptavidin (strep)-allophycocyanin, OX40-biotin, Rat anti-Mouse IgG1-FITC, CD49a-AlexaFluor 647, IFNγ-AlexaFluor 700, Ly49F-biotin (bio). TCRδ-allophycocyanin, ICOS-PE, NKG2A/C/E-FITC, TCRδ-PE-Cy5, TCRδ-PerCp eFluor 710, CD122-PerCp eFluor 710, IL-4-PE-Cy7, Streptavidin (Strep)-PE-Cy7, CD49a-Alexa Fluor 647, CD244.2-Alexa Fluor 647, TCRβ- allophycocyanin eFluor 780, CD24-eFluor 450 and CD40L-allophycocyanin were purchased from eBioscience. PNA-FITC was purchased from Vector Laboratories, Inc. CD8α-PE-Texas Red was purchased from Invitrogen Molecular Probes. CD1d-PBS57-PE and CD1d-PBS57 allophycocyanin tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility. Strep-Cascade Yellow was purchased from Invitrogen Molecular Probes. The Vγ1.1 antibody (247), a kind gift of Dr. Pablo Pereira provided by Dr. Lynn Puddington, (Univ Conn Health Ctr) was purified from antibody producing cell lines by Bio-XCell. Purified Vγ1.1 was conjugated to Alexa Fluor 647 with the Invitrogen Molecular Probes Alexa Fluor 647 Protein Labeling kit or to biotin with FluoReporter Mini-Biotin-XX Protein Labeling kit also from Invitrogen Molecular Probes. Vγ1.1-FITC was purchased from BioLegend. The following antibodies were purchased from Santa Cruz

Biotechnologies: PLZF (D-9) and normal mouse IgG. Cells (500,000-4,000,000 events) were collected on a LSRII (BD Biosciences) flow cytometer. Data were analyzed using FlowJo software (Tree Star).

Quantitative real-time PCR

Splenocytes and lymph node cells from 6-10 C57BL/10 or *Itk*$^{-/-}$ mice were pooled and $\gamma\delta$ Tcells were enriched by labeling with anti-TCRβ biotin and anti-CD19 biotin (BD Pharmigen) followed by depletion with anti-biotin magnetic beads (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 TCRγ^+NK1.1$^+$, TCRγ^+NK1.1$^-$, TCRγ^+CD4$^+$ or TCRγ^+CD4$^-$ populations on a FACSVAntage cell sorter (BD Biosciences). RNA and cDNA were prepared from sorted cells as previously described (151). Real time PCR was also performed as previously described (158) on an i-Cycler (Bio-Rad). Primer 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’; PLZF forward, 5’-TCT GAC AAA GAT GGG GAT GAT CC-3’, and reverse, 5’-CAG TAT TCC GTG CAG ATG GTA CAC-3’. cDNA clones of each gene mentioned above were used for the generation of a standard curve. A cDNA clone encoding PLZF was a kind gift from Dr. Albert Bendelac (Univ Chicago).

Western Blot Analysis

Purified cells were lysed in RIPA buffer and proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Bioscience). Membranes were blocked and incubated with anti-GATA3, anti-T-bet (Santa Cruz) or anti-HSP70 (ABR affinity bioreagents) antibodies overnight at 4 degrees C, followed by HRP-conjugated secondary antibodies (Amersham Bioscience, Santa Cruz) for 1 hour. Membranes were developed using the SuperSignal West Femta substrate (Pierce).

In vitro stimulation assays

For the analysis in Chapter II, wild type and *Itk*$^{-/-}$ TCRγ^{+}NK1.1^{+}, TCRγ^{+}NK1.1^{-}, TCRγ^{+}CD4^{+} and TCRγ^{+}CD4^{-} subsets from were activated *in vitro* with 10 μg/ml of anti-TCRγ biotin (BD Pharmingen) for 72 hours at 37 degrees, and 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 mRNA. For intracellular cytokine staining, cells from wild type and *Itk*$^{-/-}$ mice were stimulated as previously described (158). In brief, cells were stimulated with 10 ng/ml PMA (Sigma-Aldrich) and 2 μg/ml ionomycin (Calbiochem) for 4-5 hours and incubated with Golgi-Stop and Golgi-Plug (BD Pharmigen) for 4-5 hours. Cells were then surface stained for anti-TCRδ and anti-NK1.1, or anti-TCRδ, anti-Vγ1.1^{+}, anti-Vδ6.3^{+} or CD1d-PBS57 tetramer fixed, and permeabilized using a Cytofix/Cytoperm kit (BD Pharmingen), for studies in chapter II, or FoxP3 fixation/permeabilization kit (e-Bioscience) for studies in chapter III, and stained for IL-4, IFNγ and PLZF. For ICOS, CD40L and OX40 expression on $\gamma\delta$ T cells, cells were stimulated for 24 hours with 10μg/ml anti-TCRδ. Cells were then surface stained with anti-ICOS, anti-CD40L and anti-OX40 antibodies.

Calcium Flux

WT and Itk$^{-/-}$ thymocytes were first depleted for CD8 with Dynal beads (Invitrogen). Cells were then stained with surface markers for TCRδ and then loaded with Fluo-3 (FL-1) and Fura Red (FL-3) (Invitrogen). Baseline Ca^{2+} was taken for 2 min before stimulation via the TCR with 20 μg/ml streptavidin (Thermo Scientific) for 8 min. Intracellular Ca^{2+} concentration was determined by gating on γδ T cells and calculating the mean fluorescence ratio of FL1:FL3 using FlowJo. Results are representative of 2 independent experiments.

