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The Role of Itk in T Cell Development: A Dissertation

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THE ROLE OF ITK IN T CELL DEVELOPMENT

A Dissertation Presented

by

Julie Ann Lucas

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DOCTOR OF PHILOSOPHY

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IMMUNOLOGY AND VIROLOGY

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Some of the work presented in this thesis also appears in the following:

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Berg, L. J., L. D. Finkelstein, J. A. Lucas, P.L. Schwartzberg. The role of Tec family kinases in T cells. Annual Review of Immunology (23): In press, 2005.

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ABSTRACT

Itk is a member of the Tec family of non-receptor tyrosine kinases. It is expressed in T cells, NK cells, and mast cells. The purpose of this study was to determine the role of Itk in T cell development. Previous work from our lab and others has demonstrated that Itk is involved in signaling downstream of the T cell receptor and initial analysis of Itk-deficient mice revealed that these mice had some defects in T cell development. There are two stages of T cell development, the pre-T cell stage and the $CD4^+$ $CD8^+$ double positive stage, at which signals downstream of the T cell receptor are important. At the $CD4^+$ $CD8^+$ double positive stage, these signals direct two concurrent, but distinct processes known as repertoire selection and CD4/CD8 lineage commitment/differentiation. I show that there are only slight defects in development at the pre-T cell stage, presumably due to reduced TCR signaling. However these results clearly demonstrate that Itk is not essential at this stage of development. In contrast, repertoire selection, in particular positive selection, is significantly affected by the absence of Itk. Similarly, I show that Itk plays a role in lineage differentiation, although commitment to the appropriate lineage occurs normally in the absence of Itk.

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ATTRIBUTIONS

Chapter II

The expression of Tec family members in thymocyte subsets was done in collaboration with Martin Felices.

Itk$^{-/-}$*rag*$^{-/-}$ mice were provided by Michael Li and Zhong-bin Lai.

The bone marrow chimeras were made with the assistance of Morgan Wallace. In addition, both Joseph Maciaszek and Michael Brehm assisted with i.v. injections.

Chapter IV

The work in this chapter was done in collaboration with Luana Atherly.

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For my parents, I could not have done any of this without you.

I love you.

In memory of Dr. Cynthia Chambers

CHAPTER I

INTRODUCTION

INTRODUCTION

The immune system is a complex well orchestrated group of cells and organs that protect an organism from infection by bacteria, viruses, and parasites, generally referred to as pathogens. In vertebrates, the immune system includes two arms, the innate and adaptive immune systems. The innate arm of the immune system consists of cells that nonspecifically recognize foreign pathogens, phagocytose (engulf) them, and eliminate them prior to the onset of disease. The adaptive arm of the immune system is comprised of more specialized cells, known as B and T cells that react specifically to a given pathogen through a unique receptor. While innate immunity is present at birth and does not improve upon exposure to a pathogen, the adaptive arm of the immune system does change and will protect an organism from future exposure to the same pathogen. This is known as acquired or active immunity.

The ability of B and T cells to specifically recognize a given pathogen through a unique receptor is derived from a rare organization of the genes that encode these receptors. Given the innumerable potential pathogens an organism may encounter, it would be impossible to have unique genomic sequences for each receptor. Instead, the genetic sequences for the B and T cell receptors are generated by a recombination of gene segments in each individual cell during development, resulting in each cell encoding a unique receptor. The T cell receptor (TCR) is comprised of both α and β chains, which are encoded for in distinct loci within the genome. The sequence for the α chain results from the recombination of genomic segments, known as the variable (V), joining (J), and constant (C) regions, within the α chain locus. The sequence for the β chain results from

the recombination of similar genomic segments within the β locus, but there is an additional segment, known as the diversity (D) segment between the V and J regions. This process of recombining genomic segments is referred to as V(D)J recombination. Besides the recombining of these encoded segments, diversity with the TCR repertoire is generated due to imprecise joining of the various gene segments and the addition of non-templated nucleotides by the enzyme terminal deoxynucleotidyl transferase (TdT).

Inherent to the imprecision of this process is the likelihood that a number of recombination events will result in nonproductive rearrangements due to the generation of stop codons within the recombined sequence. To ensure the lymphoid organs are only populated with functional B and T cells, both of these cell types go through a series of developmental processes that require the rearrangement and expression of functional BCR and TCR genes. The locations where lymphocytes undergo this process are known as primary lymphoid organs. The primary lymphoid organ for B cells is the bone marrow and for T cells is the thymus. Once these cells become mature, they exit the primary lymphoid organs and circulate through the blood and lymph systems as well as populate the spleen and lymph nodes, the main secondary lymphoid organs.

B and T cells become activated when they recognize the presence of foreign pathogens, or antigens, through their receptors. B cells generally recognize particulate antigens that exist outside of the host cells. Once the B cells are activated through their BCR, they begin producing a soluble form of this receptor otherwise known as an antibody. These antibodies can circulate in the body and neutralize viruses or bacteria, as well as aid in their elimination by cells of the innate immune system. T cells, on the

other hand, recognize host cells that have become infected with a pathogen. This is done by binding of the TCR to molecules known as major histocompatability (MHC) molecules. There are two types of MHC molecules, class I MHC molecules are present on the surface of all nucleated cells, and class II MHC molecules are present on specialized immune cells known as antigen-presenting cells (APCs). Along with the MHC molecule, the TCR recognizes a peptide that is bound to the MHC molecule. In healthy cells, all the MHC molecules will have self-peptides bound to them, but infected cells will have both self-peptides and antigenic peptides that are derived from the invading pathogen. Once a T cell recognizes an infected cell, it becomes activated and through a variety of mechanisms will aid in the elimination of the infected cell. Thus, the T cells that develop must not only have properly rearranged TCR genes, but must also be able to bind peptide-MHC molecules present in the host while being able to distinguish those that have self-peptide and those that have foreign-peptide (1).

αβ T cell development

T cells develop from the precursor cells in distinct stages and areas of the thymus. The three main regions of the thymus are the subcapsular region, the cortex and the medulla (Figure 1.1). Prenatally, the precursors that give rise to T cells originate in the liver; postnatally, they originate in the bone marrow. Until recently, cells known as common lymphoid precursors (CLPs) were thought to be the cells that seed the thymus from the blood (2, 3). While it has been shown that CLPs have the capacity to differentiate into T cells, as well as B cells, natural killer (NK) cells and dendritic cells

(DCs), a recent study has shown that they are unable to detect CLPs either in the blood or the thymus. Instead it is thought that a precursor to CLPs, known as LSKs because they are lineage marker negative and express high levels of stem cell antigen-1 and c-kit (lin^- $Sca\text{-}1^{hi}$ $c\text{-}kit^{hi}$), are the cells that migrate to the bone marrow through the blood and enter the thymus at the cortico-medullary junction (4). LSKs are thought to give rise to the earliest T cell precursors (ETPs), which are exclusively found in the thymus. LSKs and ETPs differ based on their expression of cytokine receptors, LSKs express Flt3 and ETPs express low, but detectable levels of IL-7Rα. LSKs are multipotent and ETPs, similar to CLPs, have the capacity to differentiate into B, NK, and DC cells (3).

Once in the thymus, the blood progenitor cells migrate across the cortex to the subcapsular region. These early progenitors and the first cells that commit to the T cell lineage are more commonly known as double negative (DN) thymocytes because they lack the expression of the coreceptors CD4 and CD8. DN thymocytes can be subdivided into four distinct subsets and maturational stages (DN1-4) based on the expression of CD44 and CD25 (5). The cells that enter the thymus are at the DN1 ($CD44^+CD25^-$) stage of development, they have not yet begun to rearrange their TCR genes, and are not fully committed to the T cell lineage. In addition to becoming either αβ or γδ T cells, these cells also still have the potential to become natural killer (NK) cells, dendritic cells (DC), or B cells. As these cells migrate across the cortex to the supcapsular region, they continue to differentiate through DN2 ($CD44^+CD25^+$) to the DN3 ($CD44^-CD25^+$) stage. The transition of cells from DN3 to DN4 ($CD44^-CD25^-$) occurs in the subcapsular region, at which point the cells reverse their direction of migration and begin to move into the

cortex. At the same time, these cells begin to up-regulate the coreceptors CD4 and CD8 to become double positive (DP) thymocytes (6). Since the CD8 coreceptor seems to be up-regulated faster than CD4, an intermediate CD8 single positive (ISP) population exists between the DN4 and DP stages of development (7). During the final stages of maturation, DP cells become either $CD4^+$ or $CD8^+$ single positive (SP) and they are allowed to migrate from the cortex into the medulla (Figure 1.1 and Figure 1.2)

Figure 1.1 Cell migration during αβ T cell development [Figure adapted from (6)]

T cell precursors originate in the bone marrow, travel through the blood, and enter the thymus through post-capillary venules at the cortico-medullary junction. The earliest progenitors are DN1 cells. As they migrate through the cortex, the cells differentiate through the DN2 and DN3 stages. The DN3 to DN4 transition occurs in the subcapsular region, at which point the polarity of the maturing thymocyte reverses. Differentiation from DN4 through the ISP stage to the DP stage is accompanied by migration into the cortex. Only DP thymocytes that successfully undergo positive selection can progress to the SP stage and are allowed access into the medulla. Maturation of SP thymocytes continues in the medulla and the mature $CD4^+$ and $CD8^+$ SP cells exit the thymus to populate the periphery.

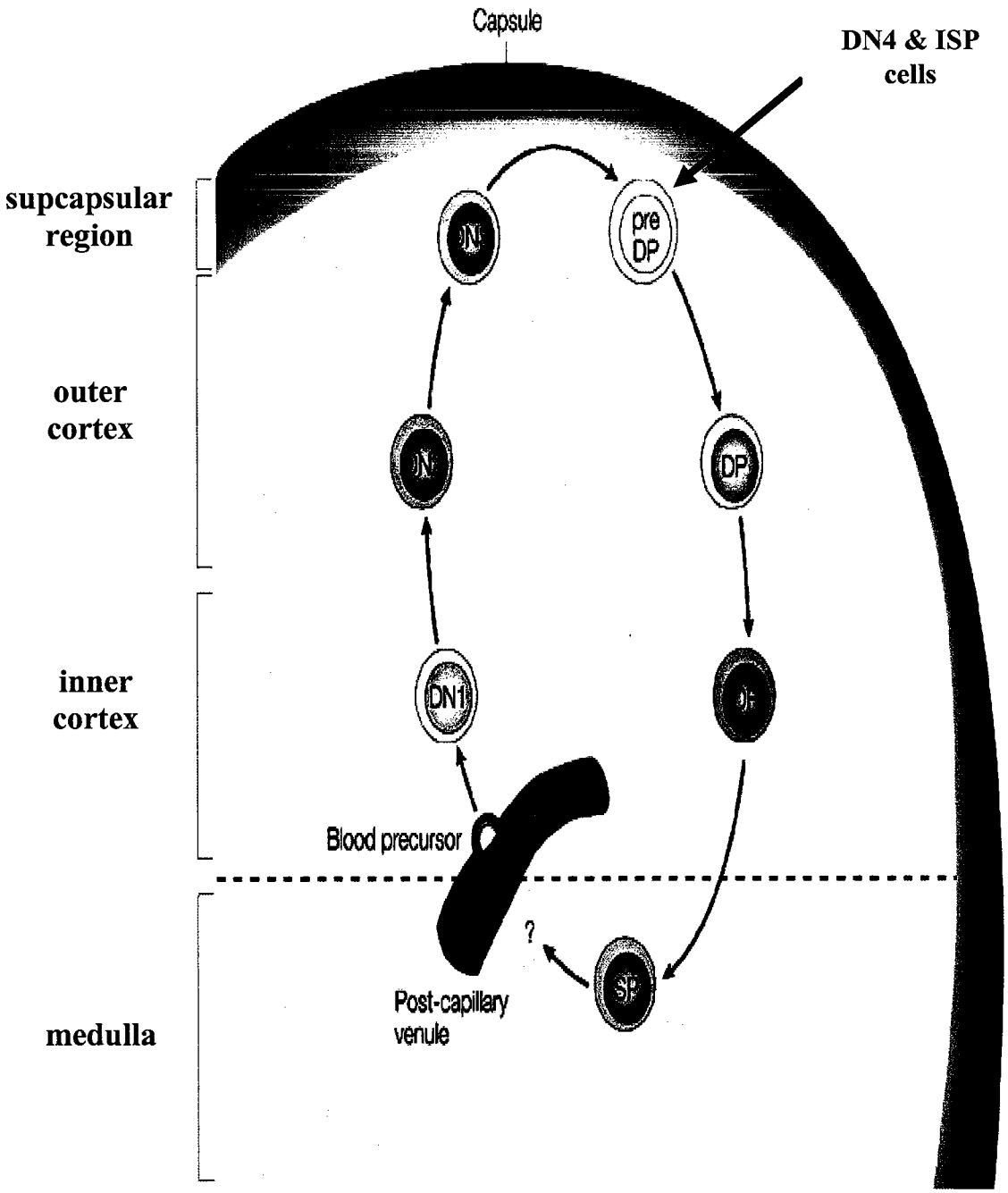
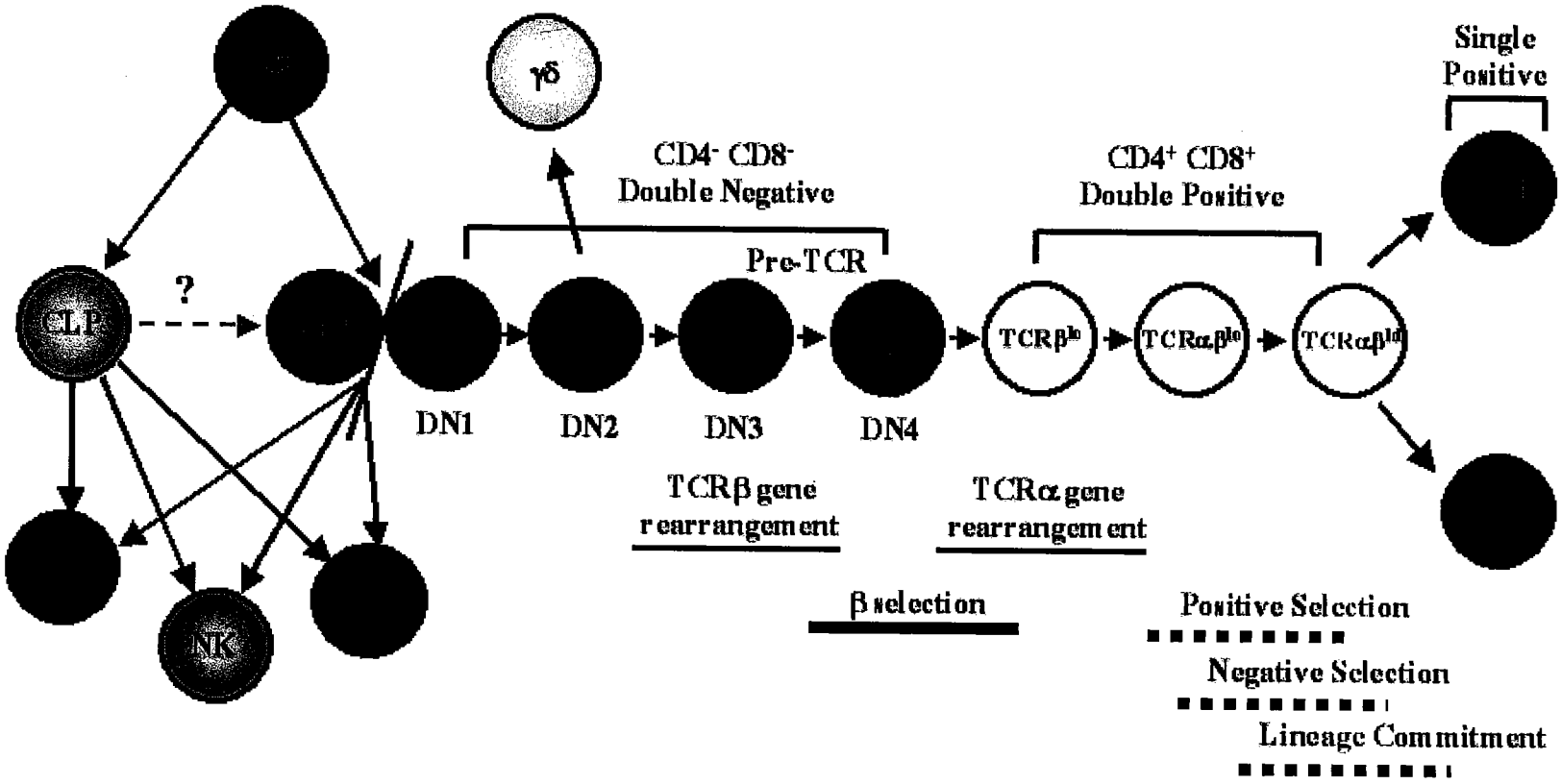

Capsule
DN4 & ISP cells
supcapsular region
outer cortex
inner cortex
medulla
DN
pre DP
DP
DN1
Blood precursor
?
Post-capillary venule

Figure 1.2 Overview of T cell developmental stages

A schematic diagram of stages of T cell development based on the expression of cell surface markers is depicted. The earliest T cell progenitors (ETPs) are double negative (DN) for the coreceptors CD4 and CD8 and they have the potential to become αβ T cells, γδ T cells, NK-T cells, B cells, NK cells, and dendritic cells (DCs). DN thymocytes can be further subdivided into four stages (DN1 – DN4) based on the expression of CD44 and CD25, and the ETPs are contained within the DN1 thymocyte compartment. Commitment to the T lineage occurs as the cells transition between DN1 and DN2. The αβ and γδ lineages diverge at the DN2 to DN3 transition. The subsequent stages of αβ T cell development are also depicted.

LSK: lineage marker negative, stem cell antigen high and c-kit high (lin^- $Sca\text{-}1^{hi}$ $c\text{-}kit^{hi}$); CLP: common lymphoid precursor; NK: natural killer cell

THYMOCYTE DEVELOPMENT



While it is known that the expression of various adhesion molecules and chemokine gradients within the thymus control this localization and migration of thymic subsets, the precise molecules involved are still poorly understood. In contrast, the molecules involved in the survival and development of the various thymocyte subsets are better defined. These transitions require signals from both cytokines and from the developing TCR. The earliest T cell precursors, those at the DN1 and DN2 stages of development, express c-kit, the receptor for the cytokine stem cell factor (SCF). Both SCF- and c-kit-deficient mice have a significant reduction in these early T cell populations, but in spite of this reduction, the size of the thymus is only slightly reduced and development appears to proceed normally (8). This seems to indicate that SCF is primarily important for the survival or expansion of these T cell subsets instead of promoting transition from one subset to the next.

The cytokine IL-7 is also an extremely important survival factor for early T cell subsets. IL-7 signals through a receptor that is comprised of the IL-7Rα chain and the common γ chain (γ_c). The γ_c is part of the receptor for a number of other cytokines, including IL-2, IL-4, and IL-15 and it signals via the kinase Jak3, which activates gene transcription via signal transduction and activator of transcription-5 (STAT5). Mice that are deficient in IL-7 (9), IL-7Rα (10, 11), γ_c (12-14), or Jak3 (15-17) have a severe reduction in thymic cellularity. In addition, although all the thymic subsets, based on CD4 and CD8 expression, are present, development past the DN2 is severely impaired. A role for IL-7 in promoting V(D)J recombination has been proposed, however the

clearest functions of IL-7 in T cell development are in promoting the survival and cell-cycle progression of early thymic subsets (18).

Whereas cytokines are clearly crucial during the DN1-DN3 stages of thymocyte development, the transition from DN3 all the way through to the DP stage of development is critically dependent on the proper rearrangement of one of the TCR β chain loci. Successful rearrangement and expression of a TCR β chain allows the formation of receptor complex known as the pre-TCR. The pre-TCR is formed by a TCR β chain, with a monomorphic α chain, pre-Tα, and non-covalently associated CD3 subunits that connect the αβ TCR chains with the signaling machinery in both the pre- and mature-TCR complexes. The formation of this signaling complex is absolutely essential since mice deficient in TCRβ (19), pre-Tα (20), and certain CD3 subunits (21, 22), as well as mice deficient in the recombination activating gene (RAG) enzymes that are essential for V(D)J recombination (23, 24), are blocked at the DN3 stage of T cell development. This checkpoint in T cell development is commonly referred to as β selection.

Initiation of pre-TCR signaling appears to be ligand independent since the extracellular part of pTα (25) and the TCR V-β (26) region are not required for the DN to DP transition. In addition, an αβ TCR transgene has recently been shown to be able to provide a pre-TCR signal in the absence of pTα and RAG even when its MHC ligand is not present (27). Besides signaling the DN to DP transition, pre-TCR signaling results in cell-cycle entry, expansion, and allelic exclusion at the TCR β locus (28-30). Allelic exclusion ensures that once there is a productive β chain rearrangement, rearrangement

ceases in order to prevent the expression of second β chain. Without this, the specificity of T cells would be compromised since more than one receptor could be expressed. At least part of the mechanism behind allelic exclusion is the down-regulation of RAG expression following pre-TCR signaling (29).

RAG expression is re-induced at the DP stage of thymocyte development in order to rearrange the TCR α chain (31). Unlike rearrangement at the β locus, productive rearrangement of the α locus is not sufficient to induce progression to the next stage of development. Instead, only an α and β chain pair that can interact with self-MHC molecules terminate recombination and continue to mature. The unique structure of the α gene locus allows multiple V-J recombination events to occur on the same allele (32, 33), which increases the likelihood that a self-restricted αβ pair will be produced, although this is still a relatively rare event (34). The binding of a mature TCR complex on the surface of a DP thymocyte to self-peptide/self-MHC complexes in the cortex of the thymus results in down-regulation of RAG gene expression, up-regulation of survival factors, and migration to the medulla. This process is known as positive selection and it requires continuous sustained signaling for several days (35). A requirement for ligand binding at this stage of development ensures that the T cells that mature will be functional since they have to capable of recognizing antigenic peptides in the context of self-MHC molecules. Those cells that fail to interact with self-peptide/self-MHC through their TCR, do not receive the survival and differentiation signals of positive selection eventually die, this is often referred to as "death by neglect."

Although recognition of self-MHC molecules is essential for antigen recognition by T cells, it is also important the self-peptide/self-MHC molecules are not stimulatory for mature T cells. If the mature T cells were stimulated by self-peptide/self-MHC molecules, it would lead to destruction of host cells and tissues by its own T cells, which would lead to widespread autoimmunity. The prevention of this is known as self-tolerance. One of the main mechanisms for maintaining self-tolerance is a developmental process known as negative selection. For the most part, negative selection occurs concurrent with positive selection, although it is possible for negative selection to occur as soon as TCR/CD3 can be detected on the surface of $CD4^{int}/CD8^{int}$ pre-DP thymocytes all the way through to the mature SP stage (36). Like positive selection, negative selection requires ligand binding and is the consequence of a strong interaction with self-peptide/self-MHC molecules present on thymic stromal and dendritic cells. Since this strong interaction would jeopardize self-tolerance, it results in a signal that leads to apoptosis and elimination of these potentially self-reactive cells. The combined processes of positive and negative selection are known as TCR repertoire selection.

Besides TCR repertoire selection, an additional process occurs at the DP to SP transition. This process is known as lineage commitment. Lineage commitment refers to the process by which DPs that express TCRs that bind to MHC class II molecules differentiate in $CD4^+$ SP cells and those DPs that express TCRs that bind to MHC class I molecules differentiate into $CD8^+$ SPs. In addition to retaining expression of the proper coreceptor, each SP population differentiates into a specialized subset of T cells. $CD4^+$ SP cells differentiate into T helper cells. They recognize antigen in the context of MHC

class II molecules on APCs and they primarily exert their effects through the production of cytokines. $CD8^+$ T cells differentiate into cytotoxic T cells and because these cells recognize MHC class I molecules, which are present on almost all cells, they can potentially kill any cell infected with a foreign pathogen. Just like repertoire selection, lineage commitment is known to be mediated in part by signals through the TCR on the DP thymocyte; and to some extent, the strength of this signal is thought to contribute to outcome of lineage commitment/differentiation (37). However, recent models suggest that cytokines may also contribute to this process (38).

TCR signaling and T cell development

As described above there are two main stages of αβ T cell development that require signals through the TCR. The first stage is at the DN3 to DN4 transition when a signal via the pre-TCR is absolutely required from further differentiation. The second stage is the DP to SP transition, where TCR signaling is critical for positive selection and the complete maturation of T cells. Just as the formation and specific components of the pre-TCR and mature αβ TCR are absolutely required for these processes, several TCR signaling molecules have also been shown to be crucial for proper T cell development. Among these molecules are tyrosine kinases, adaptor proteins, GTPases, exchange factors and transcription factors. Deletion of a number of these molecules results in a block at the pre-TCR stage. Deletion of others has a greater effect during selection and/or lineage differentiation. Prior to my PhD studies, a new tyrosine kinase from the Tec family, known as Itk, had been cloned by our lab and others (39-41). Its B cell

homolog, Btk, had been shown to be crucial for proper B cell development. In fact, mutations in Btk have been shown to be responsible for the human genetic disorder X-linked agammaglobulinemia (XLA) and the murine mutant X-linked immunodeficiency (xid) (42-45). Initial characterization of Itk-deficient mice suggested that Itk may play a role in T cell development (39). The goal of my thesis has been to identify the role of Itk throughout T cell development in order to better understand how the various TCR-mediated developmental processes are regulated.

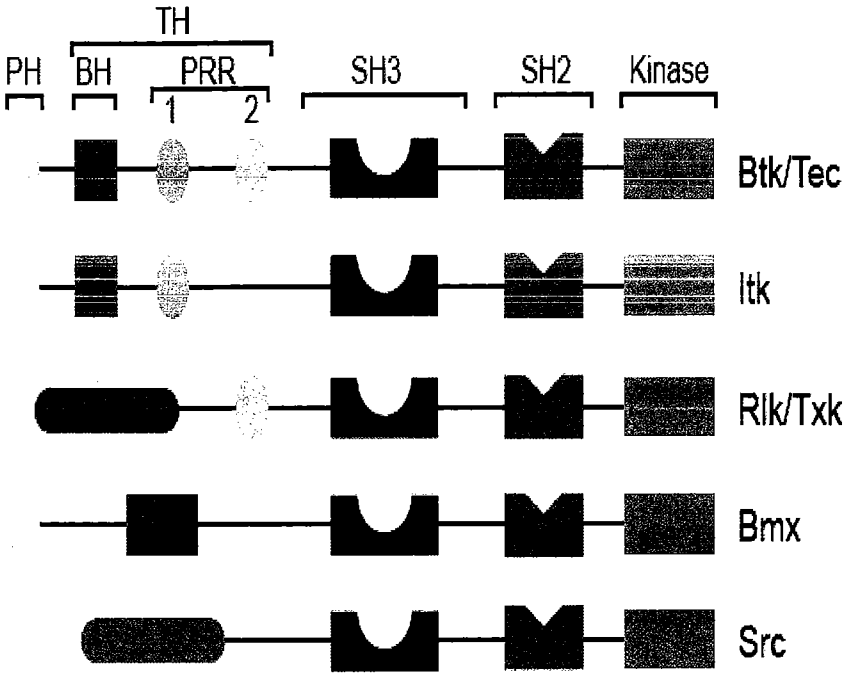
Tec Family Kinases

Six members of the Tec family of non-receptor protein tyrosine kinases have been identified. Five of the family members are expressed in hematopoietic cells, with three, Itk, Rlk and Tec, expressed in thymocytes and mature T cells. All three of these kinases are involved in signaling downstream of the T cell receptor (TCR). The domain structure of each Tec kinase family member is very similar, consisting of an N-terminal pleckstrin homology (PH) domain followed by the protein binding Tec homology (TH), Src homology 3 (SH3) and SH2 domains, and a C-terminal kinase domain. Unique to this family of protein tyrosine kinases, the inclusion of a PH domain allows recruitment of Tec kinases to the cell membrane through their binding of phosphatidylinositol (3,4,5)-triphosphate (PIP_3). Both Tec and Itk adhere precisely to this structural organization. Rlk, on the other hand, differs from Tec and Itk in two ways. First, Rlk lacks the N-terminal PH domain and instead contains a string of cysteines that can be palmitoylated. Second, a shortened form of Rlk can be generated through an alternative translational

start site, and this form of the protein can be translocated to the nucleus of T cells following activation (reviewed in (46-50).

Figure 1.3 Tec kinase's domain structures [as seen in (50)]

A schematic representation of the Tec family kinases protein domain structure is depicted. There is a pleckstrin homology (PH) the N-terminus of five of the six Tec family kinases that allows recruitment of these kinases to the cell membrane via their binding to phosphor-inositide ligands in the membrane. The sixth family member, Rlk/Txk, has a string of cysteines at the N-terminus that can be palmitoylated and direct it to the plasma membrane. Each of the Tec kinases has a Tec homology (TH) domain that is made up of some combination of a Btk homology (BH) domain and/or one or two proline rich regions (PRR). The PRRs as well as the Src homology (SH)3 and SH2 domains mediate both inter- and intra-molecular protein-protein interactions. At the C-terminus of all the family members is the catalytic kinase domain. Also depicted is a schematic representation of the prototypic Src family kinase domain structure.


TH
PH
BH
PRR
1
2
SH3
SH2
Kinase
Btk/Tec
Itk
Rlk/Txk
Bmx
Src

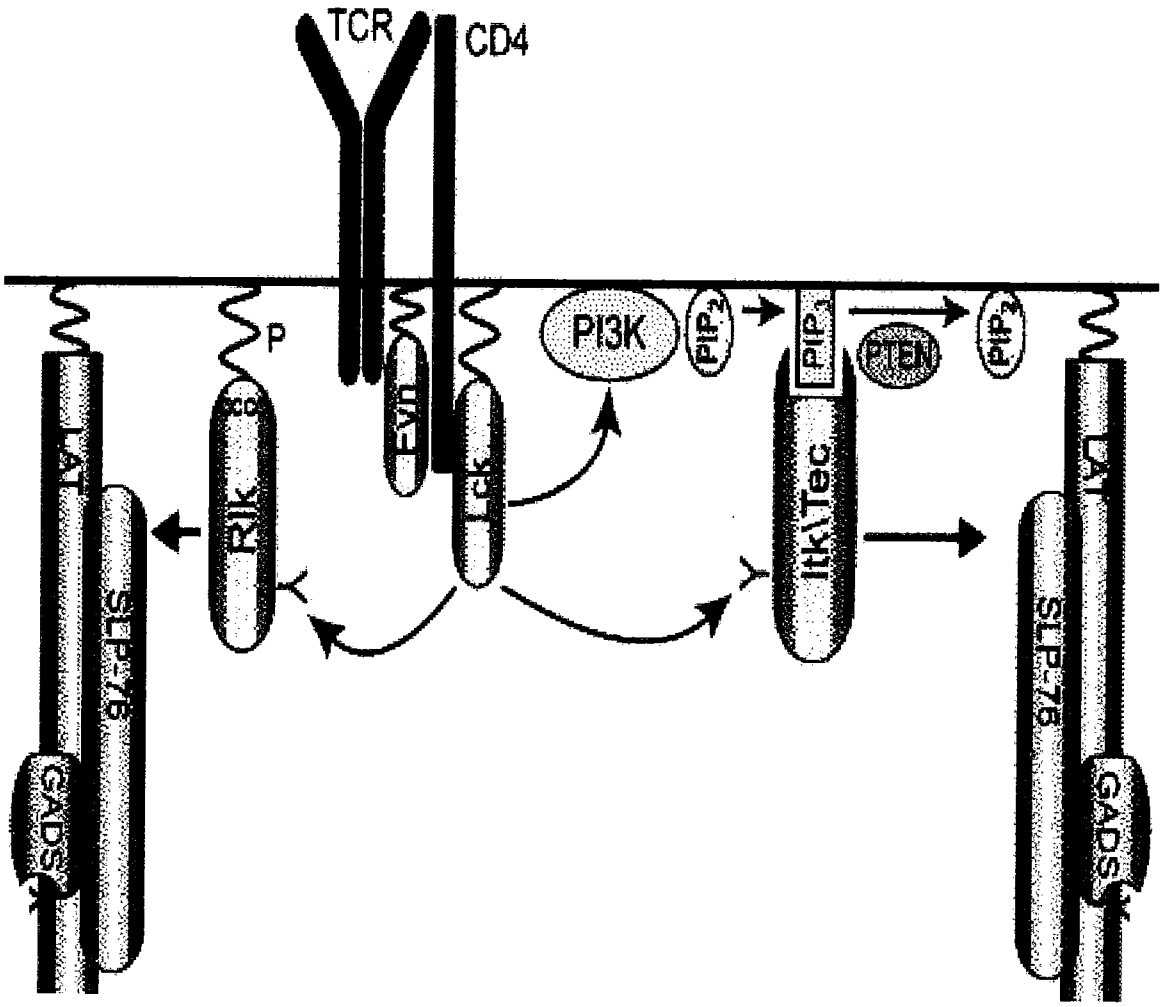
The precise course of events leading to the activation of Itk, Rlk, and Tec following engagement of the TCR has not been completely elucidated; however, localization of these kinases to the plasma membrane, as well as tyrosine phosphorylation within the kinase domain, appears to be necessary. Prior to signaling, all three kinases are predominantly cytoplasmic. Immediately following TCR stimulation, all three of these kinases are found to be associated with the plasma membrane in cultured T cell lines. This membrane association, for Itk and Tec, occurs via the PH domain (51, 52) and is dependent on the activation of phosphoinositide 3-kinase (PI3K), which converts phosphatidylinositol (4,5)-biphosphate (PIP_2) into phosphatidylinositol (3,4,5)-triphosphate (PIP_3), thereby generating the ligand for the Itk and Tec PH domains. In T cells, this process is negatively regulated by the phosphotase, PTEN, which converts PIP_3 back into PIP_2 (53, 54). Because of the absence of a PH domain, association of Rlk with the membrane is independent of PI3K activity, and instead it is due to palmitoylation of its cysteine string motif (55). Localization of the Itk and Tec to the membrane via their PH domains is crucial for their activation by tyrosine phosphorylation (51, 52, 54, 56). Similarly, the short isoform of Rlk, which lacks the cysteine string motif that is required for palmitoylation is less efficiently phosphorylated and has lower kinase activity than the long isoform, which is primarily found associated with the plasma membrane following TCR stimulation (57).

Following membrane localization, the activation of Tec family kinases in T cells requires phosphorylation by a Src kinase, possibly Lck for Itk (58) and Fyn for Rlk (55) of a conserved tyrosine within the activation loop of the kinase domain. For Itk, this

activating phosphorylation event is also dependent on the activity of zeta-associated protein of 70 kDa (ZAP-70) and linker for activation of T cells (LAT) (59). This dependence suggests that the association of Itk with the membrane is not enough to lead to its activation and, in addition, recruitment to the TCR signaling complex is required. Consistent with this notion, both Itk and Tec colocalize with the TCR following TCR stimulation (51, 59). Although the association of Itk with the TCR signaling complex requires ZAP-70 and LAT, Itk is not a substrate of ZAP-70 and does not directly bind LAT. However, Itk has been found to bind SH2 domain-containing phosphoprotein of 76 kDa (SLP-76) (54, 60), which does bind LAT via its interaction with Gads (61-64) in a ZAP-70-dependent manner (65).

Figure 1.4 Activation and recruitment of Tec kinases to the TCR signaling complex [adapted from (50)]

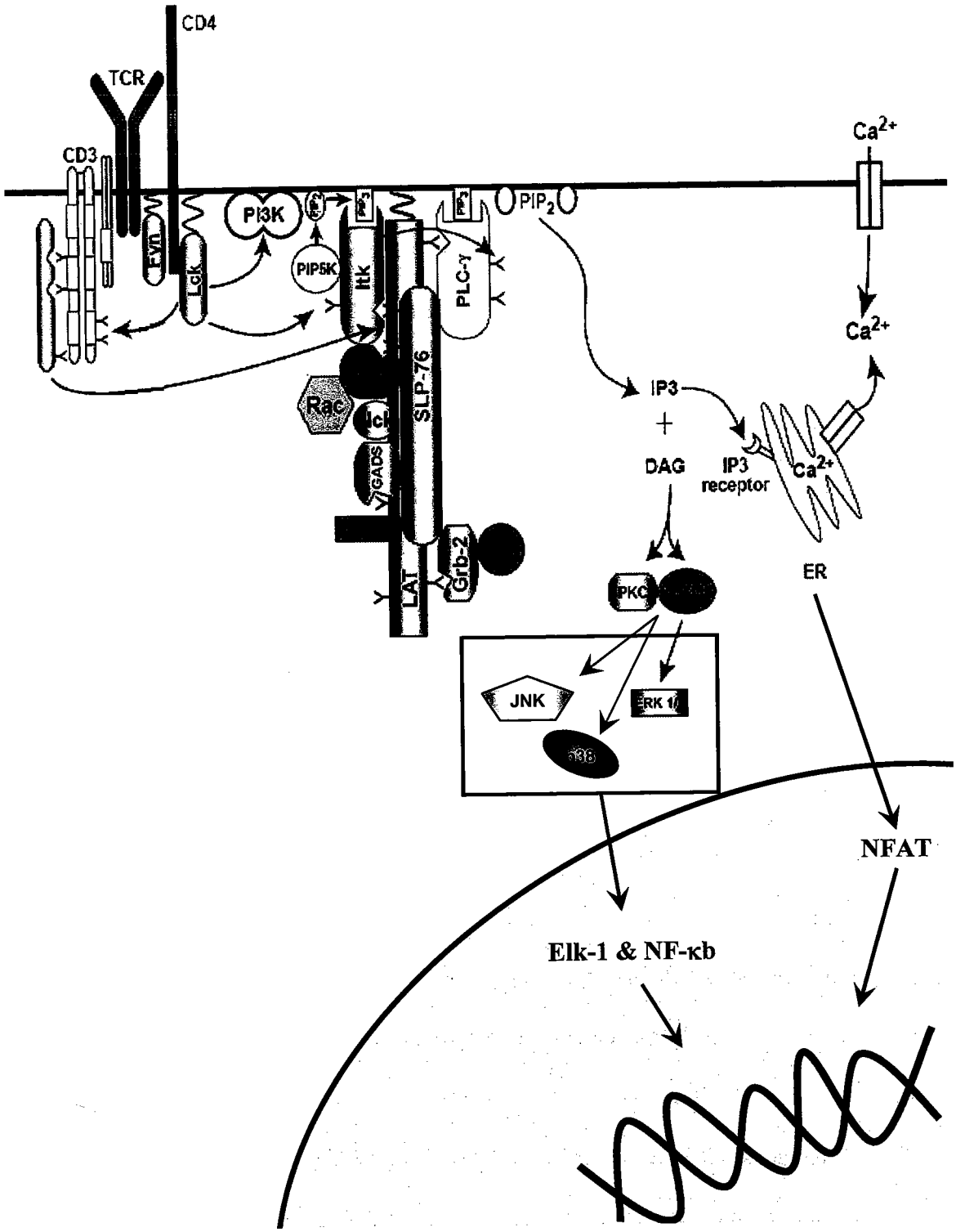
The activation of Tec-family tyrosine kinases downstream of T cell surface receptors requires at least three independent steps. (1) The localization of the kinase to the plasma membrane at the site of the activated receptor. For Itk and Tec, this localization requires the PH domain and is dependent on receptor-mediated activation of PI3K and the generation of PIP_3. For Rlk, membrane localization is mediated by palmitoylation of the cysteine-string motif and is independent of PI3K activity. (2) The phosphorylation of the kinase by a Src-family kinase, such as Lck. (3) The association of the kinase with adapter proteins, such as SLP-76, Gads, and LAT that form a complex in response to receptor stimulation. The precise order of these steps in the activation process has not been established.


TCR
CD4
P
PI3K
PIP2
PIP3
PTEN
PIP3
Evn
Lck
Rlk
Itk\Tec
LAT
SLP-76
GADS
LAT
SLP-76
GADS

Once the Tec family members have been activated and are bound to LAT via SLP-76, they are capable of phosphorylating phospholipase C-γ1 (PLC-γ1) (54). This phosphorylation leads to the activation of PLC-γ1, which then hydrolyzes PIP_2 into the second messengers inositol (3,4,5)-triphosphate (IP_3) and diacylglycerol (DAG). The production of IP_3 causes calcium mobilization in the T cells, while DAG activates protein kinase C (PKC) and Ras-GRP, thereby leading to the activation of Ras/Raf/mitogen-activated protein kinase (MAPK) pathways. Subsequent to Ca^{2+} mobilization, the nuclear factor for activated T cells (NFAT) transcription factors are dephosphorylated, translocate to the nucleus, and activate a number of genes, including those encoding cytokines. The activation of PKC and the Ras/MAPK pathways affect a number of serine/threonine kinases including Erk1/2, p38, and c-jun N-terminal kinase (JNK). These pathways culminate in the activation of additional transcription factors, such as NF-κB and Elk-1, which regulate genes involved in cytokine signaling, survival and differentiation. Thus, following the activation of PLC-γ1 by the Tec kinases, multiple processes important for T cell development, activation, effector function and homeostasis are affected.

Figure 1.5 TCR signaling downstream of Tec kinases [adapted from (50)]

Engagement of the TCR leads to rapid activation of Lck and ZAP-70, which phosphorylate numerous downstream targets, including the adaptor molecules LAT and SLP-76 that together form a platform for the accumulation of molecules into a signaling complex including: PLCγ, Grb-2, Tec kinases as well as other molecules. Itk and probably other Tec-kinases physically interact with SLP-76 and possibly LAT bringing them into this complex where they can phosphorylate PLC-γ. Phosphorylation and activation of PLC-γ is important for transducing signals that regulate Ca^{2+} mobilization (IP_3), PKC and MAPK activation (via DAG), culminating in the activation of transcription factors including NFAT, Elk-1, and NF-κb.


CD4
TCR
CD3
Fyn
Lck
PI3K
PIP5K
Itk
PLC-γ
PIP2
Ca2+
Ca2+
IP3
+
DAG
IP3
receptor
Ca2+
ER
Rac
Itk
SLP-76
GADS
LAT
Grb-2
PKC
JNK
ERK 1/
38
NFAT
Elk-1 & NF-κb

Single knockouts of the genes encoding Itk, Rlk and Tec have all been generated in mice and characterized (39, 66-68). Of these single knockouts, the loss of Itk has the most substantial effect on T cell development and function, while the loss of Rlk has a mild effect and the loss of Tec appears to have no effect. Interestingly, these phenotypes correlate with the mRNA expression levels for these genes in T cells, as recently determined by real-time polymerase chain reaction (PCR) studies in our laboratory. We find that Itk mRNA is present at the highest level, Rlk mRNA levels are two- to threefold lower and Tec mRNA is present at a level close to 100-fold lower than that of Itk (Miller, Felices and Berg, unpublished data). As none of the single knockout phenotypes leads to an absolute block in T cell signaling, it has been suggested that the activities of the remaining Tec kinases may compensate for the loss of one. The analysis of *itk*$^{-/-}$*rlk*$^{-/-}$ mice has supported this hypothesis by demonstrating that the absence of these two Tec kinases results in defects in T cell development and activation that are more severe than either single knockout and, furthermore, that this double deficiency leads to a more substantial decrease in PLC-γ1 phosphorylation/activation following stimulation of the TCR (67-69).

Tec kinases and T cell development

As mentioned previously, many proteins that are important for T cell receptor signaling have been shown to play critical roles in T cell development. For instance, disruption of the gene encoding the protein tyrosine kinase Lck (70) or ZAP-70 (71, 72) results in severe defects in T cell development. Furthermore, mutation of the gene encoding Btk, a Tec family member expressed in B cells, leads to an immunodeficiency

disease due to blocks in B cell development (44, 45, 73). Based on these findings, it was predicted that disruption of the Itk gene would lead to a block in T cell development. However, the generation and analysis of *itk*$^{-/-}$ mice revealed that T cell development was largely intact in the absence of this kinase (39, 66, 67, 69, 74). Despite the seemingly ormal T cell development in these mice, Liao et al found that when the Itk-deficient mice were crossed onto a TCR transgenic background, the development of thymocytes was blocked at the DP to SP transition due to the lack of mature SP thymocytes or peripheral T cells.

In order to better understand this observation and the role of Itk at the DP to SP transition, I began my project by crossing the Itk-deficient mice onto other TCR transgenic backgrounds. These mice allowed us to investigate the role of Itk in both repertoire selection and CD4/CD8 lineage commitment. We hypothesized that the loss of SP development in the TCR transgenics used in the study by Liao et al may be due to the fact that the transgenic TCR may have low avidity for its selecting ligand(s) in the thymus. Thus, we proposed that in the absence of Itk positive selection may be impaired, but if the developing thymocytes expressed a TCR with high avidity for its selecting ligand(s) in the thymus then selection may take place even without Itk. In all, we crossed the Itk-deficient mice onto five class II restricted TCR transgenic backgrounds and one class I restricted TCR transgenic background.

In most cases, we found that mature T cells developed in the absence of Itk, but less efficiently. In the beginning, we chose to focus our attention on class II restricted T cells since Itk-deficient mice seem to have a specific loss of $CD4^+$ T cells. This work has

been published (74) and is the main focus of Chapter 3 along with results relevant to negative selection using the same TCR transgenic mice.

Although we initially focused on the role of Itk in repertoire selection, we also examined CD4/CD8 lineage commitment and differentiation. Itk-deficient mice have a decreased CD4:CD8 ratio and this along with an emerging model of lineage commitment that suggested that the strength of the TCR/coreceptor signal determined CD4/CD8 lineage fate, led us and others to hypothesize that class II restricted T cells were developing into CD8 cells in the absence of Itk. The comprehensive analysis of five class II restricted TCR transgenic/Itk-deficient mice suggest that this is the case; however, we came to realize that the $CD8^+$ T cells in *itk*$^{-/-}$ mice have a "previously-activated" or "memory" phenotype and accumulate in the thymus. The precise cause of this still unknown, but we have determined that it is the result of altered T cell development in the thymus and discuss possible mechanisms in Chapter 4.

While most work in this thesis focuses on the role of Itk during the DP to SP transition of T cell development, we also examined the role of Itk in pre-TCR signaling during the DN to DP transition. We did not observe any obvious defects in *itk*$^{-/-}$ mice at this stage; however, we reasoned that any mild defects may not be detectable in mice that only have Itk-deficient T cells. Therefore, we made mixed bone marrow chimeras, which allowed us to examine the development of *itk*$^{-/-}$ cells in the presence of competing wild-type cells. Interestingly, the initiation of proliferation and progression from the DN3 to the DN4 stage of development seems to occur normally without Itk, but the generation or

survival of DP cells is less efficient in the absence of Itk. These results suggest that Itk plays a minor, but distinct role in pre-TCR signaling and is discussed in Chapter 2.

CHAPTER II

THE ROLE OF ITK IN PRE-TCR SIGNALING

INTRODUCTION

As described earlier, the most immature T cell precursors in the thymus lack the expression of the T cell co-receptors CD4 and CD8 and thus are referred to as double negative (DN) thymocytes. These precursor cells can be further subdivided into four stages of development, DN1 through DN4, based on the expression of CD44 and CD25. Cells at the DN3 ($CD44^{-}CD25^{+}$) stage of development are undergoing rearrangement of their T cell receptor beta chain genes. Cells progressing down the TCR αβ lineage are dependent on signals transduced through the pre-TCR, a receptor comprised of a functionally rearranged TCR beta chain paired with an invariant pre-TCR alpha chain. These pre-TCR signals induce the transition from the DN3 stage to the DN4 ($CD44^{-}$ $CD25^{-}$) stage, and subsequently to the double positive (DP, $CD4^{+}CD8^{+}$) stage.

During this developmental stage, which as stated earlier, is referred to as β selection, four concurrent, but distinctive types of signals must occur. These selection events include the induction of survival, proliferation, and differentiation, as well as, allelic exclusion and all result from signals downstream of the pre-TCR (Figure 2.1). While this process is thought to be ligand-independent (25-27), there is evidence that the pre-TCR complex needs to be transported to the surface. This evidence comes from transgenic mice that expressed a TCRβ chain with a strong endoplasmic-reticulum retrieval signal (TCRβER) on a TCRβ null background. In these mice some of the TCRβER protein is found in the cytoplasm, but none of it reaches the cell surface. While the expression of a TCRβ chain in $TCR\beta^{-/-}$ mice can relieve the block at the DN stage of development (19), the TCRβER protein cannot (75). It is likely that the requirement for

membrane localization of the pre-TCR is to bring it in proximity to crucial signaling components.

As shown in Figure 2.1, there are two families of tyrosine kinases that are critical for signaling all the selection events mediated by the pre-TCR. Establishing these molecules as crucial, as well as the role of signaling molecules downstream, was primarily the result of analyzing genetically mutated mice. Mice that are deficient in the Src kinase, Lck, or express a dominant negative form of Lck have almost a complete block in development at the DN3 to DN4 transition when pre-TCR signaling is required (70, 76, 77). In mice that are deficient in a second Src kinase, Fyn, T cell development appears to be normal (78), but mice deficient in both Lck and Fyn have a complete block at this stage (79, 80). Similarly, redundancy is observed between the Syk kinases, ZAP-70 and Syk. Like Fyn-deficient mice, mice deficient in Syk have no abnormalities in T cell development (81, 82), but Syk and ZAP-70 doubly deficient mice are completely blocked at the pre-TCR stage (83). While there is redundancy in these kinase families, based on the single knockout phenotypes, Lck and ZAP-70, are thought to be the primary kinases of each family in pre-TCR/TCR signaling. Lck phosphorylates and activates a number of substrates, two of these are the TCRζ chain and ZAP-70. Phosphorylation of the TCRζ chain provides docking sites for ZAP-70, which brings ZAP-70 in proximity with two of its substrates, the adaptor proteins, SLP-76 and LAT.

Very similar thymocyte phenotypes are observed in mice in which the adaptor proteins, SLP-76 (84, 85) and LAT(86), have been deleted. Thus, the functions of these adaptor proteins are required for pre-TCR signaling as indicated in Figure 2.1. LAT and

SLP-76 serve as scaffolding proteins that contain multiple binding sites for downstream TCR signaling molecules and thus, help bring these molecules together and presumably allows them to associate with the components of the TCR signaling complex. LAT is a transmembrane protein, but SLP-76 requires another adaptor protein, Gads, to bring it to the membrane and/or to bind LAT (62). Gad-deficient mice have a severe block at the DN to DP transition, but unlike SLP-76 and LAT knockout mice, these mice have DP and SP thymocytes, albeit at reduced levels compared to wild-type mice (87) suggesting that membrane localization and association with LAT is very important for the function of SLP-76 during pre-TCR signaling, but it is not essential.

LAT and SLP-76 facilitate the activation of a number of downstream pathways. Two of these, the Ras-MAPK and PLC-γ pathways are depicted in Figure 2.1. While induction of the Ras-MAPK pathway is sufficient for the induction of survival, proliferation and differentiation of DN thymocytes as shown by the enforced expression of an active Ras transgene in RAG-deficient thymocytes (88), it has been demonstrated to have no role in allelic exclusion (89). On the contrary, activation of PLC-γ may play a role in all the β selection events. The importance of PLC-γ in the DN to the DP has been clearly demonstrated by LAT knock-in mice that replace wild-type LAT with a LAT mutant in which the phosphorylation site that is necessary for PLC-γ binding have a profound block at the DN stage of T cell development (90, 91)

PLC-γ hydrolyzes PIP_2 into the second messengers, IP_3 and DAG. IP_3 stimulates calcium mobilization by binding to calcium channels in the endoplamic reticulum and DAG is necessary for the activation of PKC. Studies by Aifantis et al, indicate that the

mobilization of calcium leads to the activation of the transcription factors, NF-κB and NFAT, the combined effects of which should lead to the induction of survival, proliferation, and differentiation (92). The production of DAG leads to the activation of PKC by binding to a domain in PKC which increases the affinity of PKC for phospholipids in the membrane. Thus PKC is localized to the membrane, but in addition binding of PKC induces a conformational change that enables PKC to phophorylate its substrates (93).

There are numerous isoforms of PKC and so far, the study of both novel and conventional PKCs has only indicated a role for one of them in T cell development. PKC-θ, a novel PKC, has been shown to be important for signaling in mature T cells, but dispensable for T cell development (94). Likewise, T cell development was normal in mice deficient in the conventional PKC -β (95) or -γ (96). However, a role for another conventional PKC, PKC-α, which is the most highly expressed isoform, is indicated in pre-TCR signaling. This was shown by experiments in which fetal thymi were transduced with retroviruses that expressed either a constitutively-active (CA) or a dominant-negative (DN) form of PKC-α. RAG-deficient thymocytes that expressed a CA-PKC-α were able to bypass the requirement for pre-TCR signals. In addition, expression of CA-PKCα was sufficient to suspend the rearrangement of the β chain in fetal thymic organ culture (FTOC) (97). On the other hand, expression of DN-PKCα suppressed the development of DP thymocytes in FTOC. Analysis of T cell development in PKC-α-deficient mice, which have been generated (98), will help determine if PKC-α specifically is absolutely required each of the β selection events.

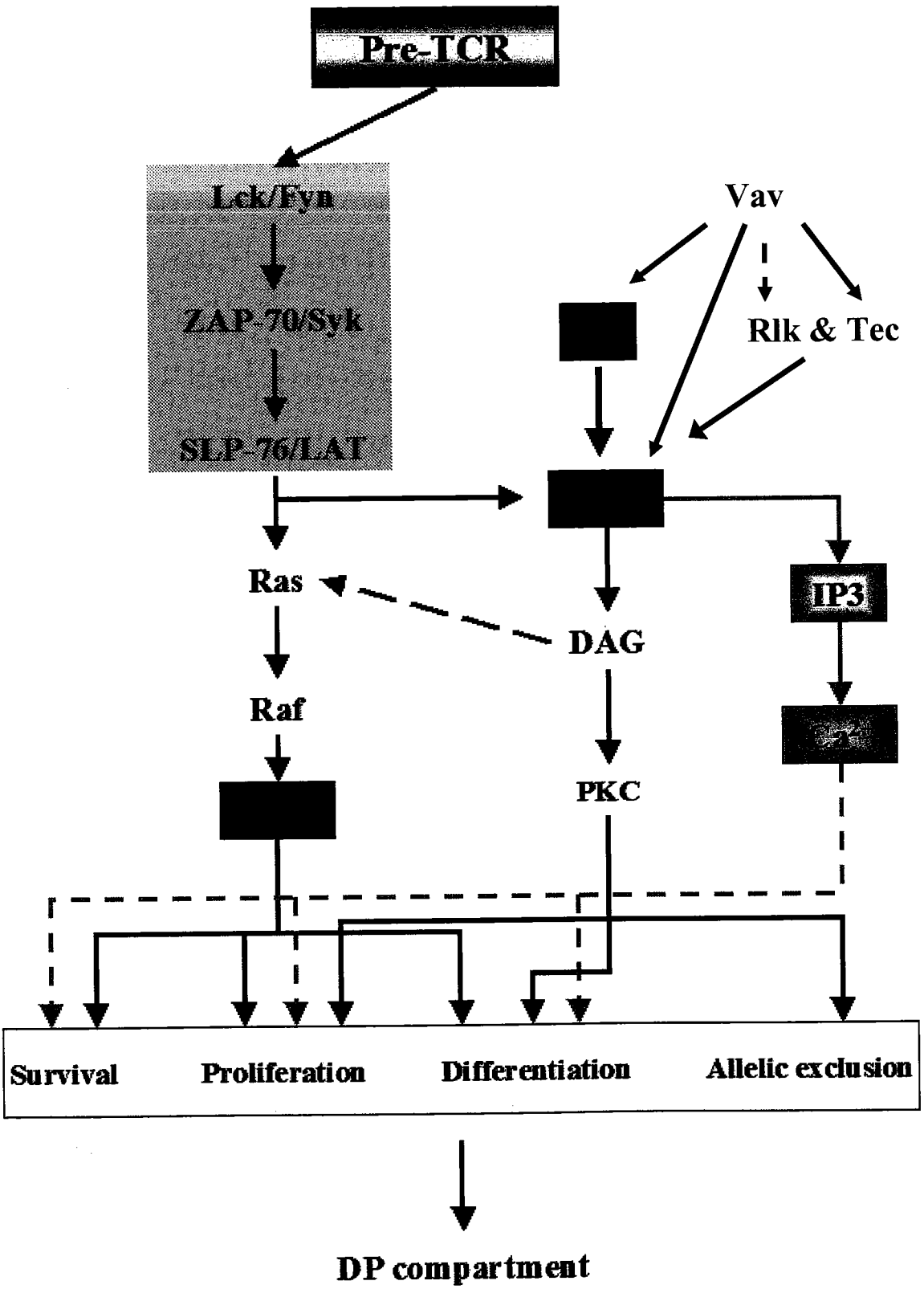
The activation of PLCγ was first shown to be reduced in mature $CD4^+$ T cells from Itk-deficient and Itk/Rlk-doubly deficient mice (66, 67). Similar results have also been seen in thymocytes from *itk*$^{-/-}$*rlk*$^{-/-}$ mice. As a consequence of this reduced PLC-γ activation, it has been shown that IP_3 levels are reduced (66) and it is presumed that DAG levels are reduced as well. In fact, pathways downstream of both of these signaling mediators are affected by the absence of Itk or both Itk and Rlk. The reduced production of IP_3 has been shown to affect calcium flux in both mature $CD4^+$ T cells and DP thymocytes. The reduced production of DAG could affect the Ras-MAPK pathway in two ways. First, there is a DAG binding site in RasGRP, an activator of Ras, which has been shown to be important in T cell differentiation and activation (99). Second, as mentioned, DAG is required for PKC activation and PKC is thought to contribute to the full activation of the Ras-MAPK pathway (100).

A role for Tec family kinases in pre-TCR signaling would seem likely in light of evidence that PLC-γ activity is important for this signal (90, 91) (Figure 2.1). Consistent with the notion that Tec kinases might play a role in pre-TCR signaling, analysis of Tec kinase gene expression indicates that these genes are expressed in early thymic progenitor cells. For instance, early studies performed by Northern blot analysis demonstrated that both Itk (40) and Rlk (101) mRNA can be detected by day 14 of fetal development, a time at which the thymus is comprised solely of DN1 and DN2 precursor cells. However, preliminary analysis of thymic development, as assessed by CD4 and CD8 expression, reveals that *itk*$^{-/-}$, *rlk*$^{-/-}$, and *itk*$^{-/-}$*rlk*$^{-/-}$ mice all have relatively normal numbers of both DN and DP thymocytes, suggesting that progression to the DP stage of

development is not significantly affected by the absence of these Tec family proteins (39, 69). In spite of this observation, we thought it was likely that Tec kinases did play some role downstream of the pre-TCR given the importance of PLC-γ, thus we decided to analyze the DN to DP transition in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ more closely.

Figure 2.1 Proposed model of pre-TCR signaling [adapted from (102)]

Some of the most important and well understood pathways of pre-TCR signaling are depicted, with emphasis on the pathways that are affected by Tec kinases. Activation of the Src kinases, Lck and Fyn, as well as the Syk kinases, ZAP-70 and Syk, and the presence and full function of the adaptors SLP-76 and LAT is absolutely required for all pre-TCR-driven selection events (highlighted in light green). Downstream of SLP-76 and LAT, pre-TCR signaling branches into different pathways. Two of the most important are shown, the Ras-MAPK and PLCγ pathways, are shown. Both of these pathways have to shown to be affected by the absence of Tec kinase signaling. The proteins or molecules that have been shown to be altered in *itk*$^{-/-}$ and/or *itk*$^{-/-}$*rlk*$^{-/-}$ T cells/thymocytes are in boxes.


Pre-TCR
Lck/Fyn
ZAP-70/Syk
SLP-76/LAT
Vav
Rlk & Tec
Ras
DAG
IP3
Raf
PKC
Survival
Proliferation
Differentiation
Allelic exclusion
DP compartment

MATERIALS AND METHODS

Mice

Itk-deficient mice were generated in our laboratory previously (66). *Itk*$^{-/-}$*rlk*$^{-/-}$ mice were a kind gift from Dr. Pamela Schwartzberg (NIH). *Itk*$^{-/-}$ mice have been backcrossed onto the C57BL/10 (B10) at least nine times *itk*$^{-/-}$*rlk*$^{-/-}$ mice have been backcrossed onto the C57BL/6 (B6) background at least five times. Wild-type mice are sometimes *itk*$^{+/-}$ littermates of *itk*$^{-/-}$ mice or pure B10 mice purchased from Jackson Laboratories (Bar Harbor, ME). CD45.1 (B6) congenic mice were purchased from Charles River Laboratories at NCI-Fredrick Animal Production Area (Fredrick, MD). Wild-type *rag-2*$^{-/-}$ mice were purchased from Jackson Laboratories. Itk-deficient *rag*$^{-/-}$ mice were generated by crossing fully backcrossed *itk*$^{-/-}$ mice to 5C.C7 *rag*$^{-/-}$ mice (Jackson labs) and were determined to be 5C.C7 negative by PCR. All mice used were between 6 - 12 weeks of age and were maintained at the University of Massachusetts Medical School animal facility under specific pathogen free (SPF) conditions.

Antibodies and DN staining

The following antibodies (Abs) and secondary reagents were purchased from Pharmingen (San Diego, CA): CD4-PE, CD8-Cy, CD8-APC, CD44-FITC, CD44-Cy, CD25-PE, CD5-Cy, CD3-bio, CD4-bio, CD8-bio, B220-bio, IgM-bio, Ter119-bio, Pan-NK(DX5)-bio, Gr1(Ly6G)-bio, Mac1(CD11b)-bio, CD11c-bio, strepavidin-allophycocyanin (strep-APC). BrdU-FITC was purchased from BD Biosciences (San Jose, CA). In order to characterize the DN thymocyte subsets, all lineage specific cells were stained with biotinylated Abs, followed by staining with anti-CD25, anti-CD44, and strep-APC. All

strep-APC^{+} events were gated out and the CD25 vs. CD44 profiles of the remaining cells was analyzed (Figure 2.2).

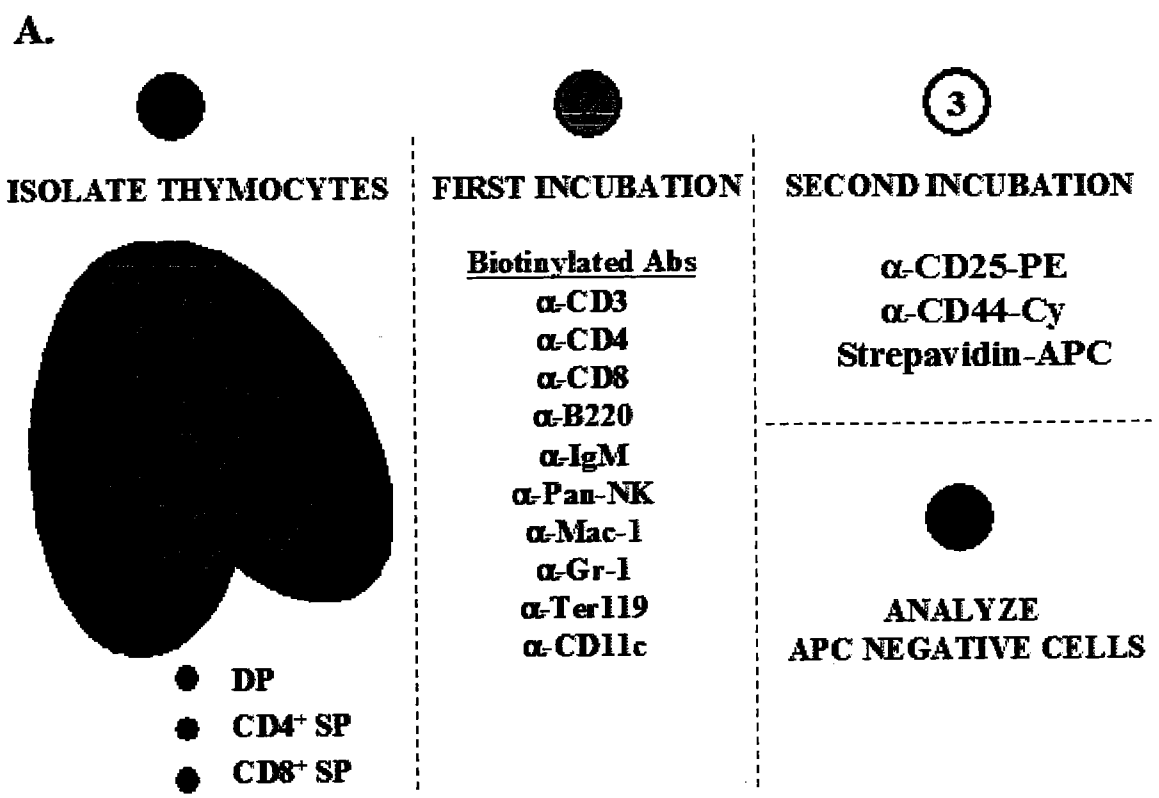
Cell preparation, staining, and flow cytometry

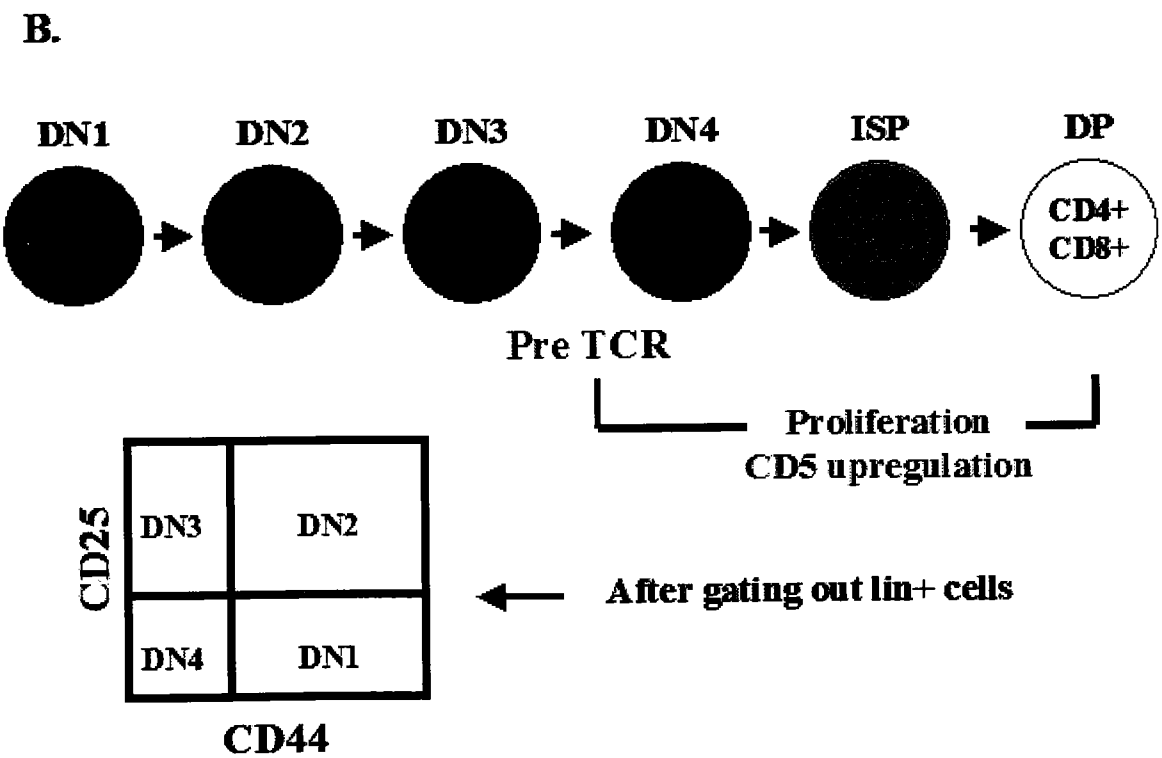
Lymphocyte cell suspensions were made from thymi by dissociation between two frosted slides in RPMI and 10% FCS. RBCs were lysed by incubation in Tris-ammonium chloride for 5 min at room temperature. Cells were washed, resuspended in FACS buffer (1x HBSS, 2% FCS, and 0.01% NaN_3), and plated in microwell staining plates at 5 x 10^5 to 2 x 10^6 cells/well. Biotinylated Abs were added and cells were incubated at 4°C for 15 – 30 min. The cells were washed and incubated with a mixture of directly conjugated Abs and strep-APC at 4°C for 30 min. After washing, cells were analyzed immediately or were fixed by the addition of 50 μl 2 - 4% Para formaldehyde and analyzed 12 – 36 h later. Cells were collected on a FACSCalibur (BD Biosciences, Mountain View, CA) flow cytometer. For the most part, 100,000 – 500,000 events were collected. Data was analyzed using both Cell Quest (BD Biosciences) and Flojo (Treestar, Ashland, OR)

Figure 2.2 Method for examining DN subsets

(A) Due to the fact that the majority of the thymus consists of DP (~80 – 85%) and SP (~10 – 15%) thymocytes, the number in one thymus is very small. Because of this, cells present in the thymus that are not of the T cell lineage, which not express CD4 and/or CD8 become a larger component of the DN subset. Thus, in order to examine the true DN compartment, we begin by staining the cells with a cocktail of biotinylated Abs to various lineage markers present on mature T cells (CD3, CD4, & CD8), B cells (B220 & IgM), NK cells (Pan-NK/DX5), macrophages (Mac-1/CD11b), granulocytes (Gr-1/Ly6G), erythrocytes (TER119), and dendritic cells (CD11b & CD11c). Subsequently, the cells are stained with anti-CD44 and anti-CD25 along with strepavidin-allophycocyanin.

(B) Analysis of the DN subset by CD44 and CD25 expression on the allophycocyanin-negative population is done as shown. The up-regulation of CD5, which occurs following signaling through the pre-TCR, is also assessed on the DN3 vs. DN4 subsets by including anti-CD44 into the biotin cocktail. Proliferation during the transition between the DN and DP stages of development will be assessed by injection with BrdU and subsequent staining of thymocytes extracellularly with anti-CD4, anti-CD8, and intracellularly with anti-BrdU.


A.
ISOLATE THYMOCYTES
FIRST INCUBATION
SECOND INCUBATION
Biotinylated Abs
α-CD3
α-CD4
α-CD8
α-B220
α-IgM
α-Pan-NK
α-Mac-1
α-Gr-1
α-Ter119
α-CD11c
α-CD25-PE
α-CD44-Cy
Strepavidin-APC
ANALYZE
APC NEGATIVE CELLS
DP
CD4+ SP
CD8+ SP


B.
DN1
DN2
DN3
DN4
ISP
DP
CD4+
CD8+
Pre TCR
Proliferation
CD5 upregulation
CD25
DN3
DN2
DN4
DN1
CD44
After gating out lin+ cells

Sorting of thymic subsets, mRNA isolation, and cDNA production

For subsets based on CD4 and CD8 expression, two B10 thymi suspensions were made and stained with anti-CD4-PE and anti-CD8-Cy and sorted into DN, DP, $CD4^+$ SP, and $CD8^+$ populations. For DN1-DN4 subsets, 25 thymi from B6 mice were pooled and DP, $CD4^+$ SP and $CD8^+$ SP cells were depleted by incubating with complement-fixing anti-CD4 and anti-CD8, followed by incubation with rabbit complement (Cedarlane Laboratories, Inc. Hornby, Ontario, Canada). Live cells were isolated by Lympholyte®-M (Cedarlane Labs) gradient, stained for CD25 and CD44 expression and sorted into all four DN subsets on a high-speed MoFlo cell sorter (Cytomation, Fort Collins, CO). Once the cells were sorted, the cell pellets were resuspended in 1 ml Trizol (Invitrogen, Carlsbad, CA) and stored at -80°C until mRNA isolation and cDNA amplification were performed. RNA was isolated by thawing the cells and lysing them by continuous pipetting and/or vortexing. Cells were incubated at room temperature (RT) for 5 min and 0.2 ml of chloroform was added and mixed by shaking vigorously for 15 sec. Cells were again incubated at RT for 2-3 min followed by centrifugation (12,000g) for 15 min. The aqueous layer was transferred to new tubes, 5 μg of glycogen and 0.5 ml of isoproponal were added, the tubes were incubated at RT for 10 min, and finally centrifuged as above. The supernatant was discarded and the RNA pellet was washed once with 1 ml of 75% ethanol followed by centrifugation (7500g) for 5 min. Residual DNA was removed by treatment with DNase (Promega, Madison, WI). After DNase treatment, 1 μg of total RNA was reverse transcribed into cDNA using Superscript II and Random Hexamers (Invitrogen) according to the manufacturer's protocol.

Real-time PCR

Real-time quantitative PCR was performed as described previously (103). All samples and standards were run in triplicate for any given experiment. The values of Itk, Rlk, and Tec were normalized to β-actin by dividing the average copy number of the respective transcript by the average copy number of β-actin. The PCR were as follows: templates were initially denatured at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, (62°C-β-actin), (60°C-Itk), (61.7°C-Rlk) for 25 s, or (54°C-Tec), and 72°C for 25 s. Primers were: Itk U 5'-CTCCGCTATCCAGTTTGCTCC-3'; Itk L 5'-GTCCTTGTTGAGCCAGTAGCC-3'; Rlk U 5'-TCAATCCAACAGAGGCGGG-3'; Rlk L 5'-CCGCTCTCTTCAGTGCCAA-3'; Tec U 5'-GGTTGGAGTGGTGAGGCTT-3'; Tec L 5'-GGTAACGATGTAGATGGGC-3'; βactin U 5'-CGAGGCCCAGAGCAAGAGAG-3'; βactin L 5'-CGGTTGGCCTTAGGGTTCAG-3'. For the generation of standard curves, plasmids containing cDNA clones of Itk (H. Wilcox), Rlk (gift from P. Schwartzberg, NIH, Bethesda, Maryland), Tec (W.C. Wang), and β-actin (gift from R. Gerstein, University of Massachusetts Medical School, Worcester, MA) were used.

Bone Marrow Chimeras

Bone marrow (BM) was isolated from femurs of CD45.1$^+$ and CD45.2$^+$ wild-type, CD45.2$^+$ *itk*$^{-/-}$, and CD45.2$^+$ *itk*$^{-/-}$*rlk*$^{-/-}$ mice and depleted for T cells using complement fixing anti-Thy1.2 Ab and rabbit complement. For non-mixed BM chimeras, 1 x 10^6 CD45.2 BM cells of each genotype were injected into separate lethally irradiated congenic (CD45.1) mice. For mixed chimeras, 5 x 10^5 CD45.1$^+$ wild-type BM cells were

mixed with 5 x 10^5 CD45.2$^+$ *itk*$^{-/-}$ or CD45.2$^+$ *itk*$^{-/-}$*rlk*$^{-/-}$ BM cells. Mice were analyzed 8 – 12 weeks after reconstitution.

BrdU incorporation

Mice were injected i.p. with 2 mg of bromodeoxyuridine (BrdU) (Sigma Aldrich, St. Louis, MO) in PBS 1 - 2 hours before harvest. Cells were plated at 6 x 10^6/well and then stained for surface antigens. Following extracellular staining the cells were washed in PBS and then fixed and permeabilized with Cytofix/Cytoperm for 20 min at 4° C. The cells were then washed with PBS and fixed again in 1%/0.1% paraformaldehyde/Tween-20. To stain for BrdU, cells were spun down and washed twice in PBS at room temperature, and subsequently resuspended in 200λ of DNase solution [750λ 5M NaCl, 105λ 1M $MgCl_2$, 250λ1mM HCl, 24mg DNase (Roche Applied Sciences, Indianapolis, IN), 23.9 ml dH_2O] and incubated at RT for 10 – 30 min. The cells were then washed and incubated in 50λ of a 1:10 dilution of anti-BrdU-FITC for 30 min. At the end of the incubation period, the cells were washed twice and analyzed by flow cytometry.

Injection of rag$^{-/-}$ *mice with anti-CD3*

Wild-type and Itk-deficient mice were injected i.p. with 50 μg of purified anti-CD3ε (2C11) from ebioscience (San Diego, CA). Mice were analyzed at day 3 and day 4 following injection for CD4 and CD8 expression, as well as CD25 and CD44 expression.