Serum analysis

Blood was collected from wild type, *Tcrd*$^{-/-}$, *Itk*$^{-/-}$, *β2m*$^{-/-}$, *Itk/β2m*$^{-/-}$, *MHC2*$^{-/-}$, *Itk*$^{-/-}$ *Tcrd*$^{-/-}$, *Itk/MHC2*$^{-/-}$, *TCRβ*$^{-/-}$, *Itk/TCRβ*$^{-/-}$, *SAP*$^{-/-}$ and *Itk/SAP*$^{-/-}$ mice. Serum was obtained by spinning the blood at 5,000 rpm for 5 min. and removing the supernatant. IgE serum was analyzed by enzyme-linked immunosorbent assay (ELISA).

Sample Preparation for Microarray analysis

Samples were prepared for microarray analysis as described previously (49). Briefly, thymocytes were pooled from 4–30 mice and enriched for γδ T cells by depletion of $CD8^+$ cells with magnetic beads and an autoMACS. Cells were then stained and sorted with a FACSAria (~2×10^4 to 3×10^4 cells; >99% pure) directly into TRIzol (Invitrogen). Independent triplicates were sorted unless indicated otherwise (complete sorting details available from the ImmGen Project). Population labels correlate with ImmGen populations as follows: MatV6.WT, T.gd.vg1+vd6+24alo.Th; TotalV6. *Itk*$^{-/-}$, T.gd.vg1+vd6+.Th.ITKko; MatCD4, T.4SP24-.Th; MatCD8, T.8SP24-.Th; NKT.44^{-}NK1.1^{-} (sorted in duplicate), NKT.44-NK1.1-.Th; NKT.44^{+}NK1.1^{-}, NKT.44+NK1.1-.Th; NKT.44^{+}NK1.1^{+}, NKT.44+NK1.1+.Th. Note that all V6 cells in *Itk*$^{-/-}$ mice are CD24^{low}.

Cloning and Sequencing

Liver Vγ1.1^{+}Vδ6.3^{+} $\gamma\delta$ T cells were sorted from wild type or *Itk*$^{-/-}$ mice. Total RNA was isolated from wild type and *Itk*$^{-/-}$ Vγ1.1^{+}Vδ6.3^{+} liver $\gamma\delta$ T cells. RNA was also isolated from 4Get and *Itk*-4Get mature Vγ1.1^{+}Vδ6.3^{+} thymus $\gamma\delta$ T cells that were GFP^{+}CD122^{-}, GFP^{+}CD122^{+} and GFP^{-}CD122^{+}. Cells were sorted either on a Moflo cell sorter (Cytomation) or a FACSVantage (BD bioscience). Reverse transcription (RT) of 0.2 µg RNA was performed with qScript DNA Supermix (Quanta™). External δTCR regions were amplified from cDNAs by PCR. For PCR, primers were targeted just before the transmembrane region of δTCR (248), Vδ forward: 5´- GAA TTC TGC AGG ATG GGG GCT TCT -3´; Cδ reverse: 5´- CTC GAG ATG CCT CCT CAC AGC CTG TTC TGT G -3´. Conditions: RT cycle: 25°C 5 min, 42°C 60 min, 85°C 5 min; PCR: 94°C 2 min, 94°C 15 sec, 63°C 15 sec, 72°C 20 sec (40 cycles), 72°C 10 min. Truncated δTCR region used in this study was cloned into pCR2.1®-TOPO® TA vector by TOPO® TA cloning kit (Invitrogen). PCR products amplified by Taq polymerase were gel-extracted (Qiagen) and 3’-T overhangs were directly ligated into the vector. All plasmids were analyzed by sequencing with M13 forward and reverse primers. For reducing sequencing error, each sequence was aligned after confirmation of 2-way (forward and reverse direction).

Data analysis and visualization

Two-tailed nonparametric Mann-Whitney tests were performed using Prism (Graph Pad software). Data for microarray were analyzed and visualized as described previously (49). Briefly, RNA processing and microarray analysis with the Affymetrix MoGene 1.0 ST array was performed at the ImmGen processing center (by ImmGen standard operating procedures; **http://www.immgen.org/Protocols/ImmGen**. Cell prep and sorting SOP.pdf; **Supplementary Notes 1 and 2**). Data were analyzed with modules of the GenePattern genomic analysis platform. ConsolidatedProbeSets, a custom GenePattern module written by S. Davis (Harvard Medical School), was used for the consolidation of multiple probe sets into a single mean probe-set value for each gene. Genes with differences in regulation were identified with the Multiplot module of GenePattern through the use of the average of all replicates. Genes were considered to be regulated differently if they differed in expression by more than twofold, had a coefficient of variation among replicates of less than 0.5, had a Student's *t*-test *P* value of less than 0.05, and had a mean expression value of more than 120 in at least one subset in the comparison. Heat maps were generated by hierarchical clustering (HierarchicalClustering module of GenePattern) of data on the basis of gene (row) and subset (column) with the Pearson correlation for distance measurement. Data were log transformed and clustered with pairwise complete linkage. Data were centered on rows before visualization with the HeatMapViewer module of GenePattern. KEGG pathway analysis (Kyoto Encyclopedia of Genes and Genomes) was used for functional classifications through the DAVID portal.

Bone Marrow Chimeras

Bone marrow cells (1×10^7) from wild type or *Itk*$^{-/-}$ donors were injected into lethally irradiated (1000 rads) B6/SJL mice (Jackson Laboratories). Ten weeks after reconstitution, donor thymocytes and splenocytes were analyzed by flow cytometery.

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