RESULTS

Analysis of thymocyte subsets in wild-type, $itk^{-/-}$, and $itk^{-/-}rlk^{-/-}$ mice

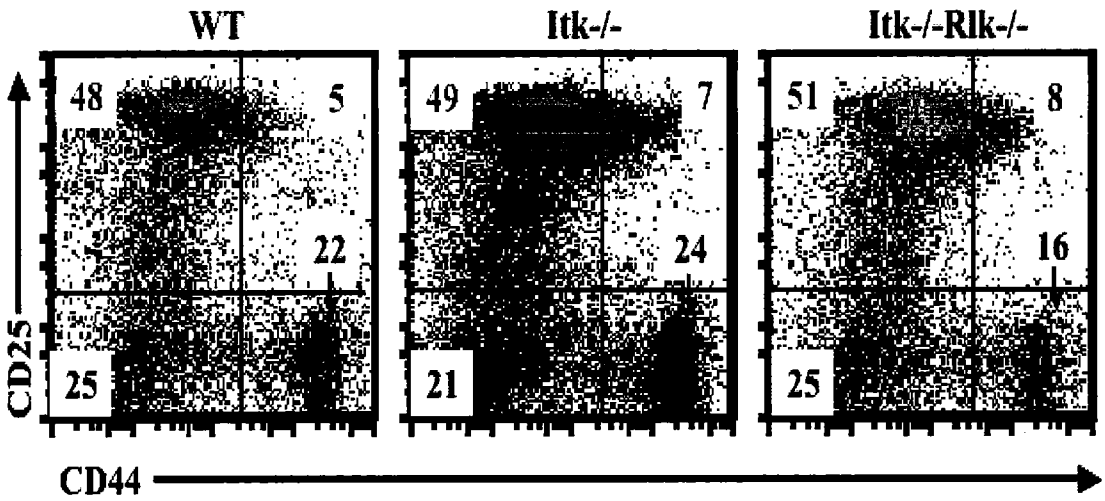
Based on CD4 vs. CD8 profiles of wild-type, $itk^{-/-}$, $itk^{-/-}rlk^{-/-}$ mice thymocyte development appears to be relatively normal, with the exception of an altered CD4:CD8 ratio, which will be discussed in future chapters (69, 74). However, in an analysis done by Schaeffer et al of the absolute numbers of each thymic subset revealed that there is a small, but seemingly significant reduction in total DP cells. Compared to wild-type mice, Itk-deficient mice have 65-70% Itk/Rlk-doubly deficient mice have 75-80% the normal numbers of DP thymocytes (69). In spite of this decrease, there does not seem to be any difference in DN cell numbers. With the exception of the small reduction in DP thymocyte numbers, there was nothing to indicate any defect in the DN to DP transition. However, in light of the predicted role of Tec family members in pre-TCR signaling, based on their role in positive selection [(69), discussed in Chapter 3] and in mature T cell activation (54, 66, 67, 103), we decided to begin by looking more closely at the individual DN subsets based on CD44 and CD25 expression, using the method described in Figure 2.2.

Initial inspection of the DN subsets in $itk^{-/-}$ and $itk^{-/-}rlk^{-/-}$ mice is in agreement with the general CD4/CD8 profiles, as the distribution of DN1-DN4 subsets is similar to that seen in wild-type C57BL/6 mice (Figure 2.3). We had first examined Itk-deficient mice and thought that one possibility for the apparent disposability of Itk in pre- TCR signaling may be due to compensation by other Tec kinase members. However, surprisingly,

subsequent analysis of *itk*$^{-/-}$*rlk*$^{-/-}$ mice revealed that the DN to DP transition was also unaffected in the absence of both Tec kinases.

Figure 2.3 DN thymocyte subsets in single wild-type, *itk*$^{-/-}$, and *itk*$^{-/-}$*rlk*$^{-/-}$ BM chimeras

Staining of DN thymocytes using the method described in Figure 2.2 is shown. The lower-right quadrant contains DN1 cells, the upper-right quadrant contains DN2 cells, the upper-left quadrant contains DN3 cells, and the lower-left quadrant contains DN4 cells. These plots are representative of 3 experiments. There is a lot of variation in the exact numbers in each quadrant, so the plot that seems to represent the best average of each genotype is depicted.


WT
Itk-/-
Itk-/-Rlk-/-
48
5
22
25
49
7
24
21
51
8
16
25
CD25
CD44

Expression of Itk, Rlk, and Tec in adult DN subsets

Due to the fact that we did not observe any impairment in the DN to DP transition in Itk-deficient or Itk/Rlk-doubly-deficient mice we were interested in determining if these kinases, as well as Tec, were expressed throughout T cell development and their relative levels. As mentioned, early studies performed by Northern blot analysis demonstrated that both Itk (40) and Rlk (101) mRNA can be detected by day 14 of fetal development, a time at which the thymus is comprised solely of DN1 and DN2 precursor cells. Therefore, we thought it was likely that these two kinases would be expressed throughout T cell development, but the expression in specific T cell subsets had not yet been done in adult mice.

Using real-time PCR analysis, we first demonstrated that all three Tec family members are present in the DN, DP, and both SP subsets (Figure 2.4A). Itk and Rlk expression is almost equivalent, although Itk expression may be slightly higher. In contrast, Tec mRNA levels are considerably lower, at least 40-fold less than Itk and Rlk levels. The levels of Tec kinases seem to be equivalent in DP and SP subsets, and slightly lower in the DN subset. However, because of the way the cells were stained and sorted the DN cells are not solely comprised of T lineage cells. Therefore, the non-T lineage cells may contribute to the levels of β-actin, thus the levels of the Tec kinases may appear to be artificially lower than they actually are.

Next, we decided to sort the DN cells into individual subsets. In order to be able to get adequate cell numbers to isolate RNA following sorting, we needed to pool many thymi and deplete the DP and SP cells with complement fixation of cells expressing CD4

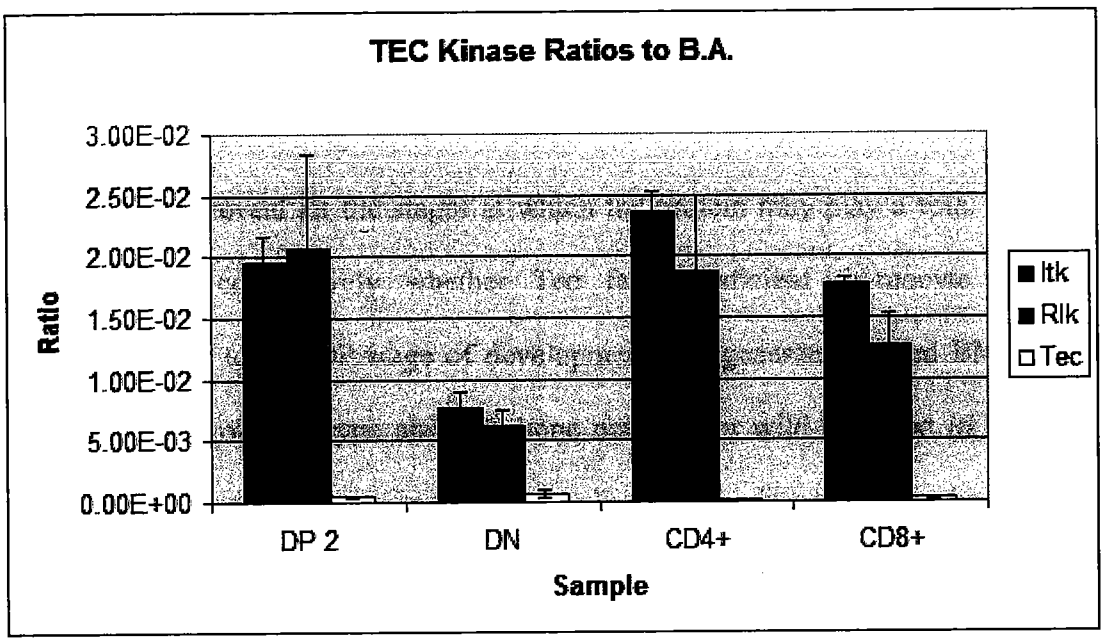
and CD8. Once the $CD4^+$ and $CD8^+$ positive cells were eliminated, the cells were stained as in Figure 2.2 and sorted into the four DN subsets. The purity of each subset was at least 92%, but most approached 98 – 99% The results of the real-time PCR analysis show that all three Tec family members are expressed in each DN subset and, as predicted, their ratios to β-actin increased with the elimination on non-T lineage cells, although to different degrees (Figure 2.4B). The ratio of Itk to β-actin, on average, increased almost 5-fold, whereas the ratios of Rlk and Tec increased 2- to 2.5-fold. The measurement of mRNA levels in the more purified DN subsets indicate that Itk is the most highly expressed, the expression of Rlk is now clearly less than Itk, and the expression of Tec is significantly lower than both Itk and Rlk. However, even though Tec has the lowest expression, its ratio with Itk and Rlk is higher than in the more mature T cell subsets shown in Figure 2.4A. Since real-time PCR measures the steady-state levels of mRNA and not the levels of protein activity in a specific subset, it might not make sense to compare the mRNA levels of one gene to another. However, these results clearly indicate that Itk, Rlk, and Tec transcripts are expressed in all thymic subsets and therefore, all three of these kinases may be playing a role in all stages of T cell development.

Figure 2.4 Expression of Tec family members in thymocyte subsets

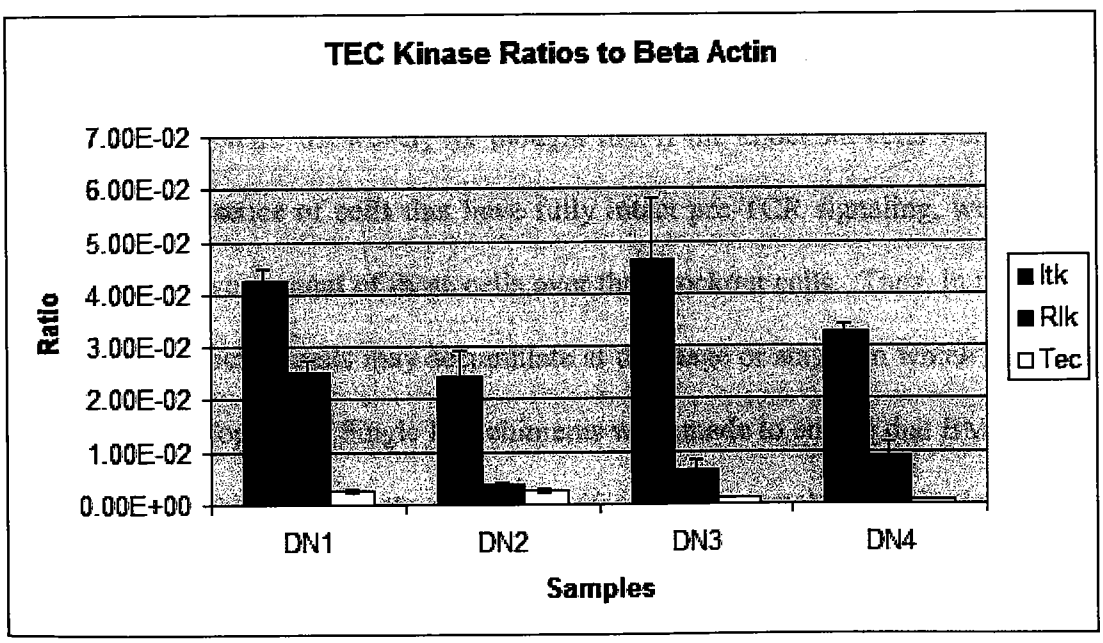
(A) DN, DP, and SP subsets by sorting wild-type thymocytes based on expression of CD4 and CD8. Levels of mRNA of Tec family members were determined by real-time PCR as described above.

(B) A pooled cell suspension from the thymi of 25 wild-type mice was enriched for DN thymocytes by complement depletion of $CD4^+$ and $CD8^+$ cells, which eliminates all DP and SP thymocytes. Cells were then stained with the protocol described in Figure 2.2 and sorted based on CD25 and CD44 expression of the allophycocyanin negative cells, into the DN1, DN2, DN3 and DN4 subsets. The levels of mRNA of Itk, Rlk and Tec in each subset was determined by isolating RNA from each subset, conversion into cDNA, and real-time PCR for Itk, Rlk, and Tec. To control for variation in the efficiency of mRNA isolation, levels of each kinase were normalized by calculating the ratio of each Tec kinase to β-actin in each sample.

A.


TEC Kinase Ratios to B.A.
3.00E-02
2.50E-02
2.00E-02
1.50E-02
1.00E-02
5.00E-03
0.00E+00
Ratio
DP 2
DN
CD4+
CD8+
Sample
Itk
Rlk
Tec

B.


TEC Kinase Ratios to Beta Actin
7.00E-02
6.00E-02
5.00E-02
4.00E-02
3.00E-02
2.00E-02
1.00E-02
0.00E+00
Ratio
DN1
DN2
DN3
DN4
Samples
Itk
Rlk
Tec

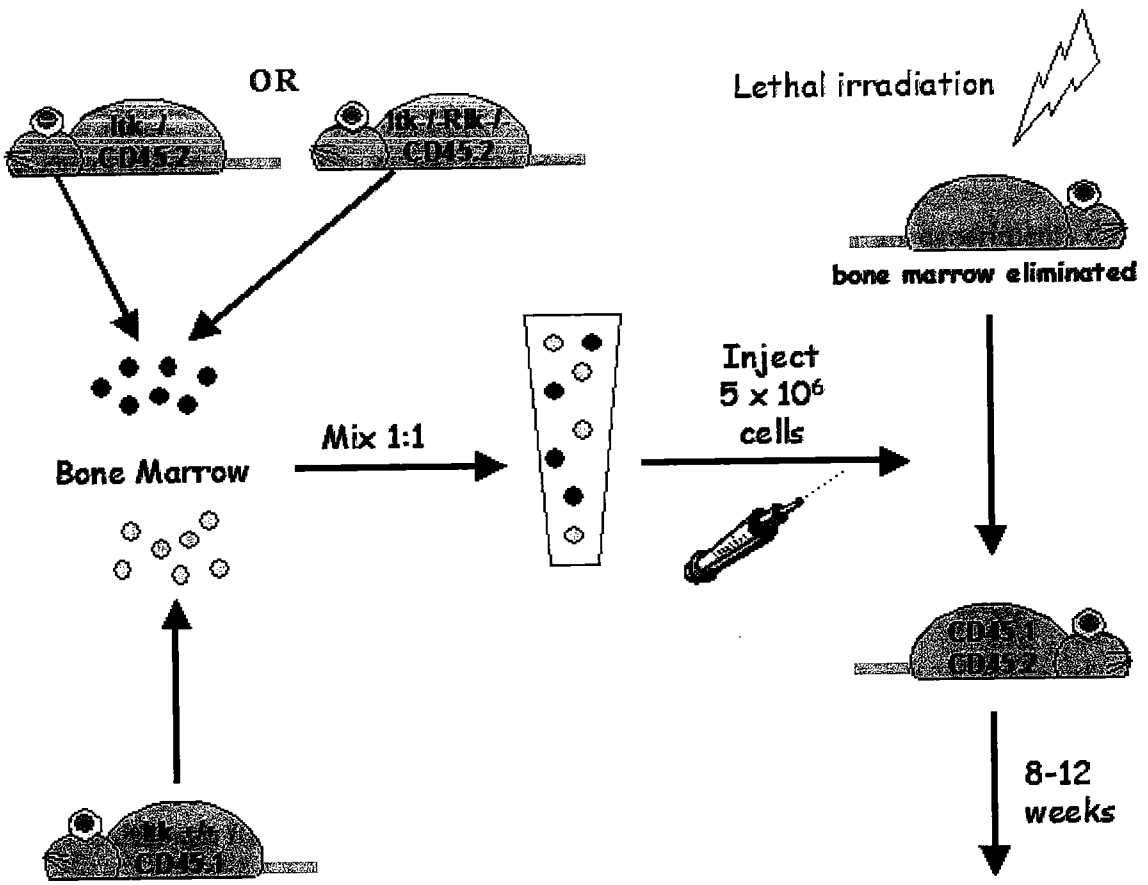
Using mixed bone marrow chimeras to determine if competition with wild-type cells reveals a defect in the DN to DP transition

Although phenotypic analysis of thymic subsets in knockout animals is useful as an initial tool for examining the role of certain proteins in lymphocyte development, it may often fail to reveal all the stages at which the protein may play a role. In order to determine more conclusively whether Tec family-deficient thymocyte precursors progress normally to the DP stage of development, we generated mixed BM chimeras with a 50:50 mix of wild-type and *itk*$^{-/-}$ bone marrow, or wild-type and *itk*$^{-/-}$*rlk*$^{-/-}$ bone marrow. The experimental design is shown in Figure 2.5. In addition to the mixed BM chimeras, we also made BM chimeras with unmixed BM of each genotype (wild-type, *itk*$^{-/-}$, and *itk*$^{-/-}$*rlk*$^{-/-}$). We reasoned that since TCR, and most likely pre-TCR signaling, was merely reduced in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ thymocytes that a block may not be evident by mere steady-state analysis of the thymic subsets, even if the transition from the DN to DP stage was less efficient. However, we thought that if the knockout cells were forced to develop in the presence of cells that have fully intact pre-TCR signaling, we might see the preferential development of these cells over the knockout cells. Thus, in this system, the Tec family deficient cells may accumulate at the stage or stages in which they play a role in T cell development. Single BM chimeras were made to ensure that BM from each genotype was capable of complete and normal (for that genotype) reconstitution.

Figure 2.5 Design of mixed bone marrow chimera experiment

Bone marrow was isolated from wild-type (B6) CD45.1, *itk*$^{-/-}$ (B10) CD45.2, *itk*$^{-/-}$*rlk*$^{-/-}$ (B6) CD45.2 mice. For mixed chimeras, wild-type CD45.1 BM was mixed 1:1 with *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ CD45.2 bone marrow. CD45.1 (B6) host mice were lethally irradiated (1000 rads) and 1 x 10^6 of the mixed BM cells were i.v. injected. Mice were analyzed as early as 4 weeks and as late as 12 weeks, but the majority were analyzed at 8-10 weeks. DN analysis was done using the previously described protocol, except in the last step CD45.1 or CD45.2 was used to distinguish between wt and ko/dko cells. Cells were also stained with Abs for CD45.1/CD45.2, CD4, CD8, and TCR. Additional analyses looking at proliferation with BrdU, and the up-regulation of CD5 at the DN4 stage, which will be described later.

Mixed Bone Marrow Chimeras



Analysis of DN subsets in mixed bone marrow chimeras

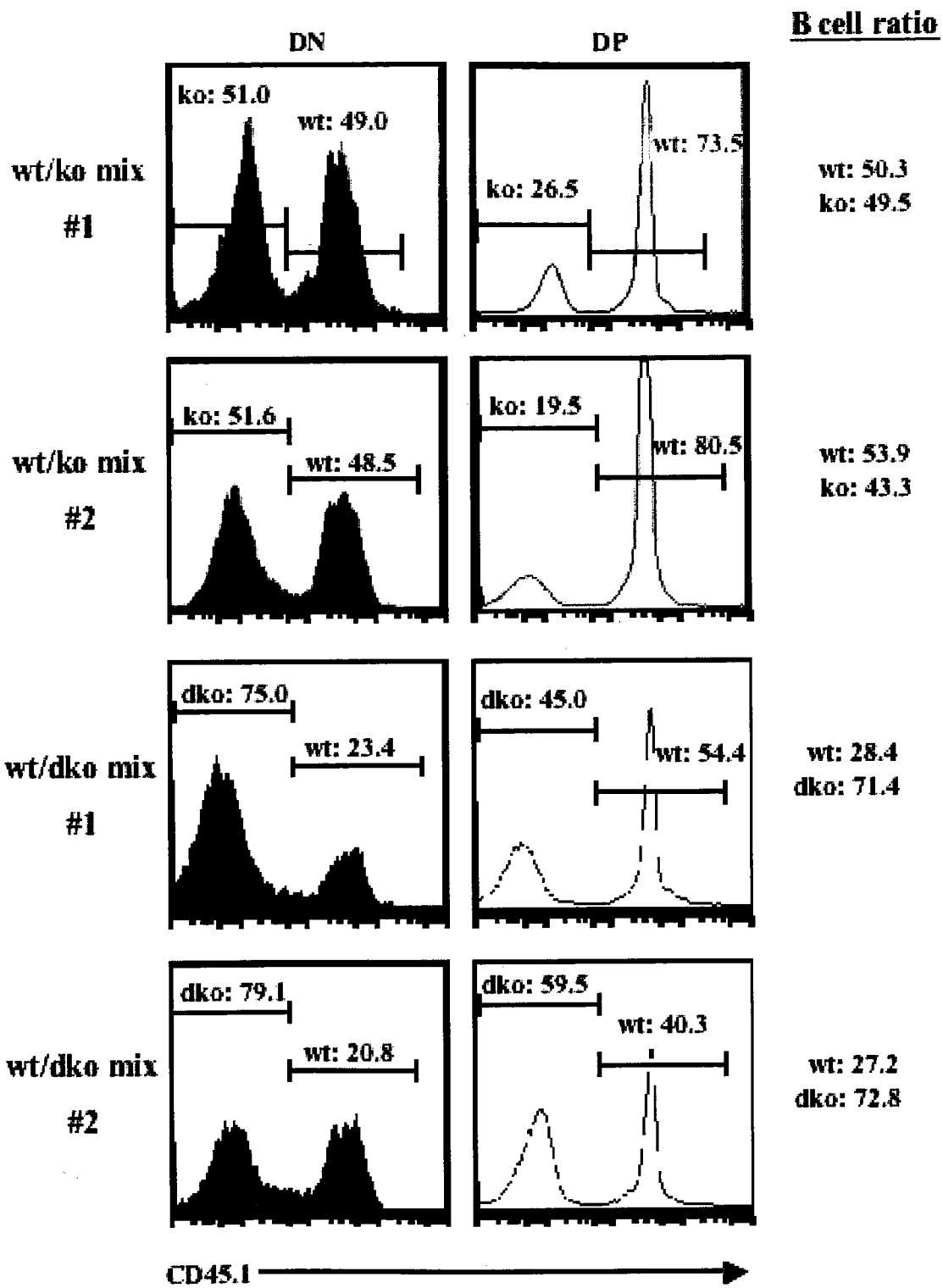
Initially, it appeared that the BM from Itk-deficient mice was partially blocked at the DN3 to DN4 transition in the presence of wild-type bone marrow. However, subsequent experiments did not repeat this observation with any certainty and the CD25 vs. CD44 profiles had a fair degree of variation among experiments and individual mice [total # of wild-type/*Itk*$^{-/-}$ (wt/ko) mixed BM chimeras = 21]. In all experiments done with wild-type and *itk*$^{-/-}$*rlk*$^{-/-}$ (wt/dko) BM chimeras (total # = 17), we never observed any blocks in T cell development in the DN to DP transition (data not shown). In addition, in the analysis of the single BM chimeras, the distribution of the DN subsets was very similar to the distribution seen in the mixed BM chimeras for a given BM genotype (data not shown). Thus, the competition between *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ BM with wild-type BM did not result in any blocks in development during the DN stages in the absence of Tec family kinases.

Although *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ thymocytes did not appear to be blocked at the DN3 to DN4 transition, it still appeared that the development of *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ DP thymocytes was less efficient in mixed bone marrow chimeras. As shown in Figure 2.6, we found that while the representation of *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ thymocytes compared to wild-type thymocytes in the DN compartment was comparable to the representation of each genotype in B cells, which should not be affected by the absence of Itk or Rlk, Tec family-deficient thymocytes were consistently underrepresented in the DP compartment compared to wild-type cells (Figure 4.6). In the wt/dko mixed bone marrow chimeras, the contribution of the *itk*$^{-/-}$*rlk*$^{-/-}$ cells to both the B and T subsets was significantly higher

than the contribution of the wild-type cells. This was true of all chimeras made over two experiments. We believe this is most likely due to errors in counting and thus, the BM mix was disproportionate, with more of the BM higher proportion of the cells being of *itk*$^{-/-}$*rlk*$^{-/-}$ origin.

Figure 2.6 Contribution of wild-type and Tec-family deficient thymocytes in mixed bone marrow chimeras to the DP subset

The contribution of $CD45.1^+$ (wild-type) and $CD45.1^-$ (ko or dko) cells to either the DN (gated on $CD4^-$ $CD8^-$) or the DP (gated on $CD4^+$ $CD8^+$) subset is depicted in the histograms. To the right of the histograms is the ratio of ko/dko to wild-type cells within the B cell compartment, which should represent the percentage of the progenitors present in the mice that was used to reconstitute the lethally irradiated mice. Two mice of each BM mix are shown. In all the BM chimeras made, the percentage of *itk*$^{-/-}$ (21 mice in three experiments) or *itk*$^{-/-}$*rlk*$^{-/-}$ (17 mice in two experiments) DP thymocytes was always significantly lower than their percentage of DN thymocytes or splenic B cells, although the amplitude of the decrease varied among experiments. Two representative mice of each BM mixture from the experiment with intermediate results are depicted.


B cell ratio
DN
DP
wt/ko mix
#1
ko: 51.0
wt: 49.0
wt: 73.5
ko: 26.5
wt: 50.3
ko: 49.5
wt/ko mix
#2
ko: 51.6
wt: 48.5
ko: 19.5
wt: 80.5
wt: 53.9
ko: 43.3
wt/dko mix
#1
dko: 75.0
wt: 23.4
dko: 45.0
wt: 54.4
wt: 28.4
dko: 71.4
wt/dko mix
#2
dko: 79.1
wt: 20.8
dko: 59.5
wt: 40.3
wt: 27.2
dko: 72.8
CD45.1

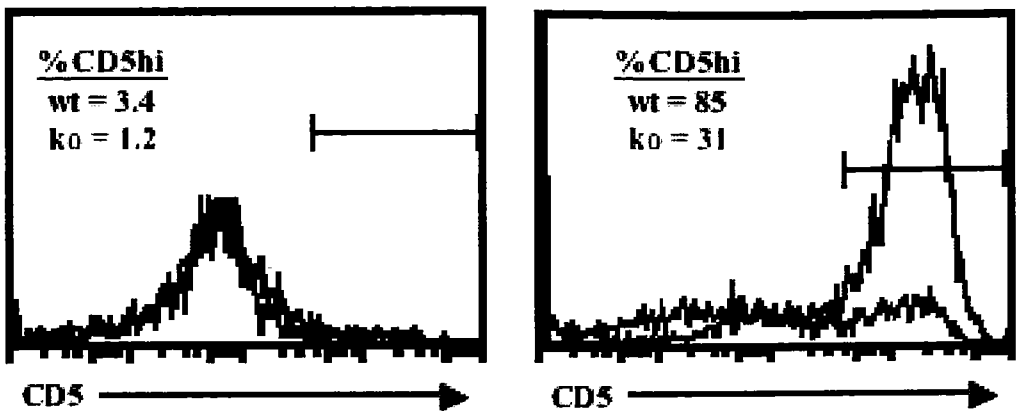
The strength of the pre-TCR signal in the absence of Tec kinases is reduced

The results obtained in the previous section were the first to indicate that the DN to DP transition is affected by the absence of Tec family kinases. As a first attempt to determine if this was due to altered pre-TCR signaling, as we presumed, we examined the up-regulation of CD5 as cells transition from DN3 to DP. CD5 is a cell-surface transmembrane protein that negatively regulates TCR signaling. It is expressed on all T cells and its expression levels are tightly regulated during T cell development. The first stage of up-regulation follows signaling through the pre-TCR. Levels are further up-regulated following selection, and the final level of CD5 has been shown to be proportionate to the avidity of the TCR expressed on the developing DP thymocyte for its selecting ligand(s) in the thymus (104). Work that was done prior to the examination of the DN subsets revealed an overall reduction of CD5 on DP thymocytes (see Chapter 3) (69, 74). Thus, we decided to examine the levels of CD5 on the DN3 and DN4 subsets in the mixed BM chimeras. In both the DN3 and DN4 subsets, the percentage of *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ cells that have up-regulated CD5 is considerably lower than the percentage of wild-type cells that have up-regulated CD5 (Figure 2.7) indicating that at least some part of the pre-TCR signal is reduced in the absence of Tec kinases.

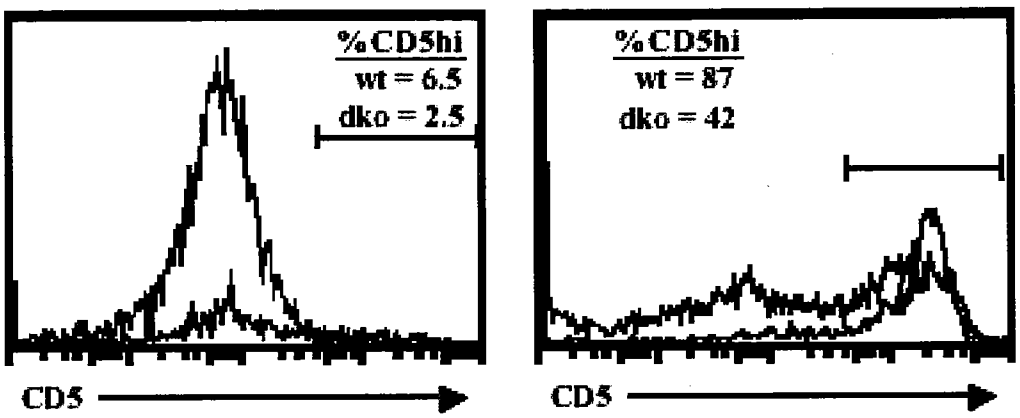
Figure 2.7 The strength of the pre-TCR signal is decreased in the absence of Tec family kinases.

In order to look at CD5 levels on DN3 and DN4 subsets, we stained the thymocytes from the mixed BM chimeras as described in Figure 2.2 except that anti-CD44-bio was added to the mixture of biotin-Abs. Thus, in addition to lineage specific cells, the DN1 and DN2 subsets were also excluded by gating out the APC^+ population. The cells were then stained with anti-CD45.1-FITC, anti-CD25-PE, and anti-CD5-Cy. The wt and ko/dko subsets were gated on CD45.1 expression and further subdivided into DN3 and DN4 subsets based on CD25 expression. Histograms of CD5 expression on DN3 and DN4 cells of wt (blue line) or ko/dko (pink line) origin from mixed bone marrow chimeras are depicted. The percentages of cells that have up-regulated CD5 are also shown.

A.


%CD5hi
wt = 3.4
ko = 1.2
CD5
%CD5hi
wt = 85
ko = 31
CD5

B.


%CD5hi
wt = 6.5
dko = 2.5
CD5
%CD5hi
wt = 87
dko = 42
CD5
wt (CD45.1)
ko/dko (CD45.2)

β-selection events are impaired following TCR signaling in the absence of Tec family kinases

We next wanted to determine which aspects of the β selection events are affected. As described in the introduction, one of the consequences of pre-TCR signaling is proliferation. This proliferation begins at the DN3/DN4 transition and continues until the cells become DP, at which point the cells become quiescent. Tec kinases could potentially affect this process since all the signals downstream of PLC-γ are believed to affect proliferation. Activation of PKC, Ca^{2+} mobilization, and the Ras-MAPK pathway all contribute to proliferation, and in the absence of Tec family kinases in T cells both Ca^{2+} mobilization (66, 67, 69, 103) and the activation Ras-MAPK pathway (measured by phospho-Erk levels) (67, 69, 103, 105) have been shown to be affected. Since PKC requires the production of DAG, one of the products of PLC-γ activation, it is presumed that this pathway should be affected as well (Figure 2.1).

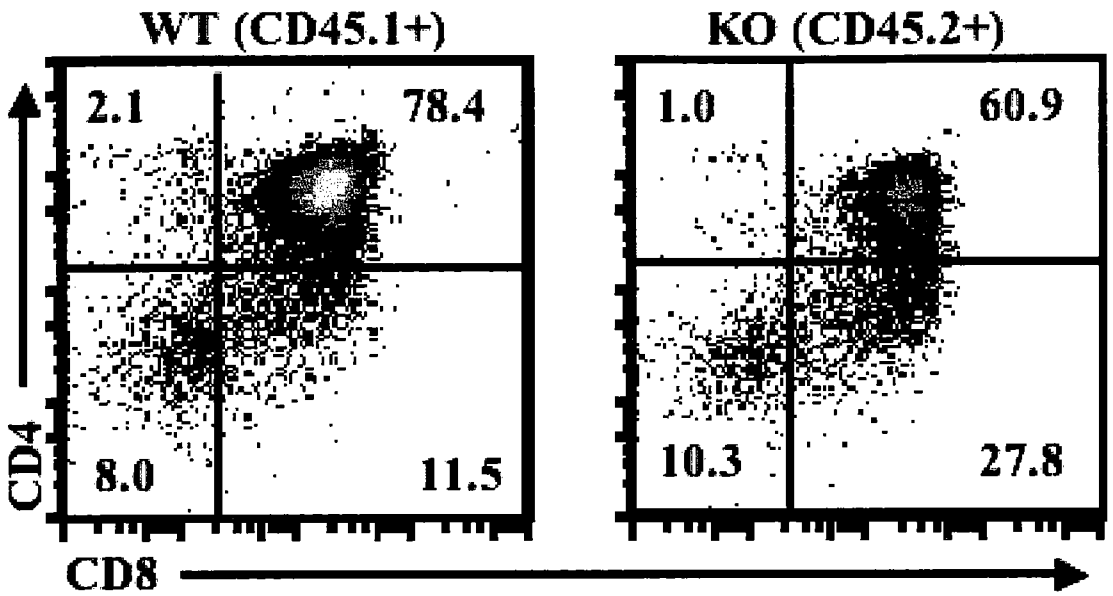
In order to measure proliferation, we injected the BM chimeras with BrdU, a nucleotide analog that can be used by cells in the place of thymidine when synthesizing DNA. BrdU can be detected by inter-nuclear FACS staining with an anti-BrdU antibody, and since only cells that are in S-phase of the cell cycle will be synthesizing DNA, this method detects any cells that are undergoing proliferation. We injected mice 1 – 2 hours before harvest. During this short pulse, the $BrdU^+$ cells represent the steady-state level of cell proliferation. As mentioned, proliferation begins at the pre-TCR stage and ends once the cells become DP. Thus, the DN4, ISP, and very earliest DP cells will be the ones proliferating and incorporating BrdU. Besides staining with anti-BrdU, the cells were

also stained with anti-CD45.1, anti-CD4, and anti-CD8. Proliferating cells were identified and gated as $BrdU^+$ cells and these cells were further sub-divided into wild-type and knockout origin based on CD45.1 expression. CD4 vs. CD8 plots of these gated sub-populations are depicted. Thus, these plots show the distribution of proliferating cells of each genotype into the following thymic subsets: DN3/DN4 ($CD4^-CD8^-$), ISP ($CD8^+$), and DP cells (Figure 2.8).

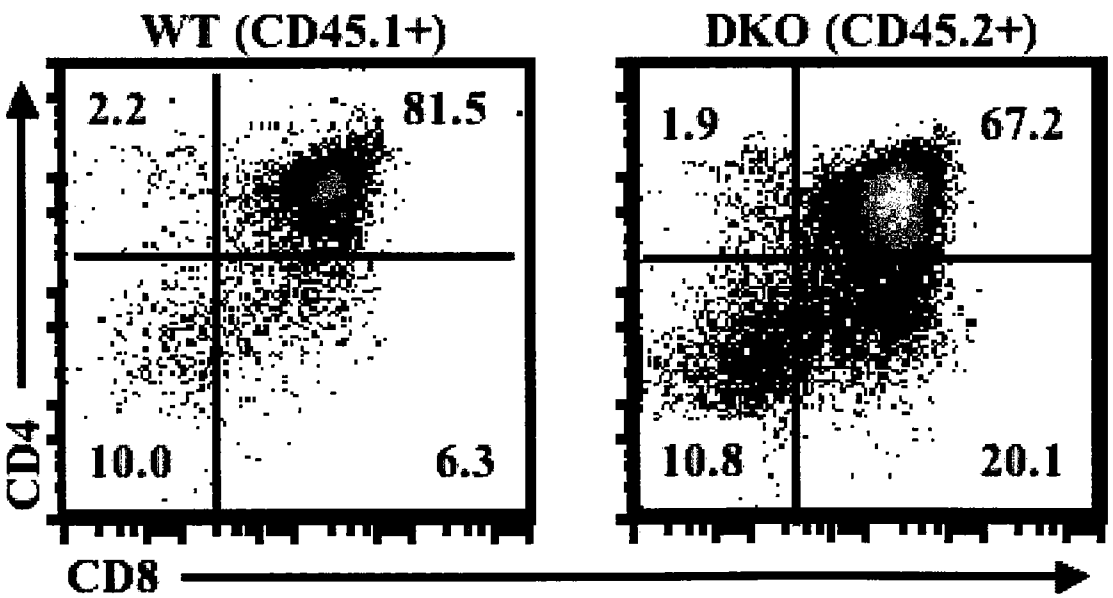
Figure 2.8 Proliferation of thymocytes in mixed bone marrow chimeras

(A) Wt/ko mixed BM chimeras and (B) wt/dko mixed BM chimeras were injected with 2 mg of BrdU. After harvest of thymocytes, cells were stained extracellularly with anti-CD45.1-PE, anti-CD4-Cy, and anti-CD8-APC. Cells were fixed/permeabilized and treated with DNase to fragment the DNA and expose the BrdU to Ab binding. Cells were then stained with anti-BrdU and analyzed immediately. Cells from each BM chimera are gated on $BrdU^+$ cells and then divided into wt ($CD45.1^+$) and ko/dko ($CD45.1^-$). CD4 vs. CD8 profiles are then shown for the $BrdU^+$ wt and $BrdU^+$ ko/dko thymocytes.

A.


Gated on BrdU+
WT (CD45.1+)
KO (CD45.2+)
2.1
78.4
8.0
11.5
1.0
60.9
10.3
27.8
CD4
CD8

B.


Gated on BrdU+
WT (CD45.1+)
DKO (CD45.2+)
2.2
81.5
10.0
6.3
1.9
67.2
10.8
20.1
CD4
CD8

In the mixed chimeras, the percentages of $BrdU^+$ cells of the wild-type and *itk*$^{-/-}$ origin were equivalent; however, when the CD4/CD8 distribution of the $BrdU^+$ $CD45.1^+$ (wild-type) amd $CD45.1^-$ (*itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$) cells were compared, the wild-type cells had a higher proportion of $BrdU^+$/proliferating DP cells and fewer immature (DN + ISP) cells proliferating than the Tec family-deficient counterparts. Averaging the percentages of DN, ISP, and DP thymocytes from 12 wt/ko and 12 wt/dko BM chimeras yielded very comparable results. The ratio of immature (DN + ISP) to mature (DP) cells of the proliferating population for each BM genotype are also remarkably comparable (see below).

$BrdU^+$ thymocytes in wt/ko mixed BM chimeras						**$BrdU^+$ thymocytes in wt/dko mixed BM chimeras**				
	DN	**ISP**	**DP**	**(DN + ISP)/DP**			**DN**	**ISP**	**DP**	**(DN + ISP)/DP**
WT (CD45.1+)	**13.2**	**17.7**	**65.9**	**0.49**		**WT**	**13.9**	**17.8**	**64.6**	**0.52**
Itk$^{-/-}$ (CD45.2)	**18.0**	**29.6**	**50.6**	**1.04**		**Itk$^{-/-}$Rlk$^{-/-}$**	**22.5**	**23.4**	**50.2**	**1.06**

The wild-type distribution and immature:mature ratio, is virtually identical for both the wt/ko and wt/dko chimeras. The same is true when comparing the distribution and ratio from ko and dko BM; although there may be a slight difference in the DN and ISP distribution. Conversion of these proportions into absolute numbers reveals that there are comparable numbers of cells of the wild-typ and Tec family-deficient origin in the immature (DN/ISP) subsets, but there are fewer $BrdU^+$ DP cells of the Tec family-deficient origin suggesting that fewer DP cells are proliferating at any given time in the absence of Itk and/or Rlk.

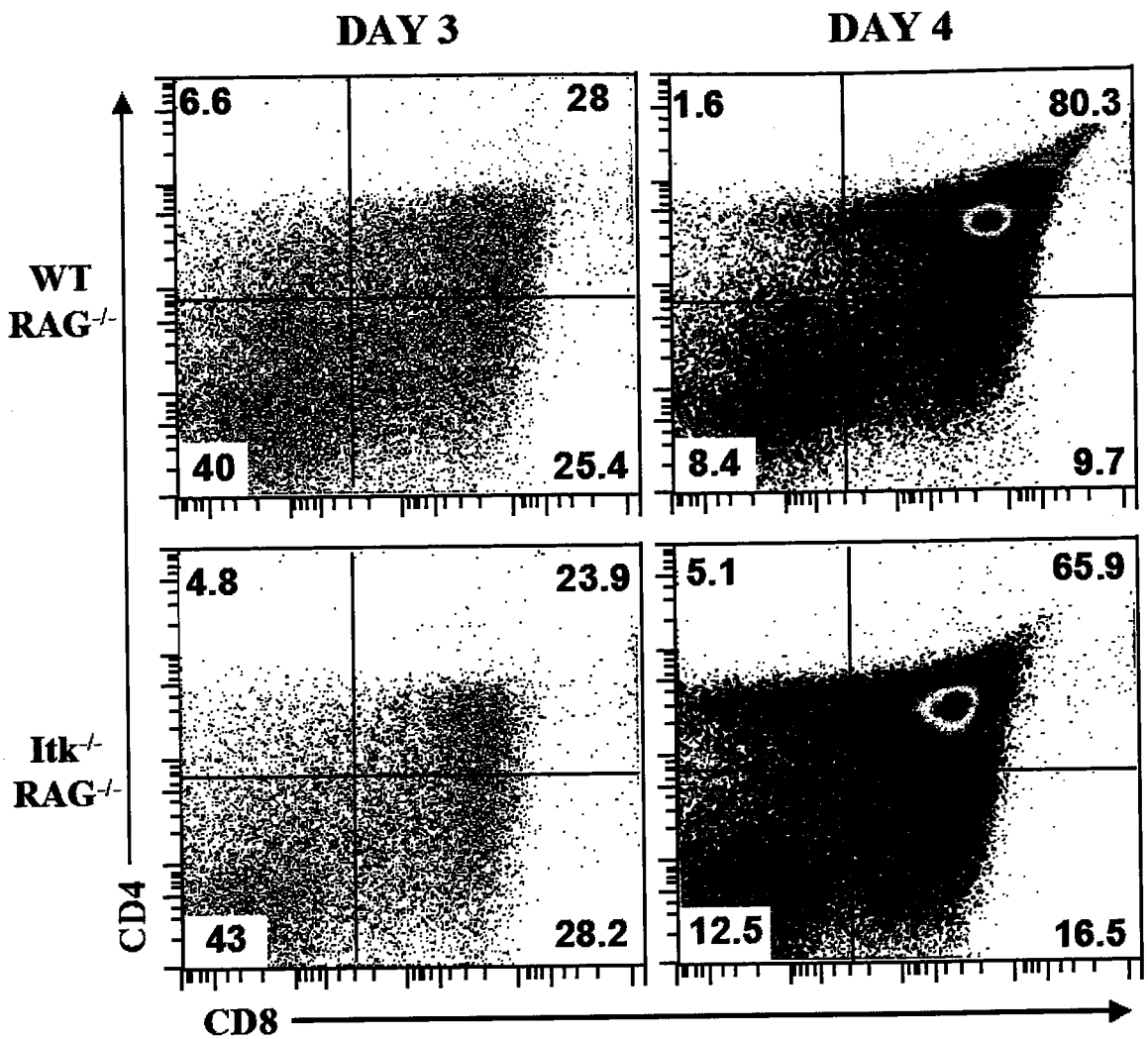
The DN to DP transition in the absence of Itk is impaired following pre-TCR stimulation

Although, analysis of the thymocyte subsets, proliferation, and up-regulation of CD5 suggested that pre-TCR signaling was reduced in Itk-deficient and Itk/Rlk-double deficient mice, all these experiments are based on the interpretation of steady-state phenotypes. Thus, we felt that these were passive ways of looking at pre-TCR signaling and it was still possible that the defect was due to something besides pre-TCR signaling, such as altered cytokine signaling. To look at pre-TCR signaling actively, we made use of a well-established observation that Rag-deficient DN thymocytes can be induced to become DP cells by CD3 stimulation. It was demonstrated that even in the absence of a rearranged β chain, low levels of CD3 components are found on the surface of *rag*$^{-/-}$ DN thymocytes and following injection with anti-CD3, DP thymocytes accumulated and the total thymic cellularity increased 100-fold (106).

Itk$^{-/-}$*rag*$^{-/-}$ were available from crosses of *itk*$^{-/-}$ mice to TCR transgenic (5C.C7) *rag*$^{-/-}$ mice and wild-type *rag*$^{-/-}$ mice were purchased from Jackson Laboratories. Four of five mice of each genotype were injected with anti-CD3-ε and two of each were analyzed at both 3 and 4 days following injection (Figure 2.9). While this is a preliminary experiment, the pattern of these results closely mimics the proliferation data. The Itk-deficient mice, at each time-point have fewer cells at the DP stage and more cells at the DN and ISP stage of development. I plan to repeat this experiment in the near future and will also cross the *rag*$^{-/-}$ mice to *itk*$^{-/-}$*rlk*$^{-/-}$ mice so that we can more directly determine whether pre-TCR signaling is more impaired in doubly deficient mice or if the phenotype is the same as Itk-deficient mice.

Figure 2.9 Fewer *itk*$^{-/-}$*rag*$^{-/-}$ DN thymocytes transition to the DP stage compared to *itk*$^{+/+}$*rag*$^{-/-}$ DN thymocytes following injection with anti-CD3

The CD4 vs. CD8 profiles of wild-type- and Itk-deficient-*rag*$^{-/-}$ mice injected with 50 μg anti-CD3 (2C11) at 3 and 4 days post-injection are shown. $CD4^-$ $CD8^-$ DN thymocytes are primarily at the DN3 and DN4 stage of development (data not shown). $CD8^+$ cells are cells at the ISP stage of development since SP cells do not develop in this system.


DAY 3
DAY 4
WT
RAG-/-
Itk-/-
RAG-/-
CD4
CD8
6.6
28
40
25.4
1.6
80.3
8.4
9.7
4.8
23.9
43
28.2
5.1
65.9
12.5
16.5

DISCUSSION

In light of the clear importance of PLC-γ in pre-TCR signaling and its apparent role in all four β selection events: survival, proliferation, differentiation, and allelic exclusion (Figure 2.1), we were interested in determining if the Tec family kinases, Itk and Rlk, whose only known substrate in T cells is PLC-γ, were also important at this stage of development. Initial characterization of the DN, DP, and SP subsets of both *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice did not suggest that there was any defect in T cell development prior to selection or lineage commitment (39, 66, 67, 69). Besides the fact that Tec kinases activate PLC-γ, Lck, LAT, and SLP-76 are all upstream of Tec kinase activation and mice deficient in these proteins have a block at the DN3 to DN4 transition. Since Tec family deficient mice have significant numbers of DP thymocytes, we knew that there was not a absolute block at the stage. However, other TCR signaling proteins, like Vav, have been shown to be important during the DN to DP transition even though *vav*$^{-/-}$ mice do not have a complete block at the DN3 stage. Instead, a 2-fold decrease of the DP subset was observed, which resulted in an 2-fold reduction in thymic cellularity when compared to wild-type mice (107, 108). There was no increase in the absolute number of DN cells, but upon analysis of the DN subsets it was revealed that the Vav-deficient mice had a higher DN3:DN4 ratio (107). As mentioned ealier, data from Schaeffer et al demonstrated that Itk-deficient mice also had a reduction in the total number of DP thymocytes, although it was much smaller than that seen in *vav*$^{-/-}$ mice (69). Thus, we decided to look more closely at this stage of development in Itk-deficient mice by

analyzing the DN subsets. However, unlike *vav*$^{-/-}$ mice, *itk*$^{-/-}$ mice had a DN distribution that was indistinguishable from that seen in wild-type mice (Figure 2.3).

We were surprised based on the role of Tec kinases in TCR signaling that we did not observe any change in the DN subsets in the absence of Itk. One possible explanation was that the other two Tec kinases, Rlk and Tec, that are expressed in T cells may be compensating for Itk. Before pursuing this further, we wanted to determine if all three Tec kinases were expressed in DN thymocytes. Using real-time PCR, we determined that indeed, Itk, Rlk and Tec, were all expressed in DN thymocytes, and also showed that they were expressed throughout development beginning at the DN1 stage (Figure 2.4). Since real-time PCR is quantitative, we were able to determine the hierarchy of mRNA expression, which is Itk > Rlk > Tec, which is identical to the hierarchy observed in peripheral $CD4^+$ T cells (109).

Having demonstrated that all the Tec kinases were present, we decided to look at the DN subsets in Itk/Rlk-doubly-deficient mice, which had been shown previously to have greater defects in later stages of thymocyte differentiation than Itk-singly-deficient mice. Once again, we did not observe any alterations in the distribution of the DN subsets (Figure 2.3). At this point, it did not appear that pre-TCR signaling was defective in the absence of Tec kinases based on knockout phenotypes. We then decided to try another approach by using mixed bone marrow chimeras. Lethally irradiated mice were reconstituted with a 1:1 mixture of wild-type and *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ BM cells. In this approach, it was possible that a minor defect in pre-TCR signaling in the absence of Tec family kinases would be revealed when the cells were in competition with wild-type

cells. Preliminary analysis of the contribution of each BM type to the DN and DP subsets revealed that significantly fewer cells of the Tec kinase deficient origin contributed to the DP compartment suggesting that the DN to DP transition was less efficient in the Tec kinase deficient progenitors compared to wild-type progenitors (Figure 2.6); however, we still did not observe any dissimilarity in the DN subsets when comparing the two types of progenitors present in each BM mix. Thus, the defect seemed to take effect after the DN4 stage of development.

Yet, analysis of CD5 up-regulation at the DN3 and DN4 stages of development indicated that the *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ cells were receiving weaker pre-TCR signals at the earliest stages of β selection (Figure 2.7). Prior to analysis of the mixed BM chimeras, they were injected with BrdU to measure proliferation of cells during the DN to DP transition. The majority of proliferation in the thymus is initiated upon pre-TCR signaling and ends as the cells become DP. Thus, cells at the DN4, ISP, and early DP stage will incorporate BrdU in a short pulse (1 - 2 hrs). When comparing the distribution of proliferating thymocytes between the two BM types in each mixed chimera, we found that the distribution of proliferating wild-type thymocytes in the DN, ISP, and DP subsets was equivalent whether these cells were developing in the presence of *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ cells. Similarly, the distribution of *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ thymocytes was also comparable, with more of the proliferating cells being at an earlier stage of development when compared to control cells (Figure 2.8). Thus, it appears that the DN to DP transition of Itk-deficient thymocytes is not more affected by the additional absence of Rlk.

From these experiments, it appears that the initiation of proliferation is normal following pre-TCR signaling in the absence of Tec family kinases, but there are defects at later stages. In this type of analysis, it is impossible to determine if the alterations in the subsets represented by the $BrdU^+$ cells is due to altered proliferation, survival or differentiation. One possibility is that on average the Tec family deficient thymocytes undergo fewer rounds of division and thus, fewer DPs are proliferating. A second possibility is that the DP cells generated from BM lacking Itk do not survive as well as wild-type DP cells, and thus proliferation of this subset is underrepresented. A third possibility is that differentiation as measured by up-regulation of CD4 to the DP stage is less efficient in the absence of Itk and thus, a normal amount of proliferation is occurring, but the cells have not fully differentiated. There are other potential possibilities, perhaps a combination of those already mentioned, but it does appear that the defect is not revealed until the later in the DN to DP transition. It is possible that similar to $CD4^+$ positive selection and lineage commitment (see later chapters) the DN to DP transition requires sustained signaling in order to complete β selection. In fact, it has been shown that this process takes 24 hours and it is possible that a sustained signal is required throughout (110). Tec family deficient thymocytes have an inability to maintain a sustained Ca^{2+} flux (69), and it has been hypothesized that the sustained signaling required to complete differentiation does not occur in as many developing cells in the absence of Tec family kinases.

Finally, in order to measure the effects of Itk on pre-TCR signaling in a more direct manner, we injected control *rag*$^{-/-}$ and Itk-deficient *rag*$^{-/-}$ mice with anti-CD3,

which is known to overcome the block at the DN3 stage in *rag*$^{-/-}$ mice and allow differentiation to the DP stage. The results of this preliminary experiment, which suggest that a smaller proportion of cells are at the fully mature DP stage following injection with anti-CD3, are in complete agreement with the analysis of proliferating (BrdU^{+}) cells in the mixed bone marrow chimeras (Figure 2.9). As this assay is a more straightforward way of evaluating pre-TCR signaling it may be useful to generate *itk*$^{-/-}$*rlk*$^{-/-}$*rag*$^{-/-}$ mice to confirm whether or not this stage of development is independent of Rlk activity. In addition, none of the experiments done in this section looked at allelic exclusion, so it might be interesting to examine the Vβ usage in Itk- deficient mice in order to ascertain whether or not Tec kinases play a role in this important aspect of β selection. Ultimately, the generation of *itk*$^{-/-}$*rlk*$^{-/-}$*tec*$^{-/-}$ mice will aid in resolving whether or not the Tec family kinases are essential at this early stage of development or if they just aid in the efficiency of this process. At present, it seems more likely to be the latter.

CHAPTER III

THE ROLE OF ITK IN T REPERTOIRE SELECTION

INTRODUCTION

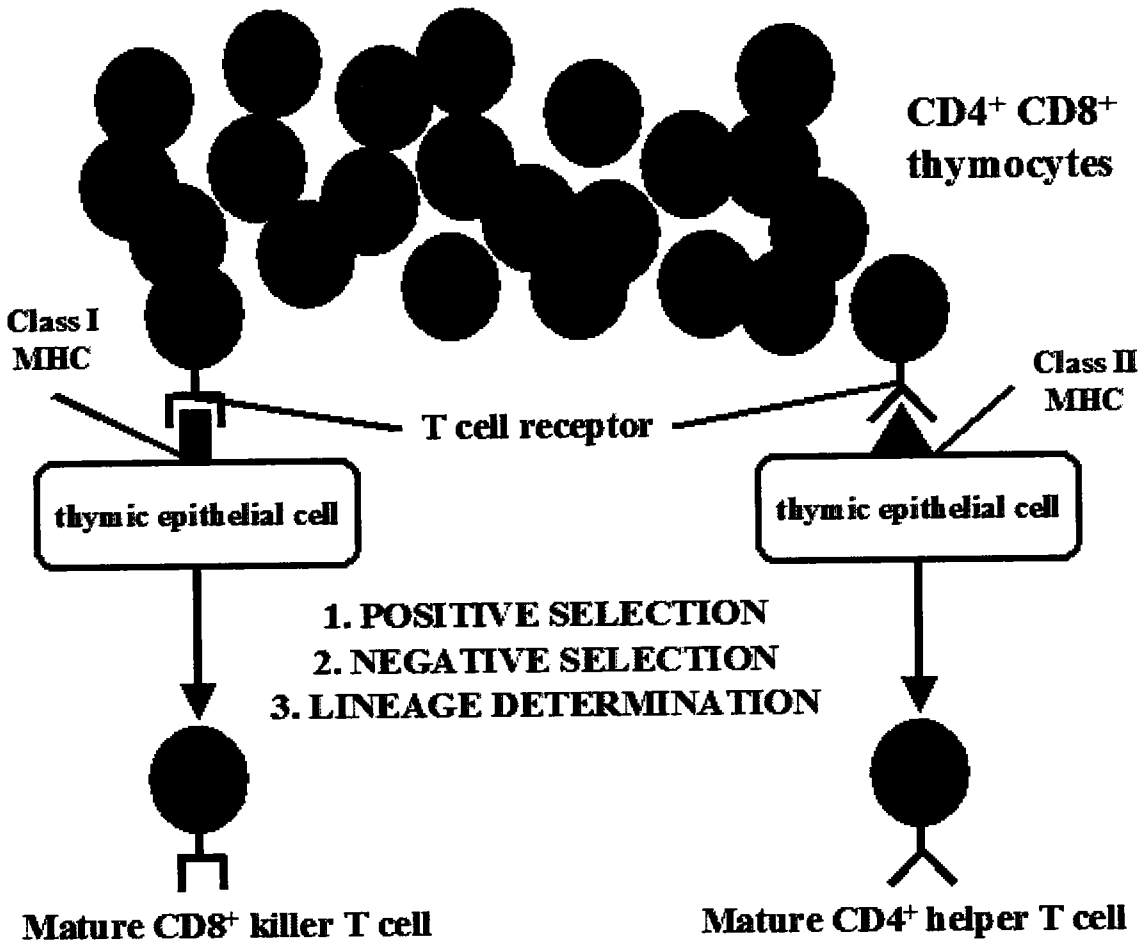
In the previous section, the process of β selection following pre-TCR signaling was discussed. The next developmental checkpoint that requires signaling from the TCR takes place at the DP stage following α chain gene rearrangement. Although some rearrangement of the alpha chain can be detected by the DN4 stage of development, the majority of rearrangement takes place once thymocytes become quiescent DP cells (111). The lifespan of an unselected DP thymocyte is approximately 3 – 4 days. During this time, α chain rearrangement continues until a functional αβ pair is formed and a TCR signal is generated. If no signal is received during this time, which is the outcome for the majority of DP thymocytes (34), the DP cell will die and this is known as "death by neglect."

At this stage in development, thymocytes must undergo two concurrent, but distinct, developmental processes to become functional and tolerant mature T cells. One of these processes is known as repertoire selection, which ensures that mature T cells can recognize foreign peptides presented by self-MHC molecules, but will not become activated when they encounter self-peptide/self-MHC complexes. The second process, lineage commitment, is the process by which thymocytes that recognize peptide in the context of MHC class I become $CD8^+$ cytotoxic T cell precursors, and thymocytes that recognize peptide in the context of MHC class II molecules become $CD4^+$ T helper cell precursors (Figure 3.1). Both of these processes are known to be dependent on TCR signaling; however, it has often been debated how the same initial signals can give rise to these different and opposing developmental outcomes. This chapter will focus on the

role of Itk in signaling during repertoire selection and its role in lineage commitment and differentiation will be discussed in Chapter 4.

Figure 3.1 Developmental processes that take place at the DP stage

DP thymocytes undergo three distinct processes. Cells that have weak or no binding avidity for self-peptide/self-MHC complexes on thymic epithelial cells do not undergo selection. However those that bind to self-peptide/self-MHC will undergo either positive or negative selection depending on the strength of that interaction (see B). Cells that undergo positive selection will also undergo lineage determination to become either a $CD8^+$ killer T cell or a $CD4^+$ helper T cell.


CD4+ CD8+
thymocytes
Class I
MHC
Class II
MHC
T cell receptor
thymic epithelial cell
thymic epithelial cell
1. POSITIVE SELECTION
2. NEGATIVE SELECTION
3. LINEAGE DETERMINATION
Mature CD8+ killer T cell
Mature CD4+ helper T cell

Current theories propose that the strength of the signal received by a DP thymocyte through its TCR and/or coreceptor can determine the developmental fate of the thymocyte with respect to both selection and lineage commitment. In the case of selection, it is believed that the avidity of the TCR for self-peptide/self-MHC complexes in the thymus will influence the intensity of the TCR signal. Thus, thymocytes that bind self-peptide/self-MHC complexes with intermediate avidity will receive intermediate signals that induce positive selection and the generation of T cells that would be useful to the individual. In contrast, if the thymocytes bind strongly to self-peptide/self-MHC complexes, a strong signal would be transduced, leading to negative selection and the elimination of potentially autoreactive cells. Consistent with this idea, there is extensive evidence that both TCR avidity for self-peptide/self-MHC and TCR signal strength influence the repertoire selection process (112). Further, it has recently been demonstrated that there is a clear link between TCR-MHC/peptide avidity and the ensuing strength of the TCR signal (113, 114). The studies presented in these references, and probably hundreds more, looking at avidity, strength of signal, and selection has led to or supported the most commonly accepted model of repertoire selection, known as the avidity model of thymocyte selection (Figure 3.2).

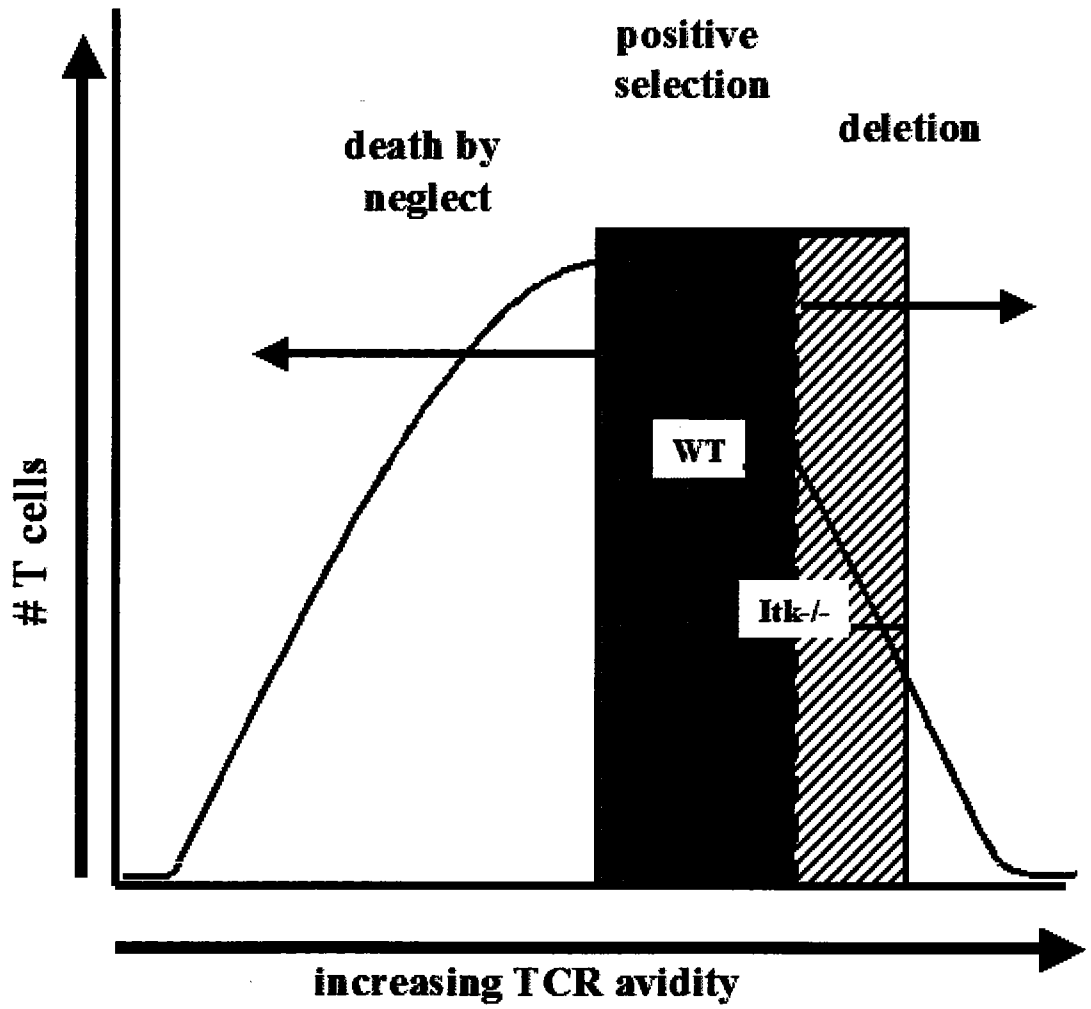
The signaling pathways shown to be important for pre-TCR signaling (see Figure 2.1) are also important for both positive and negative selection. Thus, for all the reasons discussed in Chapter 2, we would expect Tec family kinases to play a role in selection. Other signaling molecules have been shown to be at least partially dispensable during β selection, and yet, play a critical role in selection. Two examples are *ZAP-70*$^{-/-}$ mice (71,

115), which only have a block at the DN3 stage when Syk is absent as well, and *vav*$^{-/-}$ mice (107, 108). In fact, the overall *vav*$^{-/-}$ thymic phenotype is the most similar to Tec family-deficient mice.

Previously, the analysis of *itk*$^{-/-}$ mice crossed to either a class I- or a class II-restricted TCR transgenic line revealed that virtually no TCR transgenic T cells developed in the absence of Itk (39). Based on this original observation and more recent studies with these same TCR transgenics crossed to both *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice, it has been proposed that Tec family kinases play a role in setting thresholds for thymocyte development (69). Therefore we hypothesize that the avidity "window" in which cells that are positively selected has shifted in the absence of Itk. As a result, cells that are at the low end of the avidity window in wild-type mice, do not receive a proper selection signal in the absence of Itk. It also proposes that cells that would normally receive enough signal to undergo negative selection now fall into the upper end of the positive selection window. Thus, the cells that are positively selected in the absence of Itk have an overall increase in avidity when compared to wild-type cells (Figure 3.2)

Figure 3.2 The avidity model of thymocyte selection

A graph of thymocyte cell number and TCR avidity is depicted. The curve represents a Gaussian distribution of cell number and avidity, however this is completely hypothetical distribution. At the low end of the curve, cells receive little or no TCR signal as a result of low TCR avidity and therefore undergo apoptosis due to neglect. At the high end of the curve are the cells that receive a strong TCR signal due to a high avidity interaction with self-peptide/self-ligands, and thus also undergo apoptosis, but due to negative selection. In the middle are cells expressing TCRs with an intermediate avidity for self-peptide/self-MHC that receive a low but sustained level of TCR signaling and therefore are positive selected. The "window" of positive selection for wild-type cells is depicted by the two solid pink rectangles. We hypothesize that the "window" of positive selection for Itk-deficient cells partially overlaps with the wild-type window (solid dark pink rectangle), but also may include cells that would normally be negatively selected (rectangle with pink stripes).


positive
selection
death by
neglect
deletion
WT
Itk-/-
T cells
increasing TCR avidity

This chapter presents work we did to determine the role of Itk in TCR repertoire selection. To better understand the role of Tec family members in thymocyte development, we crossed *itk*$^{-/-}$ mice to a variety of TCR transgenic lines that express TCRs with different avidities for their selecting ligands in the thymus. These mice would allow us to determine whether the requirement for Tec family signals is influenced by the avidity of the TCR on the developing thymocyte. In addition, we also examined the role of Itk in deletion of three TCR transgenic lines by superantigen and deletion in a double transgenic system in which TCR transgenic mice express a second transgene that contains the cognate antigen of the TCR expressed by these mice.

MATERIALS AND METHODS

Mice

Itk-deficient mice were crossed to 2B4 (116), 5C.C7 (117), and AND (118) TCR transgenic mice, all of which were on the B10.BR (H-2^k) genetic background. The $itk^{-/-}$ mice crossed to the TCR transgenic mice had a mixed 129/B10 genetic background (H-2^b). Since the 129 strain carries two superantigens (SAgs) that delete Vβ3-expressing T cells, a PCR screen was designed to distinguish mice that carried at least one SAg from those that were SAg negative; SAg-negative mice were used for all experiments. The non-transgenic $itk^{+/-}$ and $itk^{-/-}$ mice in all figures were backcrossed to B10 mice at least eight times. Mice were analyzed between 6 and 12 wk of age, and all TCR transgenic control mice are $itk^{+/-}$ littermates. When examined, there were no significant phenotypic differences in the lymphocyte profiles of TCR transgenic $itk^{+/-}$ and $itk^{+/+}$ mice (data not shown). Mice were bred and maintained in a SPF facility at the University of Massachusetts Medical School.

Antibodies

The following mAbs, purchased from BD Pharmingen, were used for staining cells: anti-CD4-PE, anti-CD4-CyChrome (anti-CD4-Cy), anti-CD8α-FITC (53-6.7), anti-CD8α-Cy, anti-CD8α-allophycocyanin, anti-TCRβ-Cy, anti-Vβ3-PE, anti-Vα11.1, $11.2^{b,d}$-FITC, anti-CD5-Cy, anti-CD69-bio, anti-CD24 (heat-stable Ag (HSA))-bio, purified hamster anti-mouse-Bcl-2, and purified hamster anti-trinitrophenol (107.3). Hamster Abs were detected by goat $F(ab')_2$ anti-hamster IgG (H+L)-FITC (Caltag Laboratories, Burlingame,

CA). In most cases the 2B4 TCR transgenic was detected by staining with the A2B4-2 Ab (119), which was purified and conjugated to FITC in our laboratory. Biotinylated Abs were detected using strep-APC(BD Pharmingen).

Cell preparation, staining, and flow cytometry

Lymphocyte cell suspensions were made from thymus, spleen, and lymph nodes (LNs: pooled inguinal, axillary, brachial, and cervical) by dissociation between two frosted slides in RPMI and 10% FCS. Further cell preparation and staining was carried out as described in Chapter 2. In general, 10,000–50,000 live (based on forward vs. side scatter profiles) events/sample were collected for the analysis of CD4, CD8, and TCR staining in LN, spleen, and thymus preparations. For marker analysis on thymic subsets 100–200,000 events/sample were collected. Data were analyzed using CellQuest software (BD Biosciences).

Intracellular Bcl-2 staining

Cells were prepared and stained with Abs to cell surface molecules as described above. After extracellular staining, the cells were washed and fixed/permeabilized by incubation in 100 μl Cytofix/Cytoperm (BD Pharmingen) on ice for 20 min. The samples were then washed with Perm/Wash buffer (BD Pharmingen), and a hamster mAb to Bcl-2 was added. Duplicate cell samples were also stained with an irrelevant hamster Ab (anti-trinitrophenol). Cells were incubated on ice for 30 min. After washing, the hamster Abs were detected by incubation with a goat anti-hamster Ab conjugated to FITC on ice for 30

min. Cells were again washed and analyzed immediately. For samples stained with anti-Bcl-2, 200,000 events/sample were collected; 50,000 events/sample were collected for samples stained with the control hamster Ab.

RESULTS

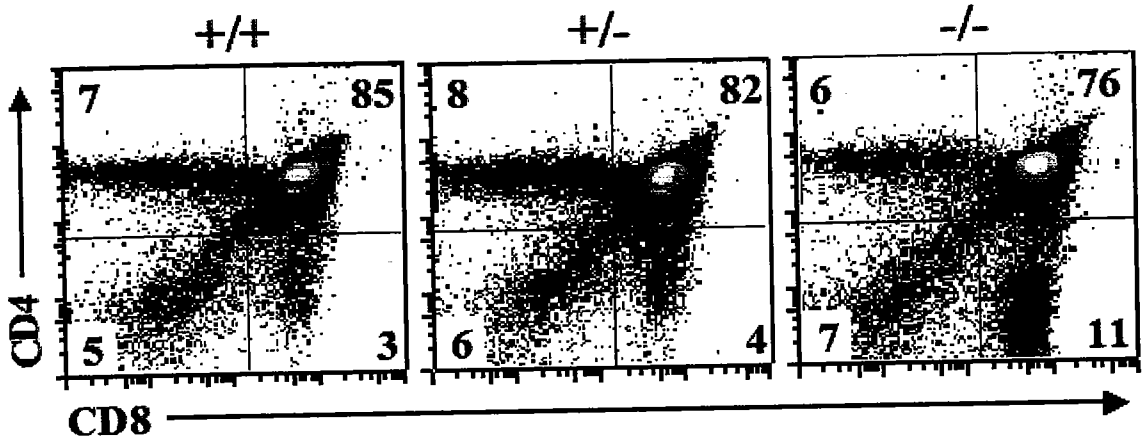
T cell development is altered in Itk-deficient mice

The examination of *itk*$^{-/-}$ mice generated in our laboratory (66) suggested that Itk plays a role in T cell development. As previously reported (39, 69) , we observed altered CD4$^+$ and CD8$^+$ T cell populations in the thymus, spleen, and LN of *itk*$^{-/-}$ mice (Figure 3.3). Although we did not observe significant differences in lymphocyte cellularity in these organs, the proportions of CD4$^+$ and CD8$^+$ T cells differ from those in control mice (*itk*$^{+/+}$ or *itk*$^{+/-}$). In the thymus, *itk*$^{-/-}$ mice have a slight reduction in the percentage of CD4$^+$ SP cells and at least a 2-fold increase in the percentage of CD8$^+$ SP cells. However, in the periphery the percentage of CD4$^+$ cells is, on the average, half that in control mice, while the percentage of CD8$^+$ T cells is essentially normal. Interestingly, with respect to absolute numbers, there is no significant reduction in the total CD4$^+$ SP cells in the thymus, but there is approximately half the normal number of CD4$^+$ peripheral T cells in *itk*$^{-/-}$ mice (39, 69). And while there is a significant increase in the absolute number of CD8$^+$ SP thymocytes (wild-type: $5.6\pm0.8\times10^6$; *itk*$^{-/-}$: $14.0\pm0.2\times10^6$; $p < 0.0019$) in *itk*$^{-/-}$ mice, the number of peripheral CD8$^+$ T cells is comparable to that of wild-type mice. Thus, in both cases cells that are present in the thymus do not appear to persist in the periphery in the absence of Itk (39). Potential reasons for this or alternative explanations will be discussed later.

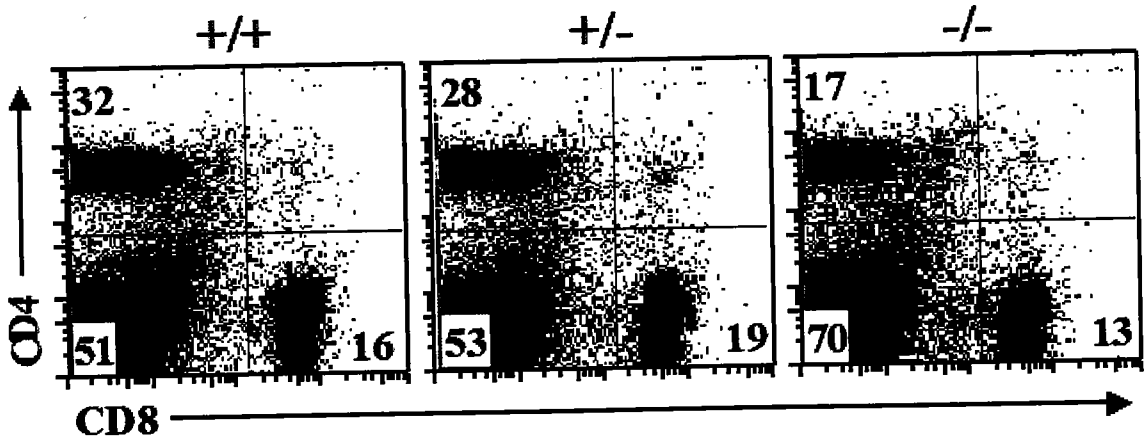
Figure 3.3 Altered T cell development in Itk-deficient mice

(A) Thymocytes and (B) lymph node cells from 8-week-old $itk^{+/+}$, $itk^{+/-}$, and $itk^{-/-}$ littermates were stained with anti-CD4-PE and anti-CD8-Cy. CD4 vs. CD8 profiles are shown.

A.



B.



Development of T cells expressing 2B4, 5C.C7, or AND TCR in H-2^k mice is affected by the absence of Itk

Based on the phenotype of the *itk*$^{-/-}$ mice, we hypothesized that the development of CD4$^+$ T cells is more affected by the absence of Itk. Therefore, to further address the role of Itk in CD4$^+$ T cell development, we crossed *itk*$^{-/-}$ mice to three different lines of TCR transgenics, specifically, 2B4 (116), 5C.C7 (117), and AND transgenic mice. Each of these transgenics expresses a TCR that uses Vα11 and Vβ3 gene segments and is specific for a moth cytochrome *c* peptide (88–103) bound to the MHC class II molecule I-E^k. However, each TCR is thought to have a different avidity for selecting ligand(s) in the thymus based on differences in the efficiency of positive selection (2B4 < 5C.C7 < AND) (120). Thus, the use of these TCR transgenic lines would allow us to determine whether the absence of Itk would differentially affect the development of cells bearing TCRs presumed to have varying avidities for selecting ligands.

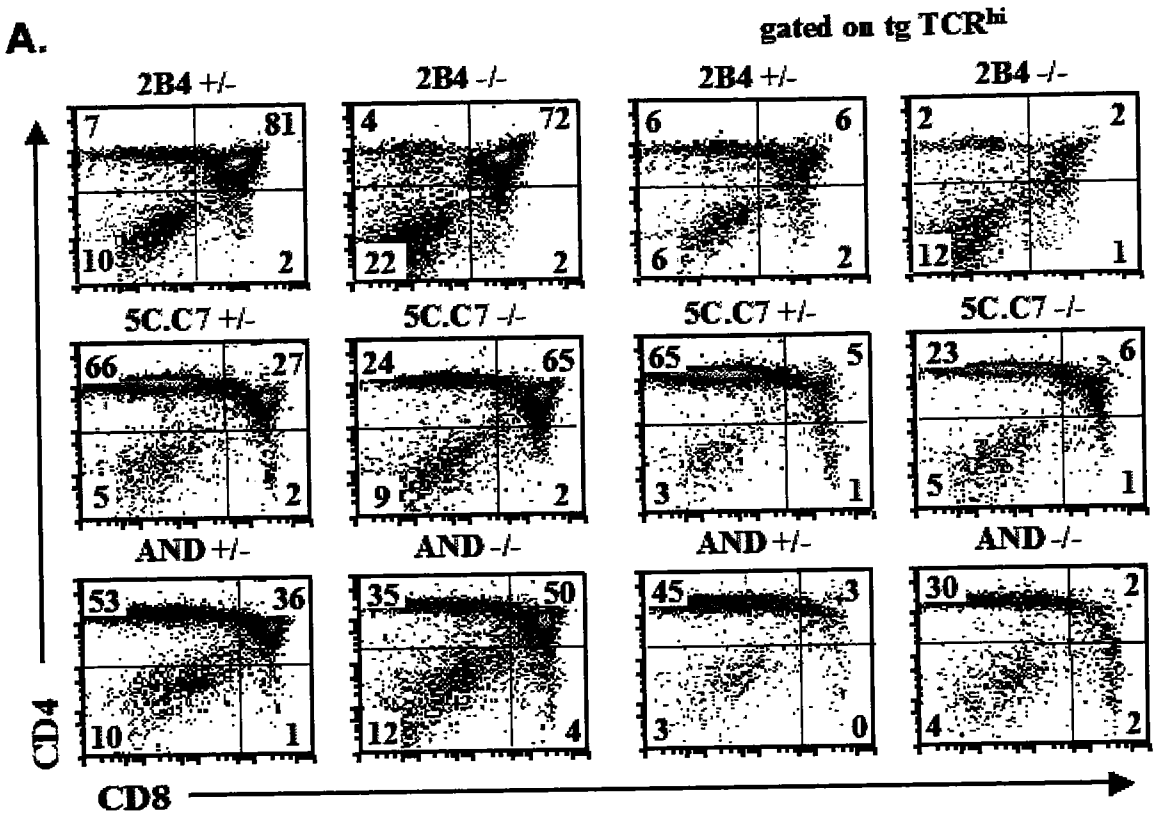
Analysis of the 2B4, 5C.C7, and AND H-2^k *itk*$^{-/-}$ mice revealed that the ability of the different TCR transgenic thymocytes to develop and populate the periphery in the absence of Itk varies in a manner consistent with the proposed avidity of each TCR for its selecting ligand(s) in the thymus. In the 2B4 and 5C.C7 transgenic *itk*$^{-/-}$ mice, the percentages and absolute numbers of CD4$^+$ SP cells in the thymus are dramatically reduced compared with the corresponding *itk*$^{+/-}$ TCR transgenic (Figure 3.4A and Table 3.1). In the AND *itk*$^{-/-}$ mice, the average percentage of CD4$^+$ SP cells in the thymus is reduced. However, as a reflection of the slight increase in thymic cellularity in AND *itk*$^{-/-}$

mice, the absolute numbers of $CD4^+$ SP cells are comparable to that seen in AND $itk^{+/-}$ mice (Table 3.1).

The hierarchy of developmental defects seen in the thymus is also reflected in the peripheral T cell populations in all three TCR transgenic lines (Figure 3.4B), and correlates with the presumed avidity of each TCR for selecting ligands. In $itk^{-/-}$ mice that express the 2B4 TCR, which is thought to be a low avidity TCR, there are very few $CD4^+$ cells that express the transgenic TCR. In contrast, there are many more TCR transgenic $CD4^+$ T cells in the $itk^{-/-}$ mice that express a high avidity TCR, such as 5C.C7 or AND. In fact, in the AND $itk^{-/-}$ mice, the percentage and absolute number of these cells are comparable to those in wild-type AND mice.

It is important to note that the mice represented in Figure 3.4 are all ~8 wk of age. In mice that are younger (~4 wk), the differences in the percentage of peripheral $CD4^+$ transgenic T cells between $itk^{-/-}$ mice and wild-type controls are more substantial in all three transgenic lines. This is indicative of the lower percentages of $CD4^+$ SP being generated in the thymus of these mice. As these mice age, the numbers of peripheral $CD4^+$ transgenic T cells in AND $itk^{-/-}$ mice increase to numbers equivalent to those found in $itk^{+/-}$ mice; however, this is not the case for the 2B4 and 5C.C7 $itk^{-/-}$ mice. These observations suggest that age is not the only factor that allows the AND $itk^{-/-}$ mice to accumulate as many $CD4^+$ transgenic T cells as wild-type AND mice (see below).

Figure 3.4 The development of MHC class-II specific T cells in the absence of Itk is affected by the avidity of the TCR for its selecting ligand(s) in the thymus. Itk-deficient mice were crossed to the 2B4, 5C.C7, and AND TCR transgenics (all on an H-2^k background). CD4 vs. CD8 profiles of (A) thymocytes and (B) lymph node cells are shown for live gated cells (left) and live TCR^{hi} gated cells (right). Each set of $itk^{+/-}$ and $itk^{-/-}$ are 8-week old littermates. Cells from non-transgenic mice were stained with CD4-PE, CD8-Cy, and CD3-APC. Cells from transgenic mice were stained with a FITC conjugated Ab that detected the transgenic TCR (anti-Vα11.$1^{b,d}$ or A2B4-2) along with anti-CD4-PE and anti-CD8-Cy.


A.
gated on tg TCR^hi
2B4 +/-
2B4 -/-
2B4 +/-
2B4 -/-
7
81
10
2
4
72
22
2
6
6
6
2
2
2
12
1
5C.C7 +/-
5C.C7 -/-
5C.C7 +/-
5C.C7 -/-
66
27
5
2
24
65
9
2
65
5
3
1
23
6
5
1
AND +/-
AND -/-
AND +/-
AND -/-
53
36
10
1
35
50
12
4
45
3
3
0
30
2
4
2
CD4
CD8

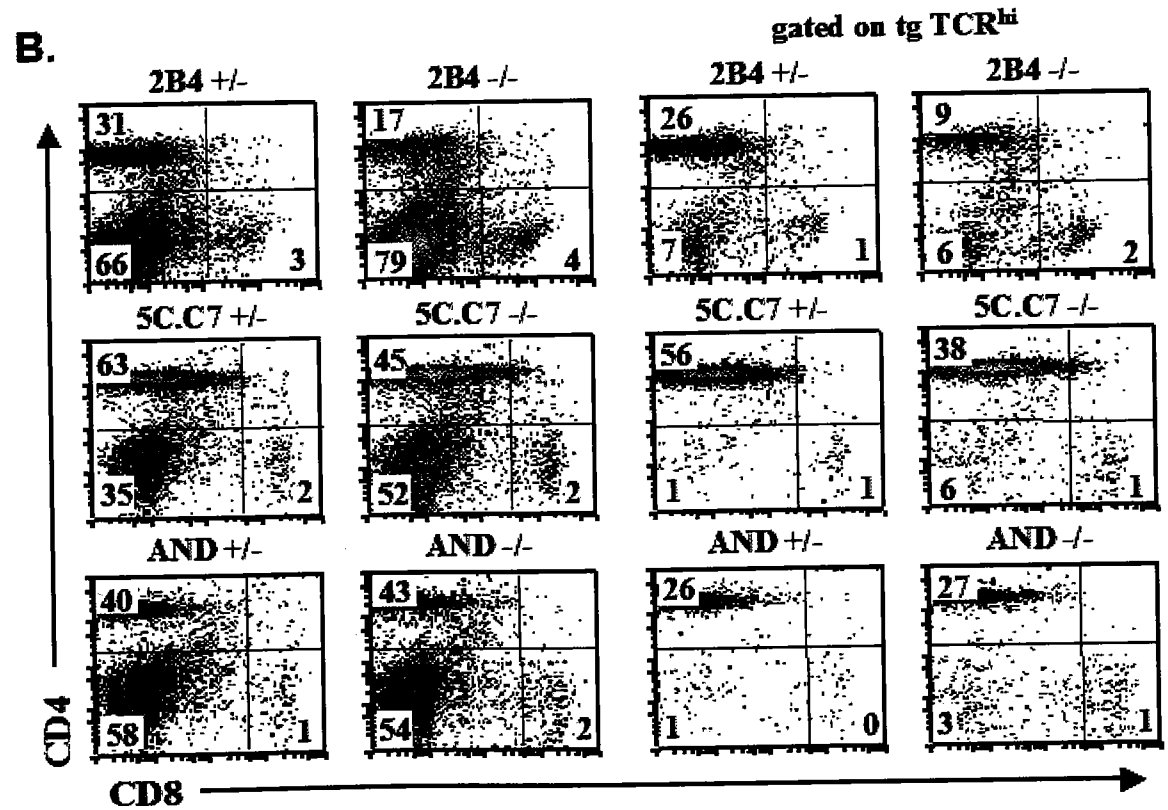

B.
gated on tg TCR^hi
2B4 +/-
2B4 -/-
2B4 +/-
2B4 -/-
31
66
3
17
79
4
26
7
1
9
6
2
5C.C7 +/-
5C.C7 -/-
5C.C7 +/-
5C.C7 -/-
63
35
2
45
52
2
56
1
1
38
6
1
AND +/-
AND -/-
AND +/-
AND -/-
40
58
1
43
54
2
26
1
0
27
3
1
CD4
CD8

Table 3.1 Thymocytes and LN cells from *itk*$^{+/-}$ and *itk*$^{-/-}$ TCR transgenic mice

The average total cell number for the thymus and lymph nodes are given. For the thymus, the percentage of DP and $CD4^+$ SP cells, as well as the calculated absolute number of $CD4^+$ cells are also given. For the lymph nodes, the percentage and absolute number of tg TCR^{hi} (as determined by staining with anti-A2B4-2 or anti-Vα11) $CD4^+$ cells are given. The values are the mean of 3 - 10 animals for each genotype with SEMs. Absolute numbers are given in millions and numbers in bold vary significantly (p value < 0.05) between the presence and absence of Itk. Similar analyses were performed on splenocytes with comparable results (data not shown).

		thymus				LNs		
	n	total #	% DP	% CD4 SP	# CD4 SP	Total #	% CD4 tg TCR^{hi}	# CD4 tg TCR^{hi}
$2B4^{k/k}$ $Itk^{+/-}$	4	**66 ± 18**	72 ± 5.5	13 ± 2.8	**8.6 ± 4.0**	14 ± 4.6	28 ± 3	**3.9 ± 1.3**
$2B4^{k/k}$ $Itk^{-/-}$	8	**24 ± 5.8**	62 ± 2.0	5.0 ± 0.4	**1.2 ± 0.3**	18 ± 2.9	6.8 ± 1.0	**1.1 ± 0.1**
$5C.C7^{k/k}$ $Itk^{+/-}$	10	136 ± 19	29 ± 2.6	61 ± 3.2	**83 ± 12**	**29 ± 5.1**	55 ± 5.3	**16 ± 2.9**
$5C.C7^{k/k}$ $Itk^{-/-}$	9	155 ± 34	66 ± 2.4	19 ± 2.4	**28 ± 5.3**	**12 ± 1.7**	32 ± 3.7	**4.0 ± 0.8**
$AND^{k/k}$ $Itk^{+/-}$	7	65 ± 9.7	30 ± 4.3	55 ± 4.2	**36 ± 7.0**	23 ± 5.7	52 ± 3.6	12 ± 4.0
$AND^{k/k}$ $Itk^{-/-}$	3	89 ± 13	42 ± 4.9	42 ± 3.6	**42 ± 4.6**	24 ± 1.3	40 ± 3.6	5.0 ± 6.4

Development of AND itk$^{-/-}$ T cells is affected by the strength and density of selecting ligands expressed by APC in the thymus

Based on the analysis of 2B4, 5C.C7, and AND *itk*$^{-/-}$ mice, it appears that the presumed avidity of the TCR for its selecting ligand(s) in the thymus plays an important role in the development of T cells in the absence of Itk. However, it is known that the development of T cells in each TCR transgenic line can be somewhat idiosyncratic, due in part to the fact that untimely transgene expression can affect development before thymocytes reach the DP stage (121). Therefore, it is possible that the developmental efficiency of thymocytes in each of the transgenics is not solely based on their given avidities for the selecting ligands. To address this concern, we chose to analyze *itk*$^{-/-}$ mice that express one of the transgenic TCRs on different MHC backgrounds in which varying levels of two different selecting ligands are expressed.

The AND TCR can be positively selected on both I-E^{k} and I-A^{b} molecules (122). However, the generation of mature CD4^{+} Vα11^{hi} T cells is greatly reduced in mice that only express the I-A^{b} molecule. These observations strongly suggest that the AND TCR has a higher avidity for I-E^{k} vs. I-A^{b} MHC molecules plus the self peptide(s) that mediates its selection. Therefore, we decided to compare the selection of AND *itk*$^{-/-}$ T cells on the H-2^{k}, H-2^{b}, and H-2$^{k/b}$ MHC backgrounds. Analysis of lymphocytes from wild-type AND mice on all these backgrounds confirms the selection hierarchy: AND$^{b/b}$ < AND$^{k/b}$ < AND$^{k/k}$ (Figure 3.5). The percentage and perhaps the absolute number of CD4^{+} cells in the thymus of wild-type AND$^{k/k}$ mice are slightly lower than those in wild-type AND$^{k/b}$ mice (Figure 3.5A and Table 3.2), but the AND$^{k/k}$ mice have more peripheral

$CD4^+$ $V\alpha11^{hi}$ cells (Figure 3.5B and Table 3.2). A probable explanation for this phenotype is that, as previously suggested (122), the interaction between AND thymocytes and their selecting ligand(s) in the thymus of $AND^{k/k}$ mice is of such high avidity that some of the cells are actually deleted in these mice. This is supported by the fact that $AND^{k/k}$ mice generally have lower total thymocyte numbers and fewer DPs compared with $AND^{k/b}$ or $AND^{b/b}$ mice (Table 3.2).

The phenotype of $AND^{b/b}$ *itk*$^{-/-}$ mice has been reported previously (39, 69), and our analysis is in agreement with these reports. Specifically, the development of transgenic T cells in these mice is dramatically reduced compared with that in $AND^{b/b}$ *itk*$^{+/-}$ mice. Despite the significant numbers of $CD4^+$ SP cells in both the thymus and LNs of the $AND^{b/b}$ *itk*$^{-/-}$ mice (Figure 3.5), very few of the $CD4^+$ cells in the periphery express high levels of the transgenic TCR, as demonstrated by staining for $V\alpha11$ (Figure 3.5B). In contrast, AND *itk*$^{-/-}$ mice that express $I\text{-}E^k$ molecules have significant numbers of $CD4^+$ $V\alpha11^+$ T cells in both the thymus and periphery. As shown in the previous section, the generation of transgenic T cells in the thymus of $AND^{k/k}$ *itk*$^{-/-}$ mice appears to be slightly less efficient than in $AND^{k/k}$ *itk*$^{+/-}$ mice, but the percentage and number of peripheral transgenic T cells are not significantly reduced, as determined statistically, which is unlike the other Itk-deficient TCR transgenics. Although time may lead to the accumulation of transgenic T cells in the *itk*$^{-/-}$ mice, it is also likely that the $AND^{k/k}$ *itk*$^{-/-}$ mice develop as many peripheral cells as wild-type AND mice, because the deletion of some of the AND thymocytes that takes place in wild-type $AND^{k/k}$ mice does not occur, or occurs to a lesser extent, in the absence of Itk.

Wild-type AND$^{k/b}$ mice have the highest number of CD4^{+} SP T cells in the thymus of all three backgrounds examined, presumably because they express the strongly selecting I-E^{k} molecules on thymic stromal cells, but due to the lower density of this selecting molecule, the partial deletion that occurs in AND$^{k/k}$ mice is absent. The percentage of CD4^{+} Vα11^{hi} T cells in both the thymus and lymph nodes of AND$^{k/b}$ *itk*$^{-/-}$ mice is one-half to one-third the number of these cells found in AND$^{k/b}$ *itk*$^{+/-}$ mice (Figure 3.5 and Table 3.2). This phenotype is, as expected, intermediate between that of AND$^{b/b}$ *itk*$^{-/-}$ mice, which have almost no mature transgenic T cells, and that of AND$^{k/k}$ *itk*$^{-/-}$ mice, which have normal numbers of transgenic T cells. Thus, the strength and density of selecting ligands or the avidity of the interactions between the T cell and thymic stromal cells influence the outcome of positive selection in the absence of Itk.

Figure 3.5 The density and avidity of the selecting ligands present in the thymus affect the development of Itk-deficient AND transgenic T cells.

Itk-deficient AND TCR transgenic mice were bred onto H-2^k, H-2^b and H-$2^{k/b}$ backgrounds. (A) Thymocytes and (B) lymph node cells from 8-week old *itk*$^{+/-}$ and *itk*$^{-/-}$ AND transgenic mice were stained with anti-Vα11.1^{b,d}-FITC, anti-CD4-PE, and anti-CD8-Cy. CD4 vs. CD8 profiles are shown for live gated (left) and live Vα11^{hi} gated (right) cells.

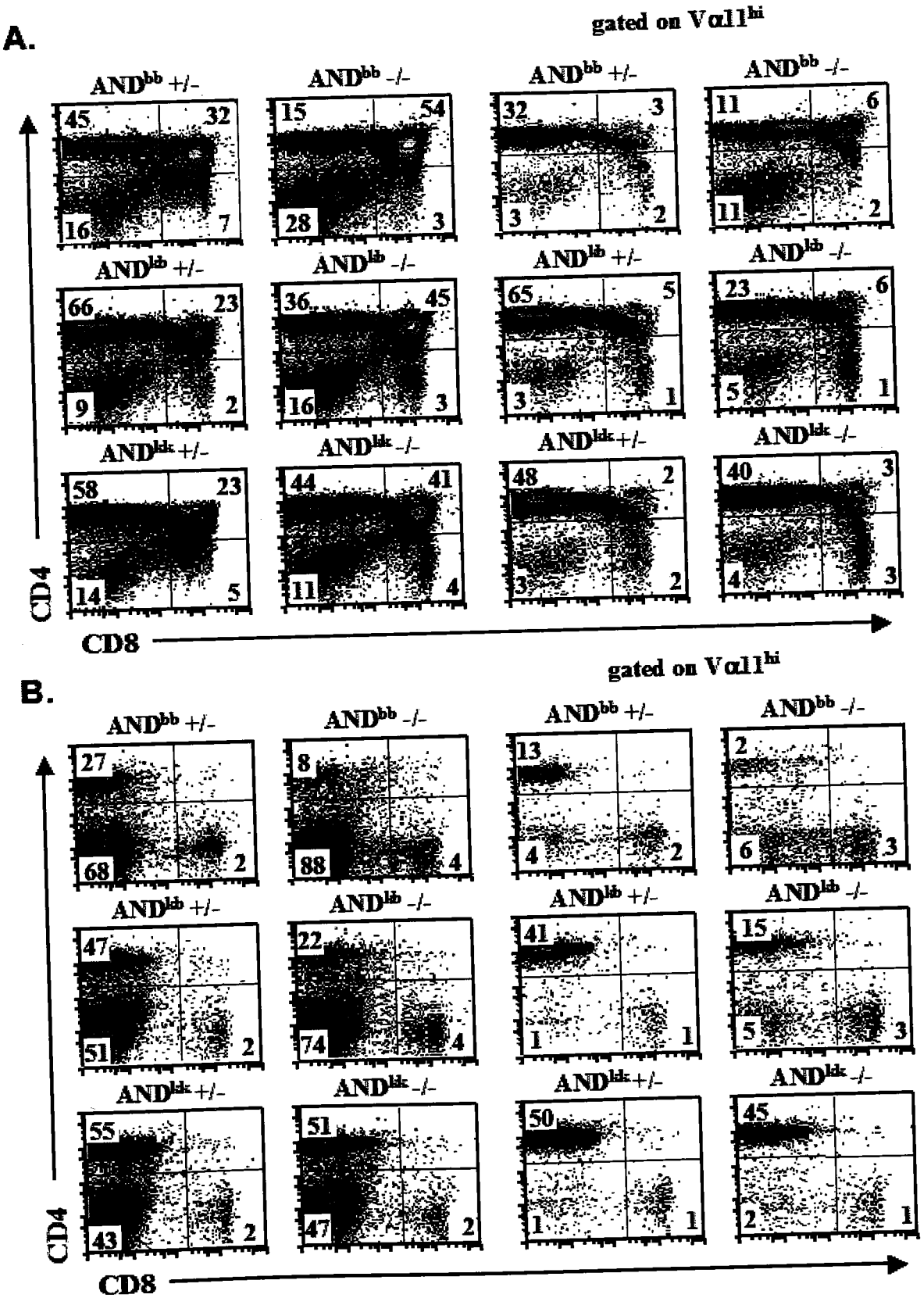

A.
gated on Vα11hi
ANDbb +/-
ANDbb -/-
ANDbb +/-
ANDbb -/-
45
32
16
7
15
54
28
3
32
3
3
2
11
6
11
2
ANDlb +/-
ANDlb -/-
ANDlb +/-
ANDlb -/-
66
23
9
2
36
45
16
3
65
5
3
1
23
6
5
1
ANDldc +/-
ANDldc -/-
ANDldc +/-
ANDldc -/-
58
23
14
5
44
41
11
4
48
2
3
2
40
3
4
3
CD4
CD8
B.
gated on Vα11hi
ANDbb +/-
ANDbb -/-
ANDbb +/-
ANDbb -/-
27
68
2
8
88
4
13
4
2
2
6
3
ANDlb +/-
ANDlb -/-
ANDlb +/-
ANDlb -/-
47
51
2
22
74
4
41
1
1
15
5
3
ANDldc +/-
ANDldc -/-
ANDldc +/-
ANDldc -/-
55
43
2
51
47
2
50
1
1
45
2
1
CD4
CD8

Table 3.2 Thymocytes and LN cells from *itk*$^{+/-}$ and *itk*$^{-/-}$ AND TCR transgenic mice on different selecting backgrounds

The percentage of DP and CD4$^+$ SP cells and absolute numbers of CD4$^+$ SP cells in the thymus are given. The percentage and absolute number of AND$^+$ (as determined by staining with anti-Vα11) CD4$^+$ lymph node cells are also given. The values are the mean of 3 – 7 animals for each genotype with SEMs. Absolute numbers are given in millions and numbers in bold vary significantly (p value < 0.05) between the presence and absence of Itk. Similar analyses were performed on splenocytes with comparable results (data not shown).

		thymus				LNs		
	n	total #	% DP	% CD4 SP	# CD4 SP	total #	% CD4 Vα11^{hi}	# CD4 Vα11^{hi}
AND$^{k/k}$ Itk$^{+/-}$	7	65 ± 9.7	30 ± 4.3	55 ± 4.2	**36 ± 7.0**	23 ± 5.7	52 ± 3.6	12 ± 4.0
AND$^{k/k}$ Itk$^{-/-}$	3	89 ± 13	42 ± 4.9	42 ± 3.6	**42 ± 4.6**	24 ± 13	40 ± 3.6	5.0 ± 6.4
AND$^{k/b}$ Itk$^{+/-}$	5	94 ± 48	25 ± 1.9	62 ± 1.7	**56 ± 27**	**38 ± 0.7**	36 ± 4.1	**14 ± 3.9**
AND$^{k/b}$ Itk$^{-/-}$	4	45 ± 16	45 ± 4.6	33 ± 4.9	**7.1 ± 2.8**	**15 ± 2.4**	17 ± 4.9	**2.7 ± 0.8**
AND$^{b/b}$ Itk$^{+/-}$	4	**89 ± 16**	35 ± 1.8	50 ± 2.0	45 ± 8.7	11 ± 2.4	15 ± 1.7	**1.5 ± 0.4**
AND$^{b/b}$ Itk$^{-/-}$	6	**34 ± 14**	60 ± 3.4	13 ± 1.0	5.2 ± 2.1	18 ± 2.5	3.8 ± 1.3	**0.6 ± 0.1**

Analyses of various developmental markers suggest that the early stages of positive selection are impaired in itk-/- thymocytes, but that the SP thymocytes generated are normal

The analysis of each of the TCR transgenic *itk*$^{-/-}$ mice revealed that the accumulation of mature CD4^{+} TCR transgenic cells in both the thymus and periphery of these mice is impaired by the absence of Itk. We hypothesized that this lack of accumulation is due to a defect in the generation or positive selection of these cells. However, it remained possible that mature CD4^{+} cells were being generated normally, but their survival was impaired in the absence of Itk. Therefore, we were interested in determining whether the survival factor, Bcl-2 is properly up-regulated in *itk*$^{-/-}$ thymocytes.

It has been shown that Bcl-2 is up-regulated in a subset of TCR^{hi} DP thymocytes and remains high in SP cells (123). This expression pattern correlates with cells that have received or are receiving positive selection signals. Figure 3.6 depicts intracellular staining for Bcl-2 in *itk*$^{-/-}$ thymocytes compared with their wild-type littermates. In non-TCR transgenic *itk*$^{-/-}$ cells, the levels of Bcl-2 are up-regulated in both the DP TCR^{hi} and CD4^{+} SP populations to the same extent as in thymocytes from a wild-type littermate (Figure 3.6A). The same is true in the analogous populations of TCR transgenic thymocytes on all the selecting backgrounds (Figure 3.6B and data not shown). These data indicate that *itk*$^{-/-}$ cells in which the early stages of positive selection are properly initiated, as determined by up-regulation of TCR, also properly up-regulate the survival factor Bcl-2. However, within the entire DP population from AND$^{k/b}$ *itk*$^{-/-}$ thymi, a

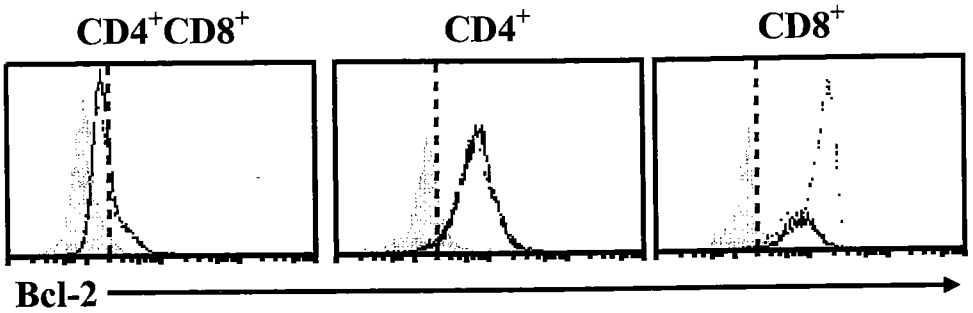
greater proportion of DP cells have background (very low) levels of Bcl-2. This is probably due to fewer cells in these mice receiving the initial signals for selection.

Figure 3.6 The survival protein, Bcl-2, is up-regulated normally during positive selection of *itk*$^{-/-}$ thymocytes

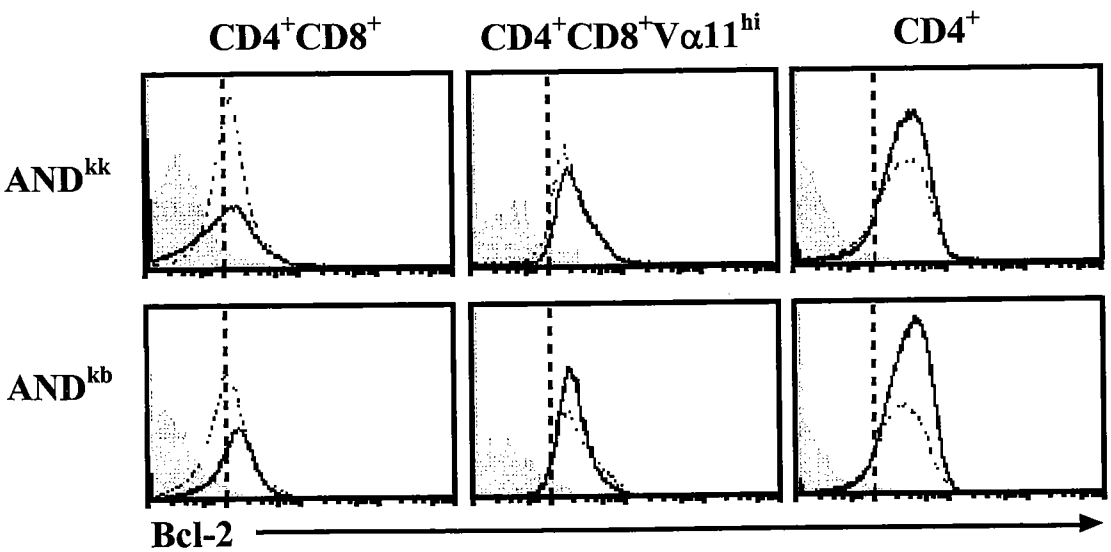
(A) Thymocytes from non-transgenic *itk*$^{+/-}$ and *itk*$^{-/-}$ were stained with CD4-PE and CD8-Cy. The cells were then fixed and permeabilized and incubated with a hamster mAb to Bcl-2 or an irrelevant hamster Ab (anti-TNP). Histograms of Bcl-2 expression on live DP, live CD4^{+} SP, and live CD8^{+} SP thymocytes from *itk*$^{+/-}$ (solid line) and *itk*$^{-/-}$ (dashed line) are shown. Non-specific staining with the irrelevant hamster Ab is also shown (gray filled histogram).

(B) Thymocytes from *itk*$^{+/-}$ and *itk*$^{-/-}$ TCR transgenic mice were analyzed similarly with the addition of anti-Vα11.1$^{b/d}$-bio followed by strepavidin-APC staining prior to fixation and permeabilization. Bcl-2 expression is shown on live DP, live Vα11^{hi} DP, and CD4^{+} SP from AND *itk*$^{+/-}$ (solid line) and AND *itk*$^{-/-}$ (dashed line) cells from both the H-2^{k} and H-2$^{k/b}$ backgrounds. Non-specific staining with the irrelevant hamster Ab is also shown (gray filled histogram).

A.


CD4+CD8+
CD4+
CD8+
Bcl-2

B.


CD4+CD8+
CD4+CD8+Vα11hi
CD4+
ANDkk
ANDkb
Bcl-2

Analysis of the Bcl-2 levels in cells undergoing selection and in cells that have completed selection suggested that *itk*$^{-/-}$ thymocytes properly up-regulated this important survival factor and thus would have comparable viability as their wild-type counterparts. Therefore, we were interested in determining whether the regulation of additional markers is normal in *itk*$^{-/-}$ thymocytes undergoing selection; this analysis would allow us to identify the stage(s) of selection in which *itk*$^{-/-}$ thymocytes are defective. Figure 3.7A depicts the stages of positive selection based on the expression of the TCR, CD69, HSA, and the coreceptors, CD4 and CD8.

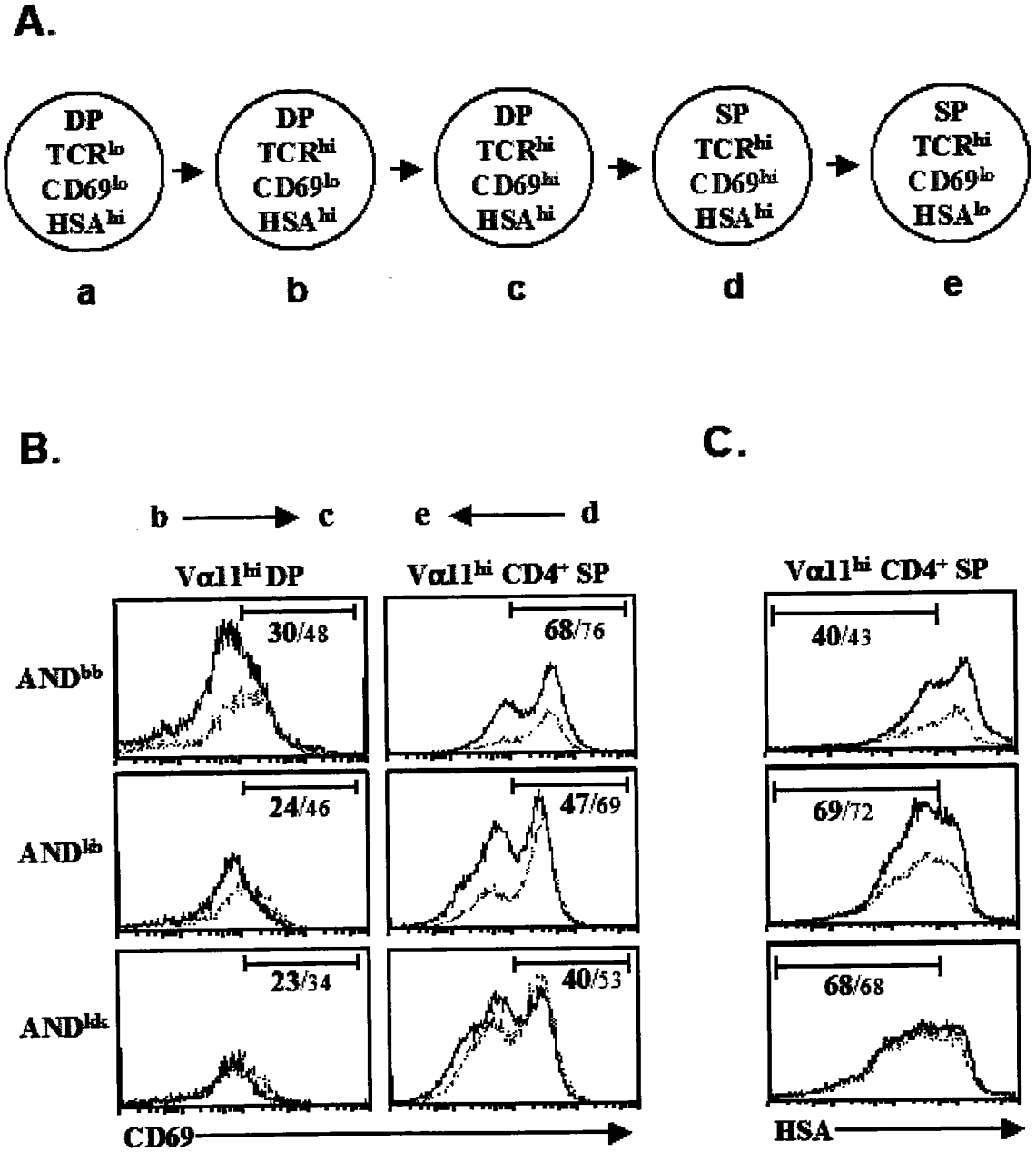
This analysis revealed that a smaller percentage of DPs have up-regulated TCR in the absence of Itk in all TCR transgenic lines (data not shown), suggesting that fewer *itk*$^{-/-}$ DP cells are receiving a strong enough signal to initiate selection. Next we wanted to determine whether Itk-deficient thymocytes would also be impaired at later stages of positive selection. Thus, we examined the pattern of CD69 expression on TCR^{hi} DP and SP thymocytes (Figure 3.7B). Thymocytes that have up-regulated CD69 are in either stage C (TCR^{hi} DP) or stage D (SP) (124). Comparing the percentage of cells in these intermediate stages of selection from wild-type AND mice on each selecting background revealed that there are fewer CD69^{hi} cells in the presence of the strongly selecting IE^{k} ligand. One interpretation of these data is that thymocytes receiving stronger signals up-regulate CD69 and then progress through stages C and D more efficiently than cells receiving weaker signals. If this interpretation is correct, we would expect that a higher percentage of TCR^{hi} DP and SP thymocytes in AND *itk*$^{-/-}$ mice would have high levels of CD69 compared with their wild-type littermates. As shown in Figure 3.7B, this is, in fact,

the case, suggesting that in the absence of Itk, thymocytes do not progress through these stages as efficiently as cells that express Itk. Finally, we examined HSA levels on the $CD4^+$ SP cells from these mice (Figure 3.7C). HSA is down-regulated in the final stage of thymocyte selection and differentiation, such that the most mature cells are HSA^- (125). Although more of the $CD4^+$ SP thymocytes in AND $itk^{-/-}$ mice are still $CD69^{hi}$ compared with those in control mice, it appears that the same proportion of the $CD4^+$ SPs have begun to down-regulate HSA in both $itk^{+/-}$ and $itk^{-/-}$ mice. This suggests that the down-regulation of HSA can take place while TCR signaling is still occurring and that this happens normally even in the absence of Itk.

Figure 3.7 Itk deficient thymocytes take longer to undergo positive selection than wild-type thymocytes

(A) Thymocytes undergo the processes of positive and negative selection after they have become DP cells (stage A). DP cells that begin the selection process first up-regulate their TCR (stage B) and then the activation marker CD69 (stage C). Cells then commit to either the CD4 or CD8 lineage and down-regulate one of the coreceptors (stage D). Cells that fully mature and complete the positive selection process then down-regulate HSA as well as CD69 (stage E).

(B) Thymocytes from *itk*$^{+/-}$ and *itk*$^{-/-}$ AND transgenic mice on all three MHC backgrounds were stained with anti-Vα11.1^{b,d}-FITC, anti-CD4-PE, anti-CD8-Cy, and either anti-CD69-bio (B) or anti-HSA-bio (C). The biotinylated Abs were detected with strepavidin-APC. Histograms of CD69 expression on thymocytes undergoing selection (Vα11^{hi} DP: stage B to stage C) and of CD69 and HSA expression on Vα11^{hi} CD4 SP (stage D to stage E) from AND *itk*$^{+/-}$ (solid line) and AND *itk*$^{-/-}$ (dotted line) are shown. The percentage of thymocytes in each histogram that are CD69^{hi} or HSA^{lo} (based on the levels found on peripheral Vα11^{+} CD4^{+}cells) for AND *itk*$^{+/-}$ (bold) and AND *itk*$^{-/-}$ (plain) are also given.


A.
DP TCR^lo CD69^lo HSA^hi
DP TCR^hi CD69^lo HSA^hi
DP TCR^hi CD69^hi HSA^hi
SP TCR^hi CD69^hi HSA^hi
SP TCR^hi CD69^lo HSA^lo
a
b
c
d
e
B.
b → c
e ← d
Vα11^hi DP
Vα11^hi CD4+ SP
AND^bb
AND^lb
AND^lk
30/48
68/76
24/46
47/69
23/34
40/53
CD69
C.
Vα11^hi CD4+ SP
40/43
69/72
68/68
HSA

The expression of CD5, a marker for TCR signal strength, is lower on thymocytes from $itk^{-/-}$ mice

CD5 surface expression is regulated throughout T cell development and correlates with the strength of the TCR signal received by the developing thymocyte (104). The studies of peripheral $itk^{-/-}$ T cells (66) and thymocytes (126) indicated that T cells get a weaker signal through their TCR in the absence of Itk; thus, we would expect that CD5 levels would be lower on thymocytes from $itk^{-/-}$ mice. This prediction was first verified by staining thymocytes from non-TCR transgenic $itk^{-/-}$ mice, which revealed that DP thymocytes have significantly lower levels of CD5 compared with DPs from $itk^{+/-}$ mice (Figure 3.8A). It is unclear at this point whether or not this dramatic reduction of CD5 expression on DP thymocytes is due to reduced pre-TCR signaling, more cells not receiving an appropriate positive selection signal, or a combination of both of these.

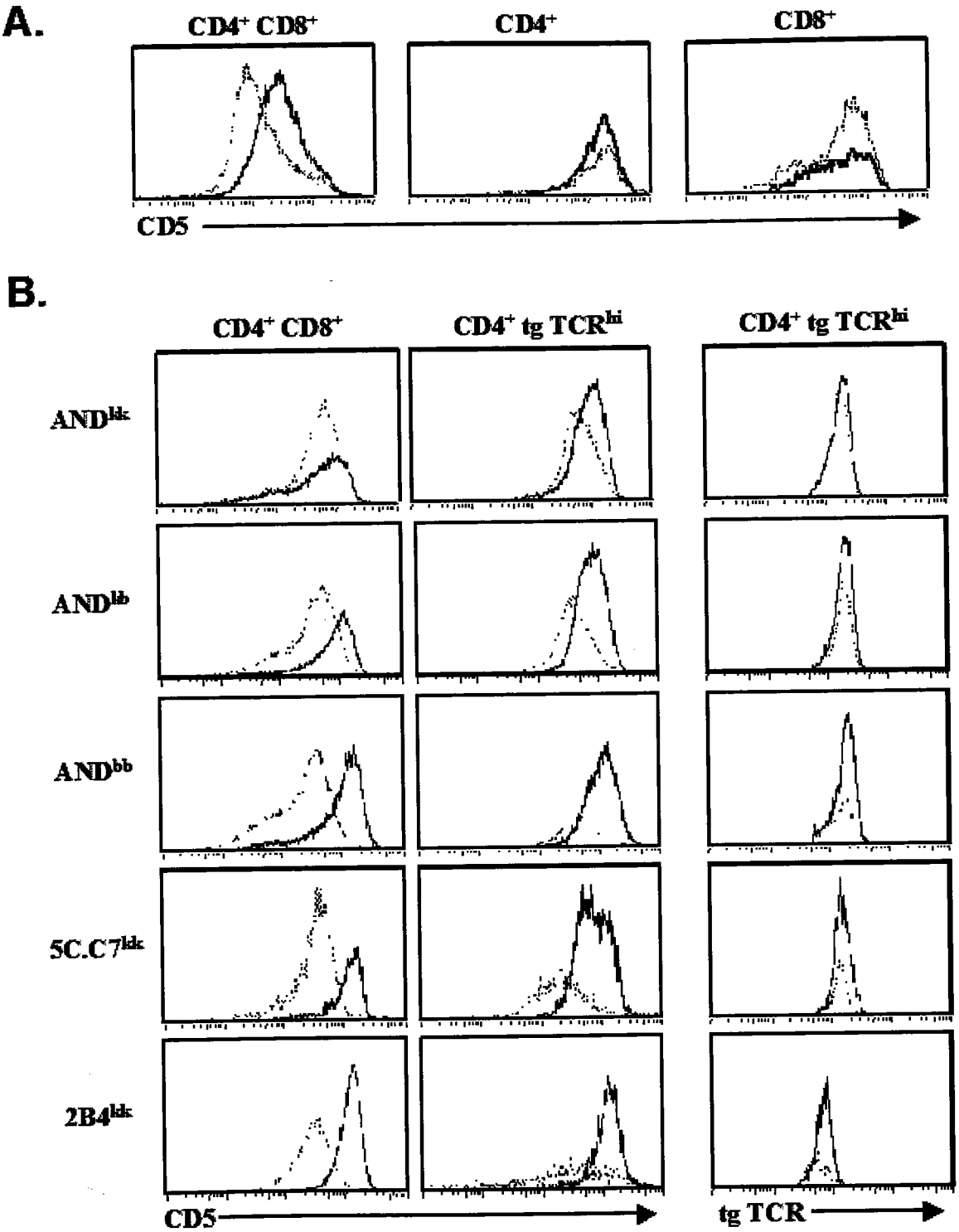
We also examined CD5 levels on DP and $CD4^+$ SP cells from all the TCR transgenic mice to compare T cells that all express the same TCR (Figure 3.8B). Similar to the non-TCR transgenic mice, all the transgenic mice lacking Itk had lower surface levels of CD5 on their DP thymocytes with the exception of $AND^{k/k}$ $itk^{-/-}$ mice. Comparison of $AND^{k/k}$, $AND^{k/b}$, and $AND^{b/b}$ mice shows that as the strength of the selecting background increases, the differences in CD5 surface expression between DPs that express Itk and those that do not is diminished. Interestingly, the CD5 surface levels do not seem to vary significantly among the wild-type AND mice with different selecting backgrounds. Unlike the non-TCR transgenic $itk^{-/-}$ mice, all the TCR transgenic $itk^{-/-}$ mice have lower levels of CD5 on their $CD4^+$ SP thymocytes, further indicating that the

strength of the signal through the same TCR is lower in cells that lack Itk. Staining with the anti-Vα11 Ab confirms that the differences in CD5 expression are not due to lower TCR levels on *itk*$^{-/-}$ thymocytes (Figure 3.8B).

Figure 3.8 The strength of the TCR signal is reduced in Itk-deficient thymocytes

(A) Thymocytes were stained with CD4-PE, CD5-Cy, and CD8-APC. Histograms of CD5 expression on live -DP, -$CD4^+$ SP, and -$CD8^+$ SP cells from $itk^{+/-}$ (solid line) and $itk^{-/-}$ (dotted line) are shown.

(B) Thymocytes from all TCR transgenic mice were stained with transgenic TCR specific Ab conjugated to FITC, anti-CD4-PE and anti-CD5-Cy and CD8-APC. Histograms of CD5 expression on live DP and live $CD4^+$ transgenic TCR^+ cells from $itk^{+/-}$ (solid line) $itk^{-/-}$ (dotted line) TCR transgenic mice are shown. The levels of the transgenic TCR on live $CD4^+$ transgenic TCR^+ cells, as detected by staining with anti-Vα11 for AND and 5C.C7 mice and anti-A2B4-2 for 2B4 mice, are also shown.


A.
CD4+ CD8+
CD4+
CD8+
CD5
B.
CD4+ CD8+
CD4+ tg TCRhi
CD4+ tg TCRhi
ANDkk
ANDkb
ANDbb
5C.C7kk
2B4kk
CD5
tg TCR

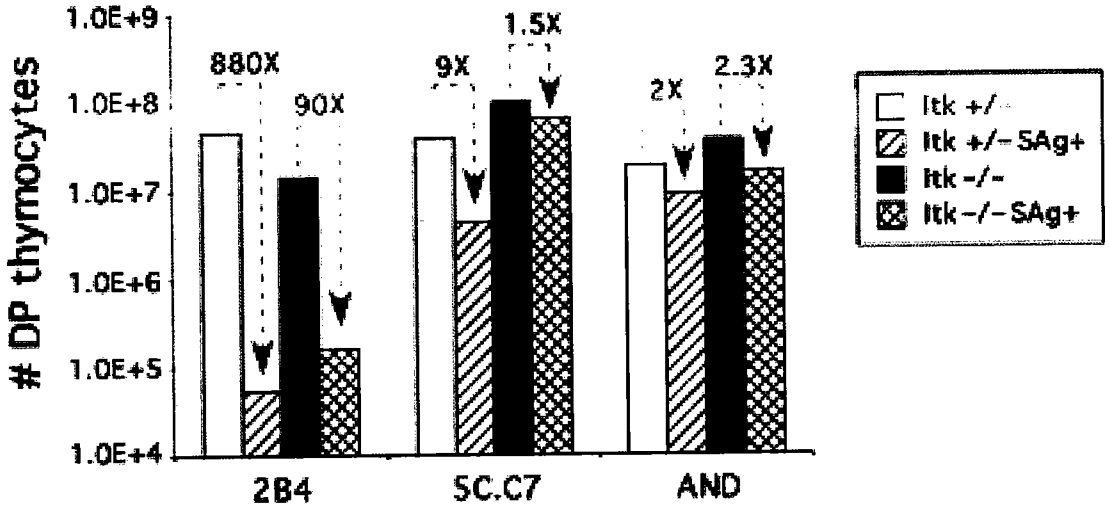
Negative selection of thymocytes with class II restricted TCRs happens later in development, but does not lead to an increase in "self-reactive" cells

We used two systems to look at the role of Itk in the negative selection of MHC class II-specific TCRs. In the first approach we examined mice that coexpress a TCR transgene (2B4, 5C.C7 or AND; all $V\beta3^+$) along with an endogenous superantigen (SAg) that specifically deletes thymocytes whose TCRs use Vβ3. For these studies, we compared the fates of TCR transgenic cells in SAg^+ mice (of 129 origin) in the presence and absence of Itk. Although each of the TCRs in this study makes use of the same Vβ region, the efficiency of SAg-mediated deletion in the different lines varied, even among Itk-sufficient mice (Figure 3.8). For instance, deletion in the 2B4 transgenic line was the most efficient, resulting in a 15-fold reduction in the total thymic cellularity in 2B4 SAg^+ $itk^{+/-}$ mice. In addition, the DP thymocytes were reduced 800-900 times and almost all of the cells that remained in these thymi were at the DN stage of development, suggesting that deletion of the transgenic cells occurs at some point between the transition from the DN to the DP stage or very soon after the cells become DP. In the absence of Itk, the 2B4 SAg^+ mice had a similar reduction in the proportions of DP and SP thymocytes, but because of a smaller decrease in thymic cellularity the total number of DP thymocytes was only reduced 80 times. In contrast, SAg-mediated deletion in the Itk-sufficient 5C.C7 and AND TCR transgenic lines seemed to occur later in development, as these mice still retained a significant proportion of DP thymocytes (Figure 3.8) and had smaller decreases in total thymic cellularity. When Itk was absent from either the 5C.C7 or and SAg^+ mice, the proportion and number of DP thymocytes were increased relative to their

wild-type counterparts; moreover, the DP cells that remained expressed higher levels of the transgenic TCR (data not shown). Interestingly, in AND SAg^+ mice the reduction in the number of DP thymocytes compared to their SAg^- counterparts was similar with or without Itk. A probable explanation for this finding is that in wild-type and mice without SAg, there is some level of deletion of the transgenic cells, presumably due to the high avidity of this TCR for its selecting ligands in the thymus (122), which does not seem to happen in AND *itk*$^{-/-}$ mice (74). Thus, the effect of the SAg on DP thymocyte numbers, in the wild-type AND mice, is masked by the self-deletion that is already occurring. From the analyses of these mice, it appears that the absence of Itk impairs the deletion of self-reactive thymocytes, and when the signal is weak, Itk-deficient thymocytes progress to a later stage of development before succumbing to negative selection signals.

Figure 3.9 Deletion of TCR transgenic thymocytes in SAg^+ Itk-deficient mice occurs later in development

The average number of DP thymocytes in 2B4, 5C.C7 and AND ($H2^k$) $itk^{+/-}$ and $itk^{-/-}$ mice with or without SAg were calculated from total thymocyte numbers and percentages of DP cells determined by flow cytometry. A minimum of three mice for each genotype were analyzed. Numbers above each pair of columns represent fold decrease from SAg^- to SAg^+ mice. Note that the Y-axis is a log scale.


1.0E+9
1.0E+8
1.0E+7
1.0E+6
1.0E+5
1.0E+4
DP thymocytes
880X
90X
9X
1.5X
2X
2.3X
2B4
5C.C7
AND
Itk +/-
Itk +/- SAg+
Itk -/-
Itk -/- SAg+

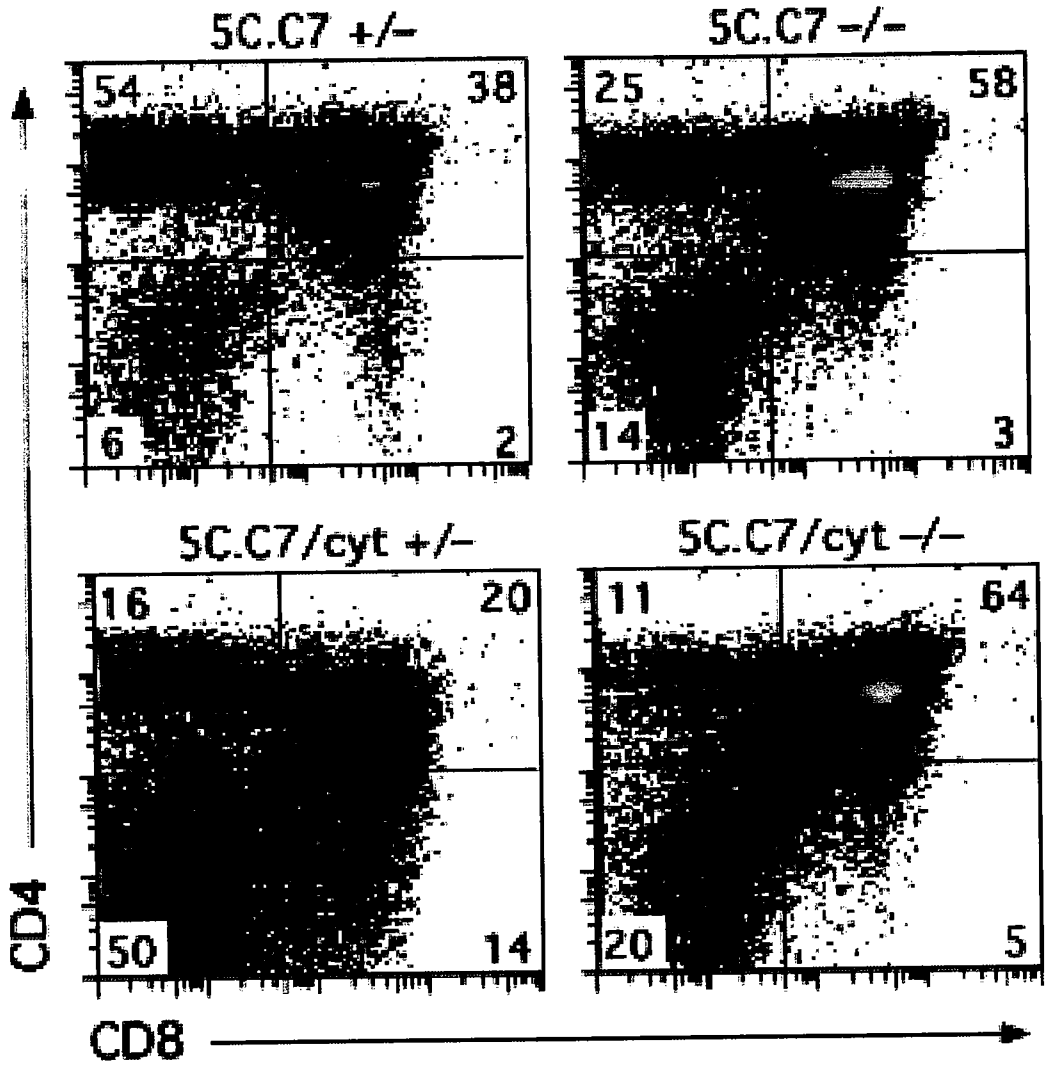
The second approach we used to examine negative selection in *itk*$^{-/-}$ mice made use of a line of transgenic mice that express a fusion protein of hen egg lysozyme and cytochrome c (HEL-cyt) under the control of the metallothionein promoter (127). These mice have low basal levels of expression of the fusion protein in the thymus, which can be increased when the mice are given Zn^{2+} to induce the metallothionein promoter. When the HEL-cyt mice are crossed to the 5C.C7 TCR transgenic line, the majority of 5C.C7^{+} T cells are deleted; however, due to the low basal expression of the antigen, this deletion is somewhat leaky and a small number of 5C.C7^{+} T cells are found in the periphery (128). Increasing the levels of the fusion protein in Zn^{2+}-treated mice enhances the extent of deletion of the 5C.C7 TCR transgenic cells. This system seemed ideal for our studies, as it provided an opportunity to test the notion that signaling through Itk is more critical when signals through the TCR are weak. Thus, we hypothesized that the leaky deletion in 5C.C7/HEL-cyt mice would be even less efficient in the absence of Itk. This prediction was borne out, as we observed increased numbers of DP thymocytes in 5C.C7/HEL-cyt mice lacking Itk when compared to its wild-type counterpart (Figure 3.9). The reduced deletion of DP thymocytes in the absence of Itk could be negated by up-regulation of antigen expression by Zn^{2+} treatment of the 5C.C7/HEL-cyt *itk*$^{-/-}$ mice (data not shown).

Despite the reduced deletion in 5C.C7/HEL-cyt *itk*$^{-/-}$ mice (before Zn^{2+} treatment), we observed fewer 5C.C7^{+} cells in the periphery of these mice compared to those expressing Itk, a finding that may relate to the reduced efficiency of positive selection accompanying the Itk deficiency. Overall, these data are consistent with all of our

findings on thymic selection in *itk*$^{-/-}$ mice, where the absence of Itk leads to a greater impairment of both positive and negative selection in response to weaker forms of stimulation. In contrast, these results conflict with those seen in the H-Y *itk*$^{-/-}$ and H-Y *itk*$^{-/-}$*rlk*$^{-/-}$ male mice, in which the decrease in deletion efficiency led to an increase in the numbers of H-Y^{+} T cells in the periphery (69). A potential explanation for this discrepancy is discussed later.

Figure 3.10 Deletion of 5C.C7 TCR transgenic thymocytes in HEL-cyt^{+} Itk-deficient mice occurs later in development

CD4 vs. CD8 profiles of thymocytes from 5C.C7 *itk*$^{+/-}$ and *itk*$^{-/-}$ mice with and without HEL-cyt transgene. Thymocytes were stained with Abs to CD4 and CD8 and analyzed by flow cytometry.


5C.C7 +/-
54
38
6
2
5C.C7 -/-
25
58
14
3
5C.C7/cyt +/-
16
20
50
14
5C.C7/cyt -/-
11
64
20
5
CD4
CD8

DISCUSSION

The analysis of mice that are deficient in various TCR signaling molecules has provided significant insight into the role of these molecules in T cell development. In this work, we utilize Itk-deficient mice crossed to several TCR transgenic lines to determine how altering the level of TCR signaling affects the processes of positive selection. We crossed *itk*$^{-/-}$ mice to three lines of MHC class II-specific TCR transgenics on three different MHC backgrounds. Previous studies have addressed the role of Tec kinases in repertoire selection using a one MHC class II- and one MHC class I- TCR transgenic system both of which are thought to have low avidities for their selecting ligands in the thymus (39, 69). Here we address the role of Itk in positive selection over a range of TCR avidities. In addition, TCR signaling is merely reduced in Itk-deficient thymocytes and not completely abolished, possibly due to weak compensation by other Tec family kinases, such as Rlk (19). Thus, *itk*$^{-/-}$ mice provide an ideal opportunity to determine how decreased activation of the PLC-γ pathway affects thymocyte selection. Our results, based on analyses of thymocytes, show that the efficiency of positive selection is reduced in all cases in the absence of Itk as we did not observe any TCR/MHC combinations in which positive selection was completely unaffected by the absence of Itk.

These studies extend previous reports of thymic selection in Itk-deficient mice by substantiating the conclusion that reduced $CD4^+$ T cell numbers result from defective positive selection, as opposed to reduced $CD4^+$ cell survival. First, we demonstrated that the survival factor, Bcl-2, is up-regulated to an equivalent degree in DP thymocytes, SP

thymocytes, and peripheral T cells from *itk*$^{-/-}$ mice compared to control mice. In addition, the expression of other surface markers that define the final stage of thymocyte maturation, such as HSA, CD69, CD44, and CD62L, is similar on cells generated in wild-type vs. *itk*$^{-/-}$ TCR transgenic mice. In the course of this analysis, we also observed that wild-type AND TCR transgenic mice expressing the highly selecting ligand, IE^k, had fewer thymocytes with high levels of CD69. Thus, in an environment where thymocytes are receiving strong selection signals, there are fewer cells in the $CD69^{hi}$ transition stage and an increase in the more mature $CD69^-$ $CD4^+$ SP subset. In the Itk-deficient AND mice there is a decrease in the overall number of TCR^{hi} thymocytes; however, among those cells, there is a dramatic increase in the proportion of $CD69^{hi}$ transition cells. This observation suggests that thymocytes receiving weaker signals during positive selection may require longer to complete the selection process. Together, these findings support the conclusion that, in the absence of Itk, fewer thymocytes undergo positive selection, and those that do take longer to become fully mature $CD4^+$ SP cells.

Overall, these data reinforce the view of positive selection as an inherently stochastic process, where only a fraction of thymocytes with appropriate TCRs receive sufficient signals to undergo selection before they succumb to programmed cell death (death by neglect). Positive selection is thought to take 1.5 - 2 days of continuous low level signaling through the TCR (129), and it has been proposed that there are a limiting number of "niches" that are present in the thymus that will support selection (130). This concept has been used to explain why, in TCR transgenic mice in which all the developing thymocytes express an appropriate TCR, the majority of cells do not actually

get selected. Thus, one explanation for the less efficient development of *itk*$^{-/-}$ thymocytes is that these cells take longer to receive sufficient signals, and due to the limiting niches, fewer cells are successfully selected before undergoing cell death. This probabilistic model of positive selection is also consistent with the outcomes we observed in *itk*$^{-/-}$ mice having a range of avidities of TCR-MHC interactions (e.g., AND$^{b/b}$ versus AND$^{k/b}$ or AND$^{k/k}$), where the higher the TCR avidity, the higher the probability that even cells lacking Itk will achieve sufficient signals to promote maturation and survival.

Consistent with the reduced degree of positive selection that has been observed, the ability of DP thymocytes to signal in the absence of Itk or both Itk and Rlk is altered. Biochemical studies verify that at least some aspects of TCR signaling are reduced in the Tec kinase-deficient thymocytes. As PLC-γ1 has been proposed to be a major substrate of Tec family kinases in T cells, Schaefer and colleagues (69) stimulated thymocytes from wild-type and *itk*$^{-/-}$*rlk*$^{-/-}$ mice with anti-CD3 Abs and found that phosphorylation of PLC-γ1 was more severely impaired in the *itk*$^{-/-}$*rlk*$^{-/-}$ thymocytes. This change in PLC-γ1 phosphorylation affects downstream pathways, namely Ca^{2+} mobilization and the Ras/MAPK pathway. For instance, in both *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ thymocytes, defects in sustained Ca^{2+} elevation, but not in the initial Ca^{2+} spike, were observed upon TCR stimulation (69). To test the role of Tec kinase signaling on the Ras/MAPK pathway in thymocyte TCR signaling, changes in the phosphorylation of the MAPKs, ERK and p38 were examined in *itk*$^{-/-}$*rlk*$^{-/-}$ thymocytes. These studies showed that there was no change in p38 activation, but reduced ERK activation was found in these cells (69).

These biochemical studies, along with the *in vivo* studies examining TCR repertoire selection, have led to the hypothesis that thresholds for positive and negative selection are shifted when TCR signaling is reduced in Tec kinase-deficient mice (69). As described earlier, it is thought that signaling through the TCR on DP thymocytes can lead to either positive or negative selection depending on the strength of the signal or the avidity of TCR for its selecting ligands in the thymus. Consequently, cells with high-avidity interactions receive strong signals and are deleted, cells with very low or no interactions do not receive any signal and die, and the cells that receive low to intermediate signals undergo positive selection. Therefore, reduced TCR signals in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ thymocytes would necessitate that the strength of the interaction necessary to achieve positive or negative selection be stronger than that required for wild-type thymocytes. This hypothesis predicts that wild-type thymocytes that would normally be positively selected may die by neglect in Tec family kinase-deficient mice, and furthermore, cells that would normally be deleted would instead be positively selected in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice. Overall, these changes would result in an altered TCR repertoire in Tec family kinase-deficient mice. Although there is no direct evidence, some of the *in vivo* studies suggest that this altered selection may be at least partially the case. The virtual loss of development in Tec family kinase-deficient AND ($H2^b$), 2B4 and H-Y TCR transgenic mice suggests that there is a loss of development of thymocytes with low-avidity TCR. Thus, the threshold for positive selection is clearly shifted.

The case of negative selection is less clear. In H-Y male mice that lack Itk or both Itk and Rlk, the number of $CD8^+$ $H\text{-}Y^+$ cells in the periphery is increased (69). On the

contrary, in both the systems we used to look at negative selection in the absence of Itk the mice had reduced numbers of peripheral $CD4^+$ $5C.C7^+$ cells (data not shown). Thus, in one system, the absence of Tec family kinases results in a shift from negative selection to positive selection, but in the other systems less efficient negative selection did not increase the positive selection of 'autoreactive' cells. One possible explanation for this discrepancy is that lowering PLC-γ1 activity has a greater effect on positive than on negative selection. Thus, changes in the outcome of negative selection when PLC-γ1 activity is lowered may only be apparent when negative selection signals are very weak. In agreement with this idea, the absence of Tec family kinases affects the activity of the MAPKs, ERK1 and ERK2 (69), which have been shown to be crucial for positive selection, while there is no effect on the activity of the p38 MAPK (69), which has been shown to affect negative selection.

An alternative explanation is that the disparity in the two systems is due to differences in the development of MHC class I- and class II-specific thymocytes. For instance, it has been shown that the maturation of $CD4^+$ T cells requires more sustained signaling than the development of $CD8^+$ T cells (38). Furthermore, maintenance of at least some TCR signals is dependent on sustained Ca^{2+} elevation, which is impaired in cells deficient in Tec family kinases due to reduced PLC-γ1 activity. Thus, Tec family kinases and PLCγ-1 activity may have a greater effect on $CD4^+$ T cell selection. In agreement with this idea, the total number of $CD4^+$ cells in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice is reduced by 50%, while there are almost normal numbers of $CD8^+$ cells. Therefore, the

changes in negative selection in the class II systems that we used might be masked due to a more substantial defect in $CD4^+$ T cell maturation or survival.

CHAPTER IV

THE ROLE OF ITK IN CD4/CD8 LINEAGE DIFFERENTIATION

INTRODUCTION

Concurrent with repertoire selection, signaling through the TCR is also thought to influence the commitment of thymocytes to either the CD8 or CD4 lineage, depending on the MHC class I versus class II specificity of their TCR. The events or signals that direct thymocytes into a specific lineage at the DP to SP transition have been more difficult to dissect than those leading to positive or negative selection. Initial studies focused on the CD4 and CD8 coreceptors, including efforts to identify unique signaling molecules/events downstream of each of these coreceptors, with the idea that the signals downstream of the coreceptor MHC interaction somehow "instruct" the cells to differentiate into the right lineage. Others proposed coreceptor down-regulation is "stochastic", and thus half of the cells of each type of MHC restriction would turn off the wrong coreceptor and would not continue to develop, however, if they turned off the right coreceptor than they would be "selected" and complete selection and differentiation. To differentiate between the instructive and selective models of lineage commitment, early work focused on enforced expression of one or the other coreceptor, the transgenic expression of chimeric coreceptor molecules that switched intra- and extra-cellular domains, analysis of various MHC deficient mice, and the "identification" of intermediate populations between the DP and SP transition. Little progress was made from these approaches, since so much of the evidence was contradictory (131).

More recent data indicate that alterations in the activities of certain signaling molecules downstream of the coreceptor/TCR may influence lineage commitment. It has been shown that both the activity of Lck (132, 133) and the Erk/MAPK signaling pathway

(134-136) affects lineage commitment, with high levels of both of these signals leading to CD4 commitment and low levels of these signals leading to CD8 commitment. These studies are consistent with early data indicating that the cytoplasmic tail of CD4 binds more efficiently to Lck that that of CD8 (137-140). Putting these observations together, one popular model of lineage commitment suggests that when thymocytes recognize class II MHC/peptide complexes, engaging both the TCR and CD4, stronger Lck signals would be induced compared with engagement of TCR and CD8 during recognition of MHC class I/peptide complexes. In addition, it has been shown that agonist signals through the TCR induce CD4 development, whereas antagonist signals induce CD8 development (141, 142). Thus, similar to repertoire selection, lineage commitment may also be dependent on the strength of TCR plus coreceptor signaling (Figure 4.1A).

Recently, a model of CD4/CD8 lineage commitment, the "kinetic model" has been proposed based on work from an *in vitro* system of T cell selection and differentiation. In this model, $CD4^+$ lineage commitment requires persistent TCR signals while $CD8^+$ SP development results from shorter TCR signals followed by subsequent gamma chain cytokine "rescue" signals that are required to complete $CD8^+$ SP maturation (38, 143, 144). The need for a $CD8^+$ T cell rescue signal is due to the fact that this model proposes that the initial TCR signals received by a DP thymocyte result in the down-regulation of CD8, regardless of MHC restriction. This will not affect development of class II restricted DP thymocytes because the continued expression of CD4 will allow TCR signals to persist. Conversely, it is proposed that due to the down-regulation of CD8 on class I restricted DP thymocytes, that TCR signaling will be brief (Figure 4.1B).

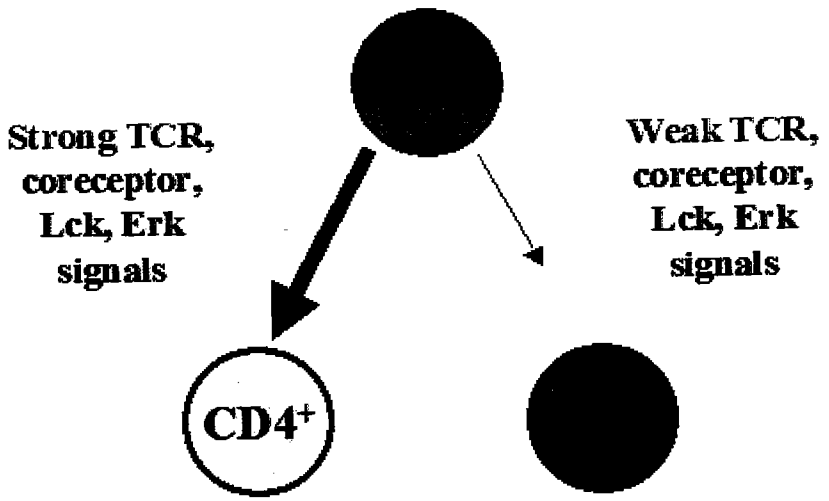
While the quantitative model does not propose any specific intermediate between the DP and SP transition, the major difference in the models is the concept that the strength of signal determines lineage outcome, whereas the kinetic model proposes that signal duration is the factor that determines lineage differentiation. However it is likely that strong signals persist and last longer, while weaker signals may not be sustained and thus would be shorter.

Figure 4.1 Models of lineage commitment and differentiation

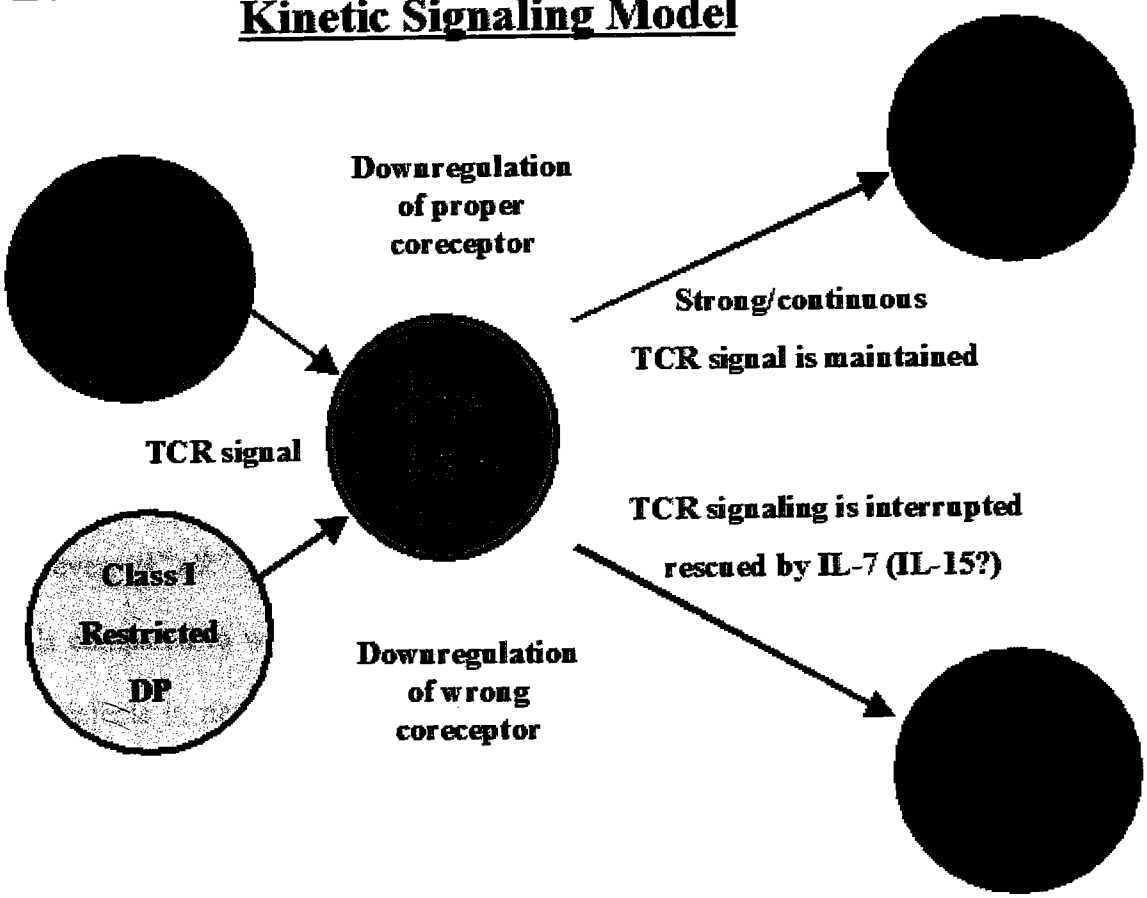
(A) The quantitative model of lineage commitment proposes that the strength of the TCR/coreceptor signal during selection/lineage commitment determines which lineage will develop. Strong signals lead to CD4 SP cell development and weak TCR signals lead to CD8 SP cell development.

(B) The kinetic model of lineage differentiation is somewhat a variation of the quantitative model of lineage commitment. In this model, intial signaling by the TCR on a DP thymocyte, irrespective of MHC restriction, results in the downregulation of the CD8 coreceptor. As a consequence, TCR signaling is interrupted in class I restricted thymocytes and their subsequent differentiation is mediated by gamma-chain cytokines, such as IL-7 and/or IL-15. For class II restricted thymocytes, the loss of CD8 will not affect TCR signaling and thus, the signal will persist and this leads to CD4 lineage differentiation.

A. **Quantitative Signaling Model**



B. **Kinetic Signaling Model**



Regardless of which model, if either, most clearly represents the signals leading to T lineage differentiation, it is clear that signaling proteins both upstream and downstream of Itk, such as Lck and Erk1/2, respectively, are important in the CD4/CD8 lineage decision (145). These data, along with the observation that *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice have altered CD4:CD8 ratios in the thymus and periphery (39, 66, 67, 69), raised the question of whether or not Tec-kinases would be involved in this developmental process. Therefore, we initially wanted to determine if CD4/CD8 lineage commitment was altered in any of the five class II TCR transgenic models used in Chapter 3. Since signals should be weaker in the absence of Itk, we thought we may see some switching to the CD8 lineage due to reduced TCR signaling.

Whether or not lineage commitment, defined as the downregulation of the proper coreceptor based on MHC restriction, is altered in the absence of Itk, there are aspects of SP thymocyte differentiation that are different in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice. For instance, *itk*$^{-/-}$ mice have roughly one-half the normal numbers of CD4^{+} T cells (39). In light of recent evidence that CD4^{+} T cell differentiation is not only dependent on the strength of the TCR signal, but also on the consistency and duration of that signal (38), the disruption of this process in the absence of Itk seems reasonable. Unexpectedly, however, *itk*$^{-/-}$*rlk*$^{-/-}$ mice have no reductions in the percentage and or numbers of CD4^{+} T cells compared to wild type mice (67, 69). This latter observation may reflect the fact that defects in negative selection balance out the defects in CD4^{+} T cell selection and differentiation in *itk*$^{-/-}$*rlk*$^{-/-}$ mice, resulting in an overall similar frequency of selectable TCRs in these mice compared to wild type controls. In contrast, *itk*$^{-/-}$ mice have near

normal numbers of $CD8^+$ peripheral T cells, together with an increase in the percentage and total numbers of CD8 SP cells in the thymus. Even more strikingly, *itk*$^{-/-}$*rlk*$^{-/-}$ mice have increased numbers of $CD8^+$ thymocytes and peripheral T cells (146). These findings indicate that Itk and/or Rlk play as yet an undefined role in $CD8^+$ T cell differentiation and/or homeostasis. Therefore we decided to investigate the phenotype and origin of the $CD8^+$ cells in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice, as well as, potential mechanisms for the altered differentiation/homeostasis.

MATERIALS AND METHODS

Mice

Itk$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice are the same as those described in Chapter 1. Wild-type littermates and non-littermate B10 mice were used as controls. *Itk*$^{+/-}$ and *itk*$^{-/-}$ 2B4, 5C.C7, and AND TCR transgenic mice are the same described in Chapter 3. *Itk*$^{-/-}$ mice were crossed to IL-15$^{-/-}$ (B6) mice obtained from Dr. Joonsoo Kang with permission from The Immunex Corporation. OT-1 TCR (B6) transgenic mice were obtained from Dr. Kenneth Rock with permission from Dr. Frank Carbone. CD45.1 (B6) congenic mice were purchased from Charles River Laboratories at NCI-Fredrick Animal Production Area (Fredrick, Maryland). All mice used were between 6 - 12 weeks of age and were maintained at the University of Massachusetts Medical School animal facility under specific pathogen free (SPF) conditions.

Antibodies and flow cytometry

Thymocytes and peripheral lymphocytes were obtained and stained as described previously (74). The following Abs and secondary reagents were purchased from BD Pharmingen (San Diego, CA): CD8α-FITC, CD4-PE, CD44-Cy-Chrome™, CD62L-PE, TCRβ-FITC, CD122-PE, HSA-biotin, CD3-biotin, TCRβ-FITC CD8-Cy-Chrome™, CD8-allophycocyanin, IFNγ-PE, Vα2-PE, Vβ5-FITC, strepavidin-allophycocyanin. Anti-Bcl-xL-PE was purchased from Southern Biotechnology Associates (Birmingham, AL). Anti-BrdU-FITC was purchased from BD Biosciences (Mountain View, CA). Ab staining was analyzed using a FACSCalibur (BD Biosciences) and data analyzed using

both Cell Quest (BD Pharmingen, San Jose, CA) and Flowjo software (Treestar, Ashland, OR).

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

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

BrdU incorporation

Mice were injected i.p. with 2 mg of BrdU (Sigma Aldrich, St. Louis, MO) in PBS 12 h before harvest. Cells were plated at 6 x 10^6/well and then stained for surface antigens. Following extracellular staining the cells were washed in PBS and then fixed and permeabilized with Cytofix/Cytoperm for 20 min at 4° C. The cells were then washed with PBS and fixed again in 1%/0.1% paraformaldehyde/Tween-20. To stain for BrdU, cells were spun down and washed twice in PBS at room temperature, and subsequently resuspended in 200λ of DNase solution [750λ 5M NaCl, 105λ 1M $MgCl_2$, 250λ1mM HCl, 24mg DNase (Roche Applied Sciences, Indianapolis, IN), 23.9 ml dH_2O] and incubated at RT for 10 – 30 min. The cells were then washed and incubated in 50λ of a 1:10 dilution of anti-BrdU-FITC for 30 min. At the end of the incubation period, the cells were washed twice and analyzed by flow cytometry.

Adoptive Transfer of $CD8^+$ thymocytes

Single cell suspensions were generated from thymi isolated from wild-type and $itk^{-/-}$ mice. $CD4^+$ and DP thymocytes were depleted by incubating with a complement-fixing anti-CD4 Ab followed by incubation with rabbit complement (Cedarlane). Live cells were isolated by Lympholyte®-M (Cedarlane) gradient, stained for CD8 and CD44 expression and sorted into $CD8^+CD44^{lo}$ and $CD8^+CD44^{hi}$ subsets. These sorted populations were then adoptively transferred (2 x 10^6 wild-type and $itk^{-/-}$ $CD8^+$ $CD44^{lo}$ thymocytes and 4 x 10^6 $CD8^+$ $itk^{-/-}$ $CD44^{hi}$ thymocytes) into separate wild-type CD45.1 congenic mice. The presence of transferred CD45.2 donor cells in spleen, lymph node, and thymus of host mice was examined from day 1.5 post-transfer to approximately 16 days post-transfer.

FTOC

Timed pregnancies were set up by mating $itk^{+/-}$ mice to $itk^{-/-}$ mice. The day of plugging was counted as embryonic day 0. Fetal embryos were typed by PCR analysis on fetal liver DNA. Thymi isolated from day 15 – day 17 fetal mice were cultured in RPMI-10 on round nitrocellulose membranes (Millipore Corp. Bedford, MA) placed on inserts made of stainless steel mesh (Small Parts Inc. Miami, FL). Thymi were cultured for 7 – 14 days at 37°C, and then analyzed by flow cytometry for CD4, CD8, and CD44 expression.

Bone marrow chimeras

(see Chapter 2 materials and methods)

RESULTS

Lineage commitment is not affected by reduced TCR signaling in the absence of Itk

In light of increasing evidence that the strength of the TCR signal influences CD4/CD8 lineage commitment, and the suggestion that Tec family kinases are important for regulating TCR signaling thresholds (69), we were interested in determining whether lineage commitment would be affected in *itk*$^{-/-}$ mice. This model of lineage commitment proposes that strong signals downstream of the TCR induce DPs to differentiate into CD4^{+} T cells, while weak signals downstream of the TCR cause DPs to differentiate into CD8^{+} T cells (37). The TCR transgenic *itk*$^{-/-}$ mice that we generated to examine the role of Itk in positive selection (Chapter 3) also provided us with an ideal system to examine how lineage commitment would be affected in the absence of Itk, as these mice represent a range of TCR avidities for selecting ligands. Specifically, we anticipated that lineage switching might occur in some of the TCR transgenics, particularly those with low selection efficiencies. In such an instance, the loss of transgenic CD4^{+} SP cells would be compensated for by an increase in transgenic CD8^{+} SP cells. This expectation would be consistent with the increased percentage of CD8^{+} SP cells seen in non-TCR transgenic *itk*$^{-/-}$ mice.

As shown in Figures 3.1 and 3.2 there were a small number of CD8^{+} SP cells in the thymus and LN of all TCR transgenic mice. The percentage and absolute number of these cells that express high levels of the transgenic TCR are shown in Table 4.1. In virtually all cases there was no significant increase in the absolute number of transgenic TCR^{hi} CD8^{+} SPs in the absence of Itk as determined by Student's *t* test ($p < 0.05$). The

only exception was in the LN of $AND^{b/b}$ mice, in which the number of $CD8^+$ cells was slightly increased. Therefore, the data from the TCR transgenic mice suggest that although the selection of $CD4^+$ T cells is reduced, lineage commitment occurs normally.

Table 4.1 CD4/CD8 lineage commitment is not altered in the absence of Itk

The percentage and absolute numbers of $CD8^+$ T cell subsets in the thymus and lymph nodes are given. The values are the mean of 3 – 7 animals for each genotype with SEMs. Absolute numbers are given in millions. Similar analyses were performed on splenocytes with comparable results (data not shown).

		thymus			lymph nodes		
	n	total #	% CD8 SP	# CD8 SP	total #	% CD8 tg TCR^{hi}	# CD8 tg TCR^{hi}
2B4$^{k/k}$ +/-	4	66 ± 18	2.3 ± 0.5	1.6 ± 0.5	14 ± 4.6	0.9 ± 0.2	0.1 ± 0.1
2B4$^{k/k}$ -/-	8	24 ± 5.8	3.0 ± 0.7	0.9 ± 0.5	18 ± 2.9	2.0 ± 0.7	0.3 ± 0.1
5C.C7$^{k/k}$ +/-	10	136 ± 19	1.9 ± 0.2	2.3 ± 0.2	29 ± 5.1	0.6 ± 0.2	0.2 ± 0.0
5C.C7$^{k/k}$ -/-	9	155 ± 34	2.1 ± 0.3	3.3 ± 0.8	12 ± 1.7	1.1 ± 0.2	0.1 ± 0.0
AND$^{k/k}$ +/-	7	65 ± 9.7	3.3 ± 0.7	1.9 ± 0.3	23 ± 5.7	1.0 ± 0.2	0.3 ± 0.1
AND$^{k/k}$ -/-	3	89 ± 13	5.8 ± 1.4	5.6 ± 1.4	24 ± 13	0.9 ± 0.2	0.1 ± 0.1
AND$^{k/b}$ +/-	5	94 ± 48	2.1 ± 0.3	1.8 ± 0.7	38 ± 0.7	1.6 ± 0.4	0.5 ± 0.1
AND$^{k/b}$ -/-	4	45 ± 16	5.5 ± 1.3	2.4 ± 0.9	15 ± 2.4	3.1 ± 1.3	0.4 ± 0.1
AND$^{b/b}$ +/-	4	89 ± 16	3.0 ± 1.5	2.1 ± 0.8	11 ± 2.4	1.4 ± 0.3	0.2 ± 0.1
AND$^{b/b}$ -/-	6	38 ± 14	3.3 ± 0.4	1.3 ± 0.6	18 ± 2.5	3.1 ± 0.5	0.6 ± 0.2

The CD8$^+$ SP thymocytes in itk$^{-/-}$ mice have a previously activated phenotype

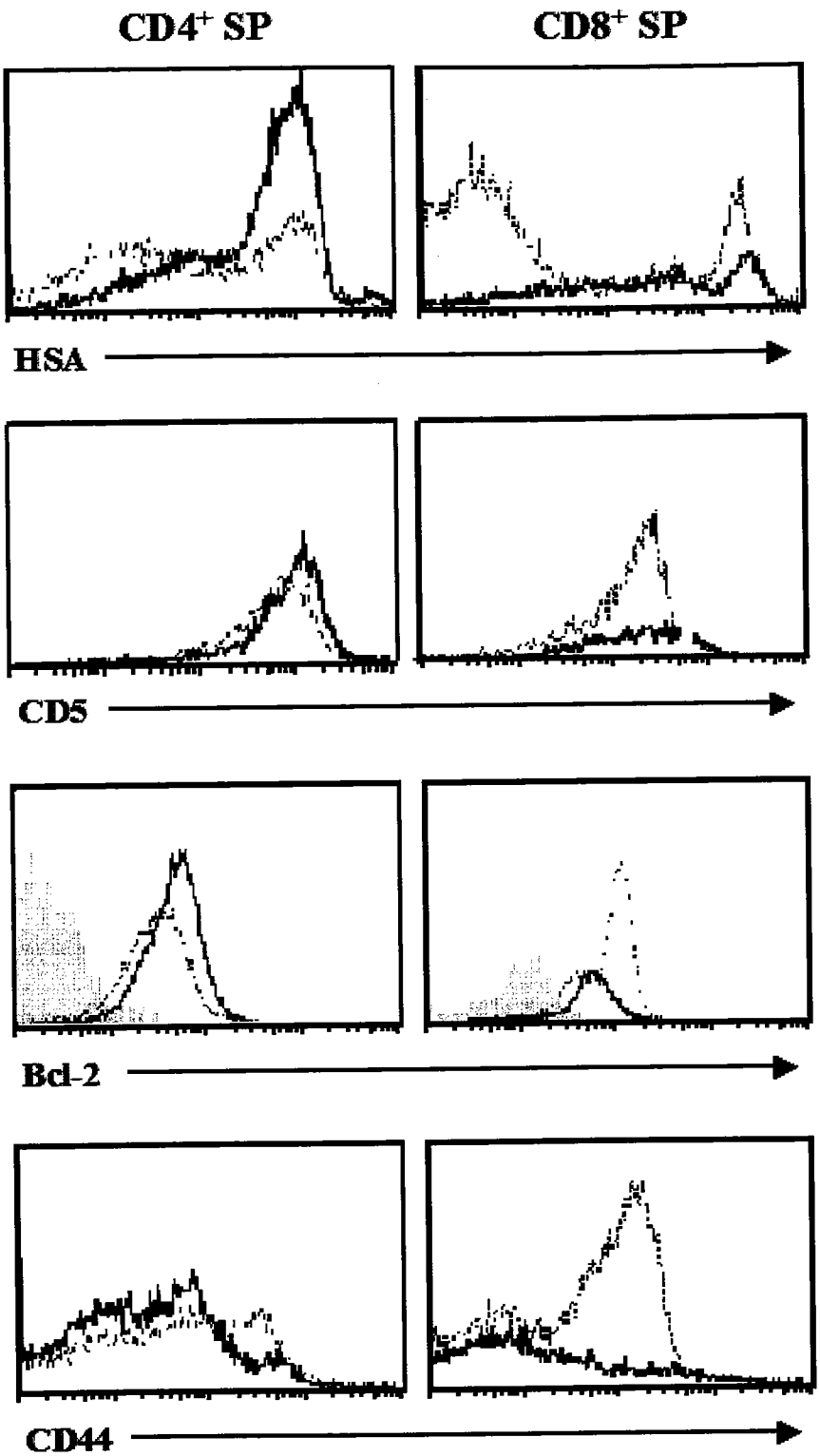
If this is indeed the case, the issue of why non-TCR transgenic *itk*$^{-/-}$ mice have such a large number of CD8$^+$ SP cells in the thymus and an altered CD4:CD8 ratio in the periphery remained unresolved. To address this question we analyzed the expression of multiple thymocyte and T cell markers on the CD8$^+$ SP cells in *itk*$^{-/-}$ mice (Figure 4.2). The phenotype that we observe is that the majority of the CD8$^+$ cells in the thymus of *itk*$^{-/-}$ mice appeared to be mature CD8$^+$ T cells rather than newly developed CD8$^+$ SP cells. For instance, the vast majority of CD8$^+$ SP thymocytes in *itk*$^{-/-}$ mice are HSA^{lo}, and thus resemble peripheral T cells rather than maturing thymocytes. Likewise, the analysis of Bcl-2 and CD5 expression on the CD8$^+$ SPs of *itk*$^{-/-}$ mice demonstrates that the majority of these cells have high levels of both of these proteins, similar to the levels found on the most mature thymocytes or peripheral T cells.

Although the analysis of these markers suggests that a significant number of CD8$^+$ SPs in the thymus of *itk*$^{-/-}$ mice have a mature phenotype, we are unable to determine whether these cells are peripheral cells that have returned to the thymus or cells that have fully matured, but are not exported from the thymus normally. It has been shown that activated peripheral T cells can re-enter the thymus (147), so we decided to analyze the expression of activation markers on these CD8$^+$ SPs. The analysis of CD69, which is expressed on activated T cells, revealed that there was no difference in the proportion of CD8$^+$ SPs that were CD69$^+$ between wild-type and *itk*$^{-/-}$ mice (data not shown). We also examined the activation/memory marker CD44. The expression of CD44 is up-regulated on cells as they become activated, but, unlike CD69, it remains high, and thus is often

used as a marker for memory T cells (148). As shown in Figure 4.2, the majority of $CD8^+$ SP thymocytes in *itk*$^{-/-}$ mice are $CD44^{hi}$, while in wild-type mice the majority of these cells are $CD44^{lo}$. Based on these results it appears that the $CD8^+$ SP thymocytes in *itk*$^{-/-}$ mice are not activated, but may have been previously. These data leave open the possibility that activated $CD8^+$ cells recirculate from the periphery to the thymus in *itk*$^{-/-}$ mice, but in a steady-state analysis show up largely as previously activated cells. Alternatively, it is possible that the $CD8^+$ SP cells acquire this phenotype in the thymus due to irregular development.

Figure 4.2 The majority of $CD8^+$ SP thymocytes in Itk-deficient mice have a mature phenotype

Thymocytes were stained with Abs to CD4 and CD8 along with either an Ab to CD5, Bcl-2, HSA, or CD44. The expression level of each of these thymocyte markers on live $CD4^+$ and $CD8^+$ SP thymocytes from 8-wk-old non-transgenic $itk^{+/-}$ (solid line) and $itk^{-/-}$ (dotted line) mice are shown. For Bcl-2 staining, nonspecific staining with the irrelevant hamster Ab is also shown (filled histograms).


CD4+ SP
CD8+ SP
HSA
CD5
Bcl-2
CD44

CD8$^+$ T Cells in both the thymus and periphery of itk$^{-/-}$ and itk$^{-/-}$rlk$^{-/-}$ mice have a previously activated phenotype and are functionally mature

As shown in Chapter 3, mice lacking Itk show an obvious reduction in the numbers of thymic and peripheral CD4$^+$ T cells. Previous studies have suggested that this loss of CD4$^+$ T cells is due to a defect in positive selection (74, 149), and further, that this defect is exacerbated in mice lacking both Itk and Rlk (7). In contrast to the deficit in CD4$^+$ T cell numbers, the proportions and total numbers of CD8$^+$ SP thymocytes and peripheral T cells in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice are not reduced compared to that of wild-type mice (Figure 4.3). Specifically, the fractions of CD8$^+$ T cells in the peripheries of *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice are comparable to those seen in wild-type mice, and in the thymus, *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ have significantly increased percentages and total numbers of CD8$^+$ SP cells (Figure 4.3 and (150); % CD8$^+$ SP thymocytes: wild-type, 3.5 ± 0.3, *itk*$^{-/-}$, 11.3 ± 0.6, $p<0.0001$; absolute numbers CD8$^+$ SP thymocytes: wild-type, 5.6 ± 0.8x10^6, *itk*$^{-/-}$ 14.0 ± 0.2x 10^6, $p<0.0019$).

In addition to the increased numbers of CD8$^+$ thymocytes in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice, analysis of activation marker profiles reveals that the majority of these cells have a mature and previously-activated phenotype (Figure 4.2A and (74)). These thymocytes are CD44^{hi} and HSA^{lo}, but are also CD69^{lo} and CD25^{lo} (Figure 4.2A and data not shown), indicating their similarity to previously activated/memory cells that usually reside in the periphery. Like the CD8$^+$ SP thymocytes, approximately 85% of the CD8$^+$ T cells in the periphery of *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice are also CD44^{hi} and have increased expression of other memory markers such as CD122, while still expressing high levels of CD62L

(Figure 4.3B). However, these peripheral $CD44^{hi}$ $CD8^{+}$ T cells do not express other markers of acute activation, such as CD25 or CD69 (data not shown).

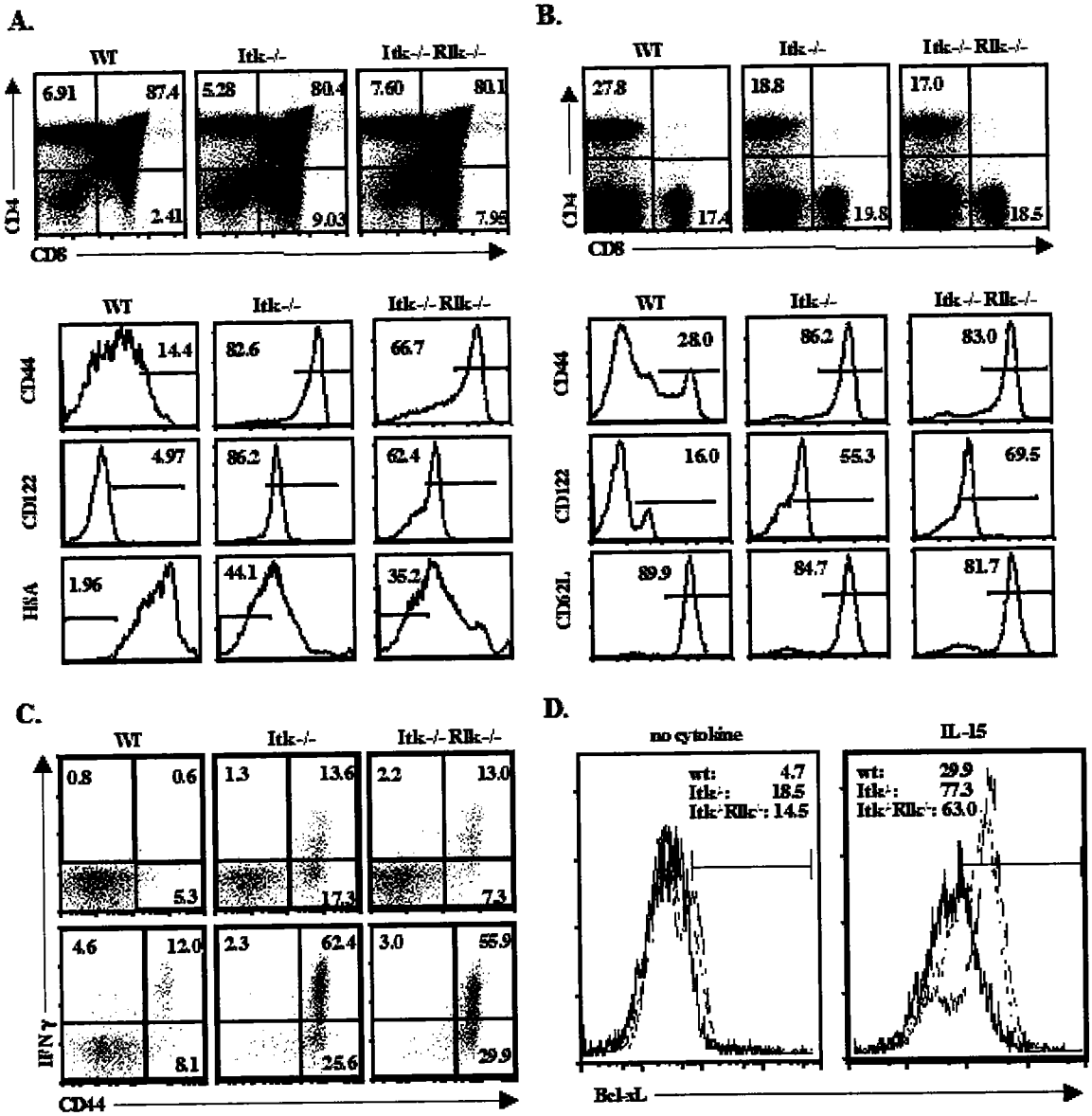
Previously-activated peripheral $CD8^{+}$ $CD44^{hi}$ T cells are characterized by their ability to secrete effector cytokines immediately *ex vivo* in response to stimulation. To determine whether the $CD8^{+}$ $CD44^{hi}$ SP cells in the thymi of $itk^{-/-}$ and $itk^{-/-}rlk^{-/-}$ mice are functionally, as well as phenotypically, similar to previously-activated peripheral cells, we examined their ability to secrete the effector cytokine, IFNγ. In response to *ex vivo* stimulation with PMA and ionomycin, a large proportion of $itk^{-/-}$ and $itk^{-/-}rlk^{-/-}$ $CD8^{+}$ $CD44^{hi}$ SP thymocytes produce the effector cytokine, IFNγ (Figure 4.3C). As these cells are absent from wild-type thymi, there was no corresponding effector cytokine production by wild-type $CD8^{+}$ SP thymocytes. Another functional characteristic of $CD8^{+}$ memory T cells is their ability to up-regulate Bcl-xL in response to IL-15 (151, 152). Therefore, we incubated thymocytes from wild-type and $itk^{-/-}$ mice in media containing IL-15 for 36 h. As shown in Figure 4.3D, the CD8 SP thymocytes from $itk^{-/-}$ and $itk^{-/-}rlk^{-/-}$ mice exhibit a dramatic up-regulation of Bcl-xL in response to IL-15. Therefore, both of these assays indicate that the $CD8^{+}$ $CD44^{hi}$ cells in the thymus of $itk^{-/-}$ and $itk^{-/-}rlk^{-/-}$ mice are functionally as well as phenotypically similar to previously-activated/memory peripheral $CD8^{+}$ T cells.

Figure 4.3 Both thymic and peripheral CD8$^+$ T cells in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice resemble previously-activated T cells

(A, B) Dot-plots show CD4 vs. CD8 profiles of wild-type, *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ (A) thymocytes or (B) lymph node cells. Numbers in each quadrant indicate percentages of each subpopulation. The histograms below show (A) CD44, CD122 and HSA expression on gated CD8$^+$ TCR^{hi} SP thymocytes or (B) CD44, CD122 and CD62L expression on gated CD8$^+$ TCR^{hi} lymph node cells. Numbers indicate percentage of CD44^{hi}, CD122^{hi}, HSA^{lo}, or CD62L^{hi} cells, respectively.

(C) Thymocytes (top panel) and splenocytes (lower panel) from wild-type, *itk*$^{-/-}$ and *itk*$^{-/-}$ *rlk*$^{-/-}$ mice were stimulated with PMA and ionomycin for 5 h and IFNγ production was assessed by intracellular staining. Dot-plots show IFNγ vs. CD44 staining on gated CD8$^+$ cells. Numbers in each quadrant indicate percentages of each subpopulation.

(D) Thymocytes from wild-type, *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice were stimulated with IL-15 for 48 h and the levels of Bcl-xL were determined by intracellular staining. Histograms show Bcl-xL expression on gated CD8$^+$ thymocytes. Wild-type sample is depicted by the thick black line, *itk*$^{-/-}$ by the thin red line and *itk*$^{-/-}$*rlk*$^{-/-}$ by the dashed blue line. Data are representative of two independent experiments.


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

Itk deficient CD44^{hi} CD8^{+} T Cells arenot actively proliferating and do not home to the thymus

CD44 expression on peripheral CD8^{+} T cells is up-regulated following a response to a foreign pathogen, or alternatively, after cells have undergone lymphopenia-induced proliferation (153, 154). To determine whether *itk*$^{-/-}$ CD8^{+} SP thymocytes and peripheral T cells were actively proliferating in response to lymphopenia-induced or infection-mediated stimuli, we analyzed BrdU incorporation of CD8^{+} CD44^{hi} cells in the thymus and spleen of *itk*$^{-/-}$ mice. Mice were injected with BrdU and analyzed 12 h later. As the majority of thymocyte proliferation occurs following pre-TCR stimulation, BrdU is primarily incorporated by TN thymocytes and cells in transition to the DP stage; following this transition, the majority of DP thymocytes are not in cycle (110). It has also been reported that a small proportion of SP thymocytes proliferate following positive selection (155). Similarly, in the periphery of unimmunized non-lymphopenic mice, there is normally very little proliferation of naïve T cells.

Following BrdU injection, a clear population of BrdU^{+} DP thymocytes is apparent in both wild-type and *itk*$^{-/-}$ mice, demonstrating that comparable levels of BrdU were present in both mice (Figure 4.4A). When CD8^{+} SP thymocytes were examined, we detected little proliferation in the mature HSA^{lo} CD8^{+} SP fraction, and interestingly, the fraction of BrdU^{+} HSA^{lo} CD8^{+} SP cells inthe *itk*$^{-/-}$ thymus was even smaller than in the wild-type thymus. Analysis of the peripheral splenic CD8^{+} T cells also revealed no increased proliferation of the *itk*$^{-/-}$ cells compared to the wild-type controls (Figure 4.4A).

These data indicate that *itk*$^{-/-}$ CD8^{+} thymocytes and peripheral T cells are not accumulating due to increased proliferation either in the thymus or the spleen.

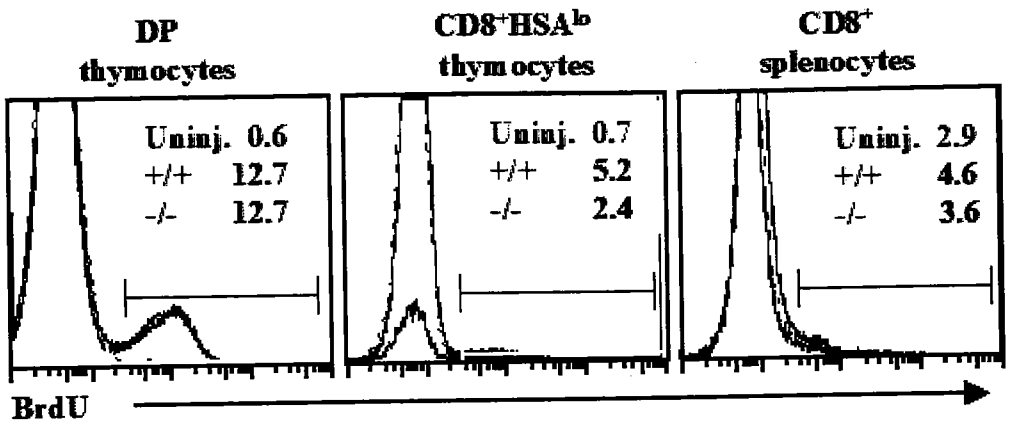
Although CD44^{hi} cells do not typically develop in the thymus, they can gain access to the thymus as a result of recirculation from the periphery (156, 157). To examine the possibility that peripheral *itk*$^{-/-}$ and CD8^{+} CD44^{hi} cells were preferentially homing to thymus from the periphery, we assessed the trafficking patterns of *itk*$^{-/-}$ cells following adoptive transfer into wild-type congenic recipients. For these experiments, purified *itk*$^{-/-}$ CD8^{+} thymocytes (CD45.2^{+}) were injected into CD45.1^{+} congenic wild-type mice, and the ability of these transferred cells to migrate back to the thymus of the recipients was assessed. As shown in Figure 4.4B, even though the transferred *itk*$^{-/-}$ CD8^{+} CD44^{hi} cells were easily detectable in the spleen and lymph nodes of the recipient mice, no transferred cells could be found in the thymus of the host mice. This was also the case for CD8^{+} CD44^{lo} thymocytes purified from either *itk*$^{-/-}$ or wild-type mice. These data indicate that the accumulation of CD8^{+} CD44^{hi} cells in the thymi of *itk*$^{-/-}$ mice is not due to preferential migration of peripheral *itk*$^{-/-}$ CD8^{+} CD44^{hi} cells to the thymus.

Figure 4.4 *Itk*$^{-/-}$ CD8^{+} cells are not actively proliferating and do not preferentially migrate to the thymus.

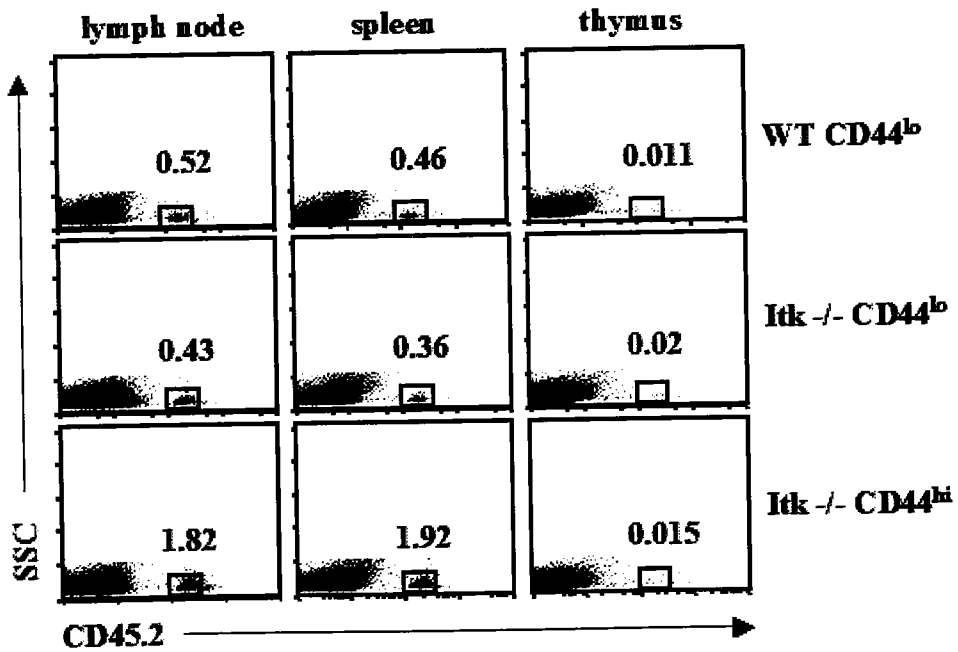
(A) Mice were injected with BrdU and incorporation was assessed 12 h later to determine the percentage of cells actively proliferating in wild-type (solid blue line) and *itk*$^{-/-}$ (solid pink line) mice. The dashed line shows cells from uninjected mice stained with the anti-BrdU Ab.

(B) Wild-type CD8^{+} CD44^{lo}, *itk*$^{-/-}$ CD8^{+} CD44^{lo}, and *itk*$^{-/-}$ CD8^{+} CD44^{hi} cells (all CD45.2^{+}) were adoptively transferred into CD45.1^{+} wild-type congenic mice and the presence of the transferred cells in various organs was analyzed 7 days later.

A.


DP thymocytes
CD8+HSAlo thymocytes
CD8+ splenocytes
Uninj. 0.6
+/+ 12.7
-/- 12.7
Uninj. 0.7
+/+ 5.2
-/- 2.4
Uninj. 2.9
+/+ 4.6
-/- 3.6
BrdU

B.


lymph node
spleen
thymus
0.52
0.46
0.011
WT CD44lo
0.43
0.36
0.02
Itk -/- CD44lo
1.82
1.92
0.015
Itk -/- CD44hi
SSC
CD45.2

The CD8$^+$ CD44^{hi} cells of itk$^{-/-}$ and itk$^{-/-}$rlk$^{-/-}$ mice are not present at birth and do not develop in fetal thymic organ culture

As shown above, the CD8$^+$ CD44$^+$ cells in *itk*$^{-/-}$ mice are not proliferating or able to recirculate to the thymus. These findings suggest that these cells arise first in the thymus and are maintained following immigration to the periphery. To examine this possibility more closely, we assessed CD44 expression on thymocytes from neonatal mice immediately after birth and additionally, looked at the ability of CD8$^+$ CD44^{hi} thymocytes to develop in fetal thymic organ culture. As shown in Figure 4.5A, CD8$^+$ CD44^{hi} thymocytes are not present immediately after birth in *itk*$^{-/-}$ mice. Similarly, CD8$^+$ CD44^{hi} thymocytes did not develop from fetal day 16 *itk*$^{-/-}$ thymi following seven days of fetal thymic organ culture (Figure 4.5A). Extension of the culture period for an additional seven days resulted in the generation of *itk*$^{-/-}$ CD8$^+$ CD44^{hi} cells, but CD8$^+$ CD44^{hi} cells also developed in the wild-type cultures at this time point, and we detected no appreciable differences in this population between wild-type and *itk*$^{-/-}$ thymic organ cultures (data not shown).

CD8$^+$ CD44^{hi} cells are first detectable in day 4 post-natal thymi and spleens of itk$^{-/-}$ mice.

Although no CD8$^+$ CD44^{hi} cells were observed in newborn *itk*$^{-/-}$ thymi or in fetal *itk*$^{-/-}$ thymi cultured for seven days, in both cases very few fully mature CD8$^+$ SP TCR^{hi} thymocytes are present at these time points, even in wild-type thymi. Therefore, to examine the timing of CD8$^+$ SP maturation, the population of the peripheral CD8$^+$ T cell compartment, and the emergence of the CD8$^+$ CD44^{hi} cell phenotype in *itk*$^{-/-}$ mice, we performed a longitudinal study of CD8$^+$ SP thymocyte and peripheral T cell development over the first seven weeks after birth of wild-type and *itk*$^{-/-}$ mice.

This analysis revealed that CD8$^+$ SP thymocytes accumulate, both in percentage and absolute numbers, in the thymus of *itk*$^{-/-}$ mice as compared to wild-type mice (Figure 4.5B). This accumulation was visible from the age of two weeks onward, and was accompanied by a relative deficit in the numbers of peripheral CD8$^+$ T cells in *itk*$^{-/-}$ mice. These data indicate that, following the first wave of CD8$^+$ T cell development (at approximately three weeks after birth), fewer *itk*$^{-/-}$ CD8$^+$ SP thymocytes either migrated to, or survived in, the periphery of *itk*$^{-/-}$ mice. Our analysis also showed that *itk*$^{-/-}$ CD8$^+$ thymocytes expressed higher levels of CD44 by four days after birth as compared to wild-type CD8$^+$ SP thymocytes, and that this increase was exacerbated over time (Figure 4.5C).

In contrast to the accumulation of CD8$^+$ CD44^{hi} cells in the thymi of the *itk*$^{-/-}$ mice as compared to wild-type mice, the CD8$^+$ cells emerging into the periphery of both wild-type and *itk*$^{-/-}$ mice at day 4 have distinctly similar CD44^{hi} profiles (Figure 4.5C). This is likely due to the fact that the first migrants into the periphery of young mice undergo

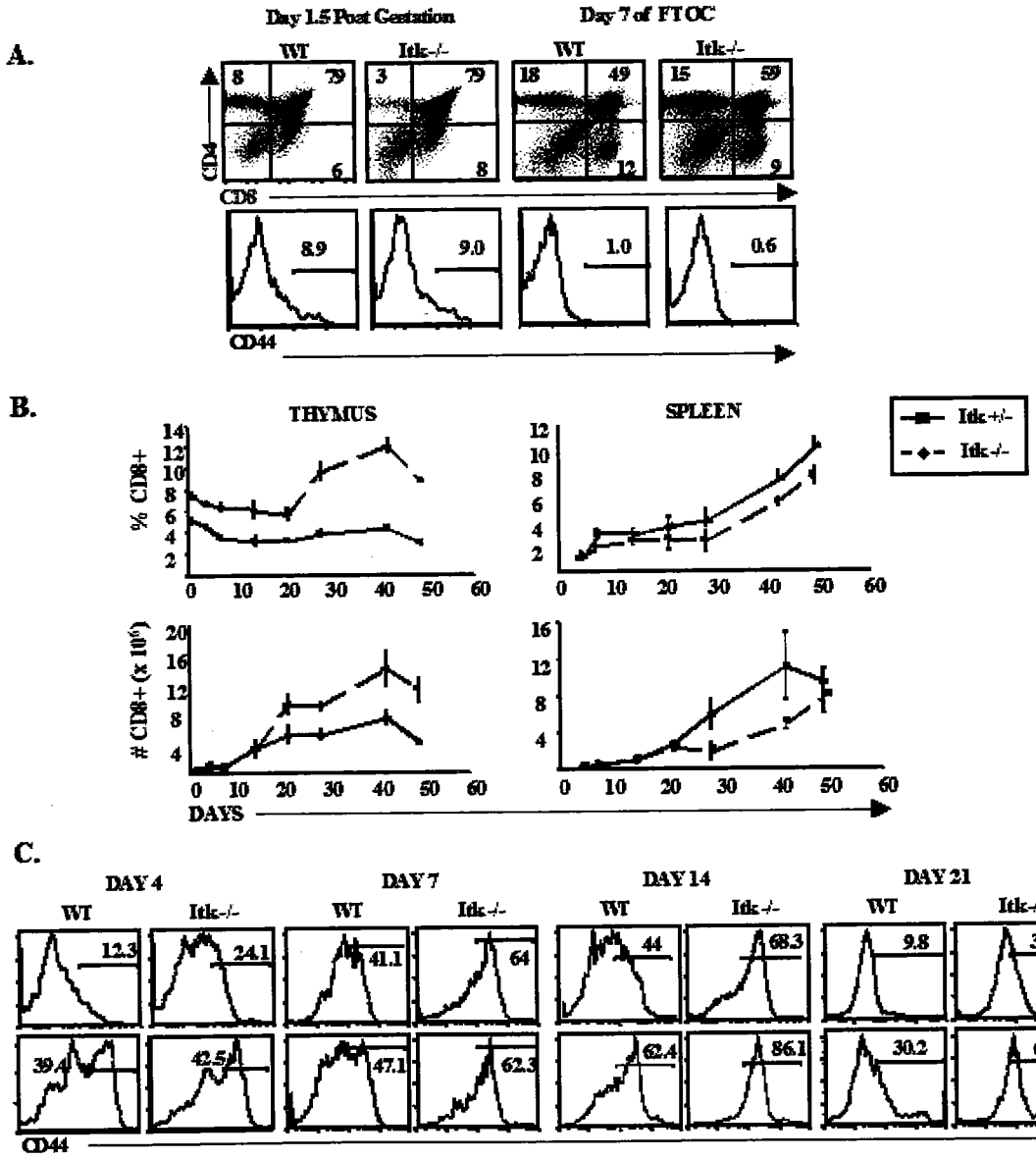
lymphopenia-induced proliferation in response to the "space" in the peripheral lymphoid organs (158). These $CD44^{hi}$ cells remain to constitute the fraction of previously activated cells typical of peripheral lymphoid organs (159). However, the frequency of these cells gradually diminishes in wild-type spleens, as cells continually migrate from the thymus into the periphery, filling up space and preventing further proliferation of the newly emigrated cells from the thymus. The preferential accumulation of $CD8^+$ SP thymocytes in the thymi of *itk*$^{-/-}$ mice as compared to wild-type mice, as well as the persistence of this population over time in the periphery of *itk*$^{-/-}$ mice, strongly suggests that the $CD8^+$ phenotype typical of *itk*$^{-/-}$ mice develops first in the thymus.

Figure 4.5 The altered phenotype of *itk*$^{-/-}$ CD8^{+} thymocytes is detectable by two weeks post-gestation

(A) Wild-type and *itk*$^{-/-}$ neonatal day 1.5 thymocytes (left two panels), and cells from fetal day15-16 thymi cultured *in vitro* for 7 days (right two panels), were stained with Abs to CD4 and CD8. The numbers in each quadrant indicate the percentage of cells in each subpopulation. Histograms of CD44 expression on gated CD8^{+} SP thymocytes are depicted below. Numbers indicate the percentage of CD8^{+} SP thymocytes expressing high levels of CD44.

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

(C) CD44 expression on CD8^{+} CD44^{hi} gated thymocytes and splenocytes from 4 day-, 1 week-, 2 week-, and 3-week old mice. Numbers indicate the percentage of CD44^{hi} cells among the gated population. Data are representative of a minimum of two experiments, each performed with a minimum of 3 mice per group.

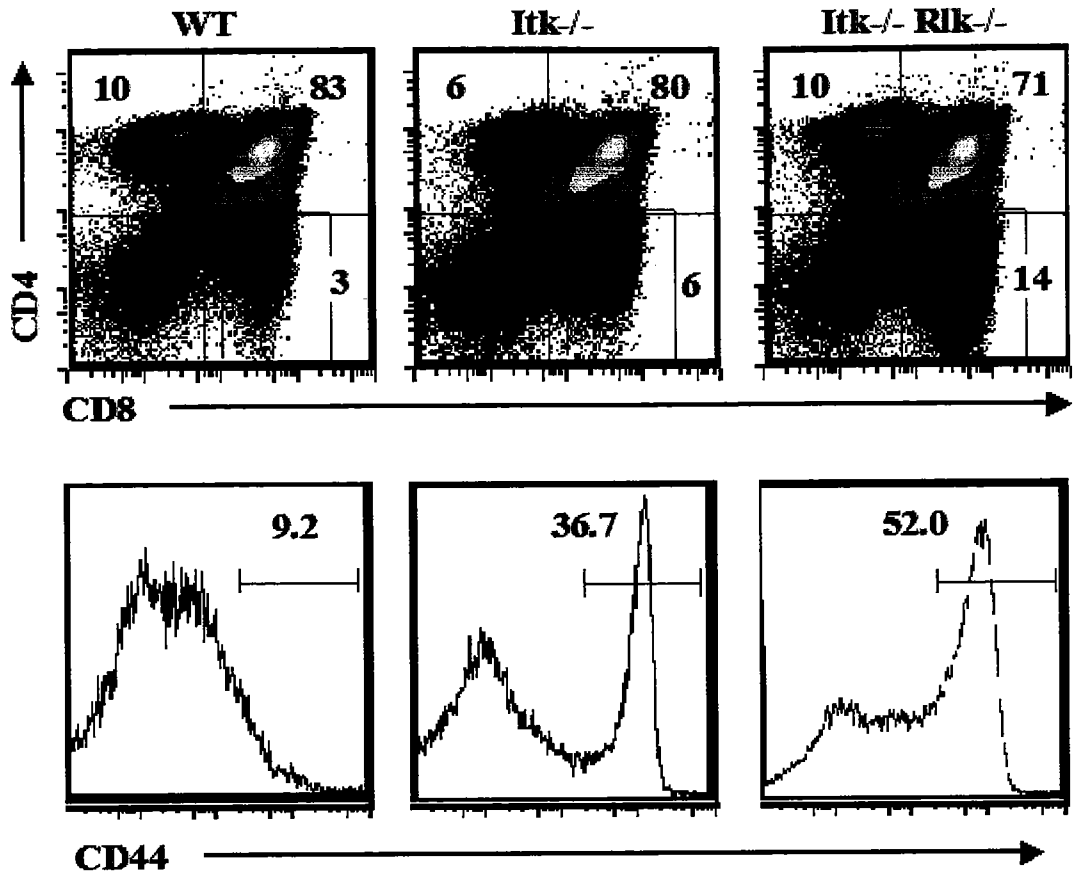

A.
Day 1.5 Post Gestation
Day 7 of FTOC
WT
Itk-/-
WT
Itk-/-
8
79
6
3
79
8
18
49
12
15
59
9
CD4
CD8
8.9
9.0
1.0
0.6
CD44
B.
THYMUS
SPLEEN
Itk+/-
Itk-/-
% CD8+
# CD8+ (x 10⁶)
DAYS
C.
DAY 4
DAY 7
DAY 14
DAY 21
WT
Itk-/-
12.3
24.1
41.1
64
44
68.3
9.8
38.4
39.4
42.5
47.1
62.3
62.4
86.1
30.2
64
thymus
spleen
CD44

The altered phenotype of CD8$^+$ thymocytes and peripheral T cells in itk$^{-/-}$ and itk$^{-/-}$rlk$^{-/-}$ mice is hematopoietic cell-intrinsic.

In addition to homeostatic proliferation, CD8$^+$ T cells have been shown to up-regulate CD44 expression and to become phenotypically and functionally mature in response to cytokines. In particular, the CD8$^+$ populations found in both IL-7 (160) and IL-15 (161) transgenic mice are remarkably similar to the CD8$^+$ cells present in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice. Therefore, we chose to examine whether the altered development of *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ CD8$^+$ T cells was due to defects intrinsic to the developing T cells, or alternatively, due to an altered cytokine environment in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice. To address this question, we generated BM chimeric mice in which wild-type, *itk*$^{-/-}$, or *itk*$^{-/-}$ *rlk*$^{-/-}$ BM was injected into lethally-irradiated wild-type congenic mice. Following reconstitution, the emergence of CD44^{hi} cells in the CD8 SP compartment of the thymus was readily apparent in mice reconstituted with *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ bone marrow, but not in thymi of mice reconstituted with wild-type BM (Figure 4.6). These findings indicate that the altered CD8$^+$ T cell development in the absence of Itk, or Itk and Rlk, is not due to abnormalities of *itk*$^{-/-}$ or *itk*$^{-/-}$*rlk*$^{-/-}$ non-hematopoietic stromal cells.

Figure 4.6 Altered $CD8^+$ T cell differentiation in the absence of Tec family kinases is intrinsic to bone marrow-derived cells

Wild-type, *itk*$^{-/-}$, and *itk*$^{-/-}$*rlk*$^{-/-}$ BM chimeric mice were analyzed 12 weeks following reconstitution. Dot plots show CD4 versus CD8 staining in donor-derived cells ($CD45.2^+$) within the thymi of indicated BM chimera. The numbers in each quadrant indicate the percentage of cells in each subpopulation. The histograms below show CD44 expression $CD45.2^+$ $CD8^+$ SP thymocytes. Numbers indicate the percentage of CD44hi cells among the gated population. Data are representative of four wild-type and *itk*$^{-/-}$ chimeras and eight *itk*$^{-/-}$*rlk*$^{-/-}$ chimeras analyzed.


WT
Itk-/-
Itk-/- Rlk-/-
10
83
3
6
80
6
10
71
14
CD4
CD8
9.2
36.7
52.0
CD44

$CD8^+$ $CD44^{hi}$ cells develop in the thymus but not the periphery of $itk^{-/-}$ $IL\text{-}15^{-/-}$ mice

Recently, several reports have described $CD8^+$ T cells with a previously-activated/memory $CD44^{hi}$ phenotype, similar to that of $itk^{-/-}$ and $itk^{-/-}rlk^{-/-}$ $CD8^+$ $CD44^{hi}$ T cells, in mice with hyperactive cytokine signaling. For example, $CD8^+$ T cells from Suppressor of Cytokine Signaling-1 $(SOCS\text{-}1)^{-/-}$ (162) and $SOCS\text{-}1^{-/-}$ $IFN\gamma^{-/-}$ mice (151, 152, 163, 164), as well as mice that express a constitutively-active STAT5b (CA-STAT5b) transgene (165), are all intrinsically hyperresponsive to cytokine stimulation. These findings correlated nicely with evidence demonstrating that IL-7 and IL-15 are important in the generation and/or maintenance of "memory" $CD8^+$ T cells in the periphery (166-168). In addition, $(SOCS\text{-}1)^{-/-}$, $SOCS\text{-}1^{-/-}$ $IFN\gamma^{-/-}$, and CA-STAT5b transgenic mice all have increased percentages of $CD8^+$ T cells in the thymus and spleen, a majority of which express high levels of CD44, CD122, and Ly6C; furthermore, the $CD8^+$ SP thymocytes in these mice are all hyperresponsive to stimulation with the common γ chain (γ_c) cytokines, IL-7 and IL-15.

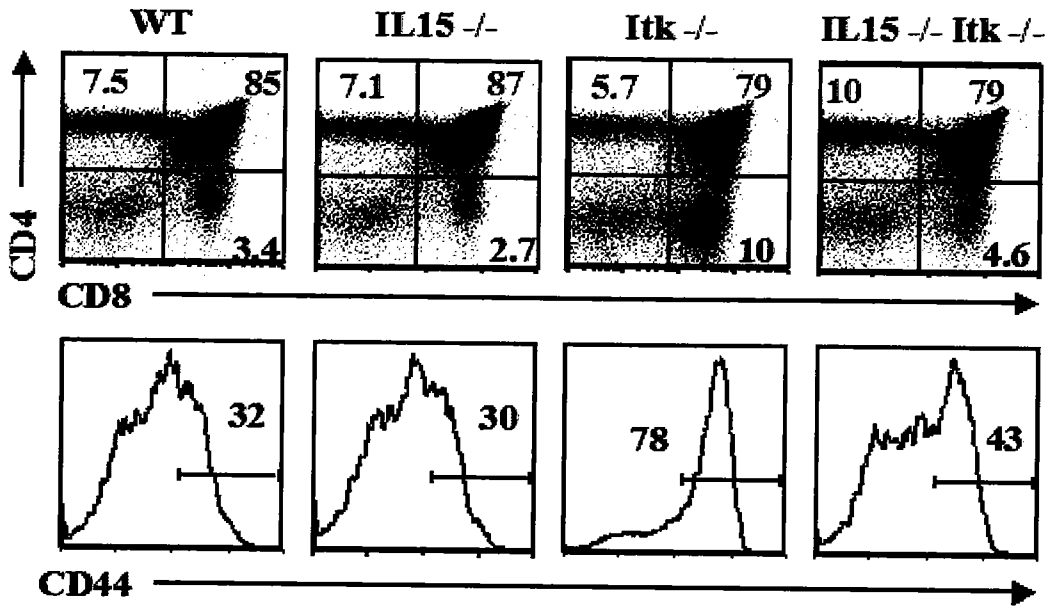
To determine whether IL-15 is required for the development and/or maintenance of $CD44^{hi}$ $CD8^+$ SP thymocytes and peripheral T cells in $itk^{-/-}$ mice, we crossed $itk^{-/-}$ mice to $IL\text{-}15^{-/-}$ mice. We reasoned that if IL-15 was indeed important for the generation of the $CD8^+$ $CD44^{hi}$ SP thymocyte population in $itk^{-/-}$ mice, then removal of IL-15 should prevent the accumulation of these cells. Additionally, since IL-15 is necessary for the maintenance of $CD8^+$ $CD44^{hi}$ cells in peripheral lymphoid organs, a selective loss of $itk^{-/-}$ $IL\text{-}15^{-/-}$ $CD8^+$ peripheral T cells compared to $CD8^+$ SP thymocytes would provide further support for the conclusion that this population of T cells arises during development in the

thymus. As shown in Figure 4.7, *itk*$^{-/-}$ *IL-15*$^{-/-}$ mice are nearly devoid of CD8^{+} T cells in the periphery, but still retain a substantial population of CD8^{+} SP cells in the thymus. Of the CD8^{+} SP thymocytes in *itk*$^{-/-}$ *IL-15*$^{-/-}$ mice, a larger percentage of these cells are CD44^{hi}, compared to those in wild-type mice (Figure 4.7). In fact, there are twice as many CD8^{+} CD44^{hi} cells in the *itk*$^{-/-}$ *IL-15*$^{-/-}$ mice compared to wild-type mice as calculated by absolute numbers (data not shown). However, although these cells are still present as a clear population, the percentage of CD8^{+} SP thymocytes in the thymus of *itk*$^{-/-}$ *IL-15*$^{-/-}$ mice, as well as the proportion of these cells that are CD44^{hi}, are reduced compared to that seen in *itk*$^{-/-}$ mice. In absolute numbers, the number of CD8^{+} SP thymocytes in *itk*$^{-/-}$ *IL-15*$^{-/-}$ mice is about one third of that seen in *itk*$^{-/-}$ mice, and is compare to the number seen in wild-type and *IL-15*$^{-/-}$ mice (data not shown). Thus, the *itk*$^{-/-}$ CD8^{+} SP cells do not accumulate in the absence of IL-15. Together, these data strongly suggest that the CD44^{hi} CD8^{+} SP phenotype observed in *itk*$^{-/-}$ mice develops first in the thymus, but also indicate that IL-15 signaling contributes to the accumulation of CD8^{+} SP thymocytes normally observed in the thymus of *itk*$^{-/-}$ mice.

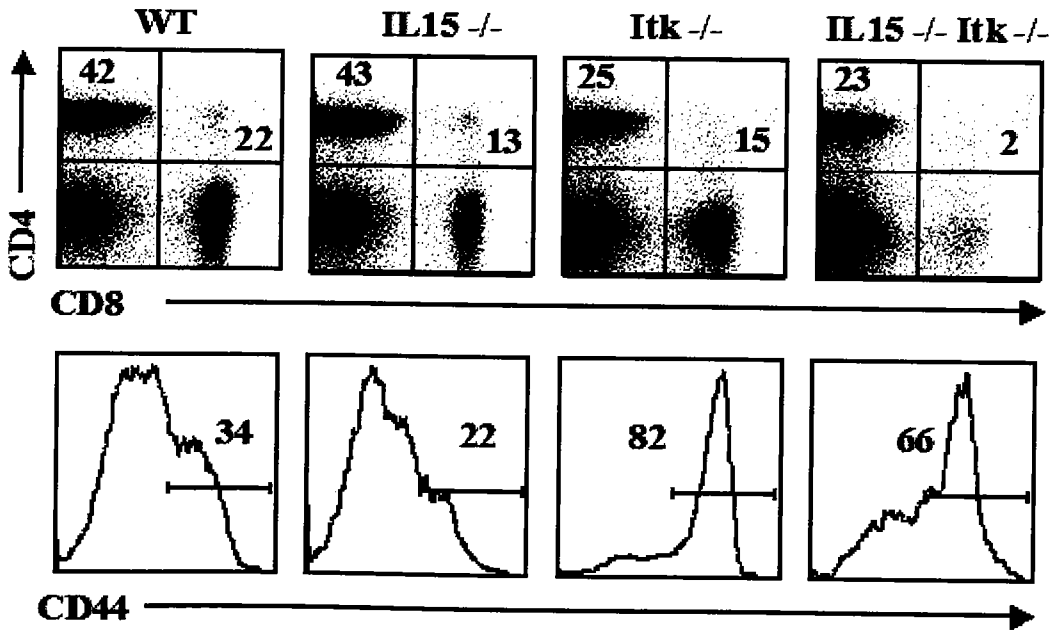
Figure 4.7 $CD8^+$ $CD44^{hi}$ cells develop in the thymus of *itk*$^{-/-}$ *IL-15* $^{-/-}$ mice

Thymocytes and lymph node cells isolated from wild-type, *itk*$^{-/-}$, *IL-15*$^{-/-}$ and *itk*$^{-/-}$ *IL-15*$^{-/-}$ mice were stained for expression of CD4, CD8 and CD44. The CD4 versus CD8 profile of wild-type, *itk*$^{-/-}$, *IL-15* $^{-/-}$ and *itk*$^{-/-}$ *IL-15* $^{-/-}$ (A) thymocytes and (B) lymph nodes is shown, with the numbers in each quadrant indicating the percentage of cells in each subpopulation. The histograms below show CD44 expression on gated $CD8^+$ populations, with the numbers indicating the percentage of $CD44^{hi}$ cells among the gated population. Data are representative of four independent experiments.

A.


WT
IL15 -/-
Itk -/-
IL15 -/- Itk -/-
7.5
85
3.4
7.1
87
2.7
5.7
79
10
10
79
4.6
CD4
CD8
32
30
78
43
CD44

B.


WT
IL15 -/-
Itk -/-
IL15 -/- Itk -/-
42
22
43
13
25
15
23
2
CD4
CD8
34
22
82
66
CD44

Altered CD8 T cell differentiation in the absence of Itk is dependent on TCR specificity

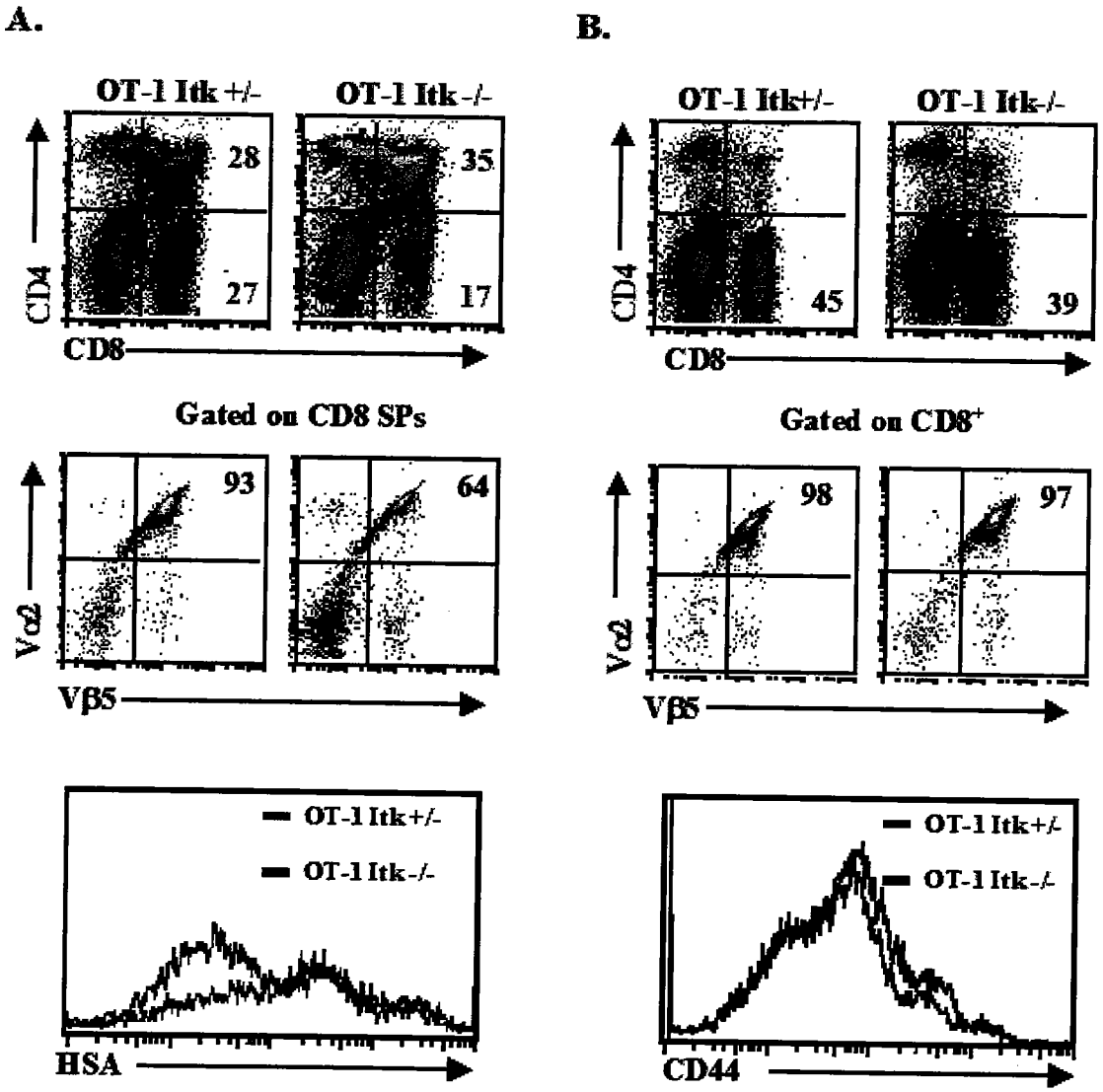
Based on the similarities between $CD8^+$ T cells lacking Itk and Rlk and those with hyperresponsiveness to common γ_c cytokines, we postulated that *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ $CD8^+$ T cells may have altered responses to cytokine signals received during the late stages of $CD8^+$ SP thymocyte differentiation. For instance, it is possible that Tec-family kinases directly regulate γ_c cytokine receptor signaling pathways. A more likely possibility is that Itk and/or Rlk activation by TCR signaling during positive selection and CD4/CD8 lineage differentiation regulates the expression of a gene involved in IL-7 and IL-15 signaling. In this latter case, one might expect that the accumulation of $CD44^{hi}$ $CD8^+$ SP thymocytes would not occur in *itk*$^{-/-}$ mice crossed to an MHC class I-specific TCR transgenic line, if the TCR chosen falls in the high avidity range of the positive selection window. In this case, very strong TCR signaling during thymic selection would likely overcome the deficiency resulting from the absence of Itk and/or Rlk, and lead to adequate induction of these putative regulatory genes.

To test this possibility, we crossed *itk*$^{-/-}$ mice to the TCR transgenic OT-1^+ line. Previous data have indicated that the OT-1 TCR has a relatively high avidity for positively-selecting ligands in the thymus (169). In support of this notion, we found that the maturation of $CD8^+$ T cells with high levels of the OT-1 TCR was only slightly reduced in the absence of Itk (Figure 4.8). In fact, the total cell number, as well as the total number of OT-1 $CD8^+$ T cells, in the thymus and the spleen, were comparable between OT-1 *itk*$^{+/-}$ and OT-1 *itk*$^{-/-}$ mice and there was only a slight reduction in the lymph node OT-1 $CD8^+$ T cells in the absence of Itk. This is in contrast to previous data

showing that the $CD8^+$ T cells expressing the H-Y TCR fail to be selected in the absence of Itk (149, 150). In addition, the CD44 expression profiles of wild-type versus *itk*$^{-/-}$ $CD8^+$ SP thymocytes and peripheral $CD8^+$ T cells were identical (Figure 4.8). These data indicate that when positive selection signals are only marginally dependent on the presence of Itk, $CD8^+$ differentiation proceeds normally and there is no accumulation of $CD44^{hi}$ $CD8^+$ SP thymocytes or peripheral T cells.

Figure 4.8 $CD8^+$ OT-I TCR transgenic *itk*$^{-/-}$ T cells develop normally

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


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

DISCUSSION

An interesting characteristic of thymocyte development in the *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ thymus, is that, in contrast to the deficit in maturation of CD4^{+} SP cells, CD8^{+} SP cells arise in increased numbers and acquire a CD44^{hi} phenotype. This phenotype is also observed on peripheral CD8^{+} T cells in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice, which in addition to being CD44^{hi} and CD62L^{hi}, also express increased levels of the activation/memory marker, CD122 (Figure 4.3B). We have shown that this increase in CD8^{+} SP thymocytes is not due to switching of MHC class II-specific cells from the CD4 into the CD8 lineage (74). Subsequently, we went on to explore the origin of the CD8^{+} CD44^{hi} SP cells that develop in the thymus of *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice and these studies suggest a potential role for Itk and/or Rlk in CD8 lineage differentiation processes.

In agreement with the kinetic signalling model of CD4/CD8 lineage differentiation, a role for cytokines in CD8^{+} development has been confirmed in studies using SOCS-1$^{-/-}$ or SOCS-1$^{-/-}$ IFNγ$^{-/-}$ mouse models. Interestingly, the CD8^{+} SP thymocytes and peripheral CD8^{+} T cells in these mice are CD44^{hi} and appear phenotypically similar to those in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice. In addition, the CD8^{+} cells lacking SOCS-1 are hyperresponsive to stimulation with IL-7 and IL-15, and thymi of SOCS-1$^{-/-}$ and SOCS-1$^{-/-}$ IFNγ$^{-/-}$ mice cultured in the presence of these cytokines generate more CD8^{+} SP cells than wild-type thymi. CD8^{+} thymocytes from SOCS-1$^{-/-}$ mice also maintain an elevated level of STAT-5 phosphorylation over a longer time period than CD8^{+} SP thymocytes from wild-type mice (152, 162). In concordance with these results, the CD8^{+} T cells from CA-STAT-5b transgenic mice, in which there is constitutive

activation of the signaling pathways downstream of the γc-cytokine receptors, are phenotypically identical to those in SOCS-1$^{-/-}$ mice (165). These experiments demonstrate a potential role for IL-7 and IL-15 in the regulation of the CD8^{+} lineage differentiation, and indicate that the inability to regulate the signaling of these cytokines can accelerate the maturation of CD8^{+} SP thymocytes and induce up-regulation of CD44.

These data on the role of γc cytokines in CD8^{+} T cell maturation prompted us to consider whether γc cytokines might be involved in the accumulation of CD8^{+} CD44^{hi} cells in the thymus and periphery of *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice. We initiated our investigation by generating *itk*$^{-/-}$ *IL-15*$^{-/-}$ mice. Analyses of the CD8^{+} T cell populations in the thymus and the periphery of these mice led us to conclude that the CD8^{+} CD44^{hi} cells in *itk*$^{-/-}$ mice most likely arose during thymocyte development, specifically during CD8^{+} lineage maturation. This conclusion was based on the data showing that a smaller fraction of CD8^{+} CD44^{hi} SP thymocytes were generated in the thymus of the *itk*$^{-/-}$ *IL-15*$^{-/-}$ mice, as compared to *itk*$^{-/-}$ mice, and that virtually no CD8^{+} CD44^{hi} cells remained in the periphery of the *itk*$^{-/-}$ *IL-15*$^{-/-}$ mice (Figure 4.7).

Although the similarities in CD8^{+} phenotype suggest that the response to cytokine signaling during CD8^{+} T cell differentiation may be altered in the absence of Itk and Rlk, the mechanism by which this might be occurring is still under investigation. Analysis of BM chimeras suggests that the defect in CD8^{+} T cell differentiation of *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ thymocytes is intrinsic to the developing thymocytes and is not due to altered cytokine production by non-hematopoietic cells (Figure 4.6). Our experiments also showed that CD8^{+} SP thymocytes begin accumulating in the thymus of *itk*$^{-/-}$ mice at 2 - 3 weeks post

birth (Figure 4.5). One possibility is that these cells have a defect in migration in response to signals, such as chemokines, that normally induce thymocyte emigration. This possibility is consistent with recent data demonstrating defects in chemokine-induced migration by *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ T cells (170, 171). As the accumulated *itk*$^{-/-}$ CD8^{+} SP cells most likely reside in the medulla of the thymus, it is possible that these cells would then be subjected to maturational stimuli for a longer period of time than wild-type CD8^{+} SP cells, causing them to completely down-regulate HSA and abnormally up-regulate CD44, all before exit to periphery. An alternative, but not mutually-exclusive hypothesis is that *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ CD8^{+} SP thymocytes are intrinsically altered in their responsiveness to cytokines. This could come about due to weaker TCR signals during CD8^{+} SP positive selection and lineage commitment that might lead to reduced levels of genes, such as SOCS-1, that negatively-regulate cytokine signaling.

Whether or not Tec family kinases in T cells are directly involved in cytokine signaling, or indirectly affect the responsiveness of developing thymocytes to cytokine signals, remains to be determined. Interestingly, Tec family kinases have been implicated in cytokine signaling in non-T cell types (172, 173). However, our data examining *itk*$^{-/-}$ OT-1 transgenic mice, which are thought to express a TCR with high avidity for its selecting ligand(s), indicate that the abnormal CD8^{+} SP thymocytes development we observe is secondary to defects in TCR signaling during selection and differentiation, as the phenotype is resolved when the developing thymocytes express this specific TCR. This data is consistent with unpublished data from the Schwarzberg lab in

which Itk-deficient mice that express a transgene for a hyper-reponsive form of ERK2 during T cell development also seem to develop normal $CD8^+$ T cells (personal communication). Therefore, we hypothesize that *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ $CD8^+$ SP thymocytes undergo lineage differentiation in response to reduced TCR-mediated signals, and that this, in turn, leads to altered cytokine responsiveness by indirectly affecting the gene expression, protein stability, or protein activity of molecules directly involved in cytokine signaling.

Due to the extremely similar phenotypes observed between SOCS-1 deficient and Tec family deficient $CD8^+$ T cells, we initially hypothesized that this reduction in the strength of TCR signaling may directly affect SOCS-1 expression or activity in $CD8^+$ SP thymocytes, leading to a hypersensitive response to IL-7 or IL-15 during the lineage differentiation process. Indeed, there has been data suggesting that the proper transduction of signals downstream from the TCR is necessary for maintaining the level of SOCS-1 protein expression. In cells lacking the negative regulatory transcription factor JunD, there is a decrease in SOCS-1 at the RNA level. As such, we thought it was possible that in the absence of Itk and Rlk which regulate the expression of the jun and fos family members, expression of JunD is decreased, thereby affecting SOCS-1 expression (174). However, preliminary quantitative real-time PCR data (not shown) examining SOCS-1 mRNA levels in DN, DP, $CD4^+$ SP, and $CD8^+$ SP thymocytes from *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice indicate that SOCS-1 expression is comparable between wild-type and Tec kinase-deficient cells.

Alternatively, SOCS-1 expression in *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ CD8^{+} SP thymocytes may be regulated post-translationally by a protein such as Pim-1. Pim-1 is a serine/threonine kinase and is expressed primarily in cells of the hematopoietic lineage. Expression of Pim-1 mRNA is markedly induced following TCR cross-linking. Pim-1 is also induced in response to cytokine signaling and is involved in the regulation of SOCS-1 protein stability. Pim-1 has a role in thymocyte development as it is expressed during the late TN stage of thymocyte development where it participates in the transition from the TN4 to DP stage of thymocyte development (175-177). Diminished production of Pim-1 in the absence of Itk and Rlk would potentially lead to the impaired stability and function of SOCS-1, lowering SOCS-1 protein expression in CD8^{+} SP thymocytes and resulting in hyperresponsiveness to cytokine stimuli. Further studies will be required to investigate this possibility, as well as other potential mechanisms by which Tec family kinases may be affecting proteins involved in cytokine signaling.

In addition to the altered CD8^{+} phenotype we observe in Itk-deficient mice, we have also observed that there is a significant increase in the absolute number of CD8^{+} T cells in the thymus of these mice when compared to wild-type mice; however, there is no significant difference in peripheral CD8^{+} T cell numbers. We initially hypothesized that this may be due to reduced survivability of peripheral CD8^{+} T cells in the absence of Itk. However, adoptive transfer experiments, in which we compared the persistence of CD8^{+} T cells from wild-type versus *itk*$^{-/-}$ or OT-1 versus OT-1 *itk*$^{-/-}$ mice after transfer into congenic hosts, did not indicate any difference in the ability of cells to survive when Itk was missing. In the non-TCR transgenic experiments, the *itk*$^{-/-}$ cells were also sorted into

$CD8^+$ $CD44^{hi}$ and $CD8^+$ $CD44^{lo}$ populations and the levels of CD44 on the cells also did not seem to affect the ability of *itk*$^{-/-}$ $CD8^+$ cells to populate and/or persist following transfer (data not shown).

Alternatively, as mentioned above, $CD8^+$ SP cells may be getting "stuck" in the thymus of *itk*$^{-/-}$ and *itk*$^{-/-}$*rlk*$^{-/-}$ mice. Tec family-deficient thymocytes have been reported to have impaired responses to both SDF-1 (171) and TECK (170); however, the precise chemokines that mediate thymic emigration are unknown. Still another possibility is that the $CD8^+$ T cells that develop in the absence of Tec family kinases may populate alternative or additional peripheral organs. Therefore, the increase of $CD8^+$ SP cells in the thymi of Tec family-deficient mice may not be represented completely by the $CD8^+$ T cells found merely in the spleen and lymph nodes. More about these possibilities and future experiments planned to test them is discussed in Chapter 5. Clearly, a number of questions remain with regard to the mechanisms that guide $CD8^+$ T cell development in the absence of Itk and/or Itk/Rlk.

CHAPTER V

DISCUSSION

DISCUSSION

The work in this thesis addresses the role of the Tec family kinase, Itk, in T cell development. Tec family kinases are primarily expressed in hematopoetic cells. Generally, cells express more than one family member, but the levels of expression as well as the precise combination of Tec family kinases expressed varies in different cell types. The reason for the expression of multiple family members in one cell type remains to be determined. Btk, Tec, Rlk, and Itk have all been shown to be able to affect PLC-γ activation downstream of antigen receptors in both B and T lymphocytes; as well as in mast cells; and Tec members appear to be exchangeable with respect to Ag receptor signaling since transgenic expression of another family member can often restore defects by the absence of another (178-180). While it is possible that Tec family kinases are performing redundant functions, the differences in regulation, expression patterns, cellular localization, and binding partners vary among Tec family members suggests that these kinases may play distinct roles as well. In order to determine if one of the other Tec family members expressed in T cells compensated for the absence of Itk, we incorporated *itk*$^{-/-}$*rlk*$^{-/-}$ mice into the pre-T cell and lineage differentiation experiments wherever possible.

When Itk was first cloned from T cells, it was thought that deletion of the gene for this protein would cause defects similar to those observed in B cells when Btk was mutated. The generation and analysis of Itk-deficient mice revealed that its role in T cells was either less important than the role of Btk in B cells, or there was more compensation by the other Tec family members in T cells. While both Tec- and Rlk-singly deficient

mice have no apparent abnormalities in immune cell generation or function, the double deficiency of Btk and Tec causes even more severe defects than Btk alone (68) just as there are greater T cell defects in Itk/Rlk double-deficient mice. Despite this similarity Btk/Tec double-deficient mice show blocks in B cell development not observed in Itk/Rlk double-deficient mice. One possible explanation is that T cells express three Tec kinases, while B cells only express Btk and Tec. The other possibility is that activation of PLC-γ is, at least in part, mediated by non-Tec family signaling molecules. The generation of Itk/Rlk/Tec triple-deficient mice would aid in distinguishing between these possibilities, however this approach has been hampered by the fact that Rlk and Tec are closely linked on chromosome 5 in mice (181, 182) and therefore, the generation of the triple knockouts can not be achieved by simple intercrossing of the three single knockouts.

Recently, the GTP exchange factor, Vav, has been shown to be important for the activation of PLC-γ in DP thymocyte via both PI3K-dependent and PI3K-independent pathways (183) . The precise mechanism by which this occurs remains to be determined, but it was also shown that the phosphorylation of both Itk and Tec was reduced in the absence of Vav. Thus, one possibility is that the reduced phosphorylation of PLC-γ is secondary to the reduced activation of PI3K seen in these cells, which is required for the full activation of these Tec family members by recruiting these kinases to the cell membrane via their PH domain. Alternatively, Vav has been shown to bind both Itk and Tec, and since the activation of Tec kinases is inhibited by inter- and intra-molecular interactions that keep the Tec kinases in an inactive state (50), it is possible that the binding of Vav disrupts these interactions, thus aiding in the activation of the Tec

kinases. The PI3K-independent pathway may involve the activation of Rlk, which is independent of PI3K activation, however citing insensitive reagents, the authors were unable to determine if Rlk phosphorylation was affected by the absence of Vav.

Besides the effect Vav plays on the activation of Tec kinases, Vav may also directly influence PLC-γ activity. Like the Tec kinases, PLC-γ has a PH domain and thus, the reduced PI3K activity in the absence of Vav, might interfere with the recruitment of PLC-γ to the membrane. In addition, the authors demonstrated that in a PI3K-independent manner, the association of PLC-γ with SLP-76 and Gads is disrupted in the absence of Vav (183) and the inability of PLC-γ to be recruited to the SLP-76/LAT complex may affect its ability to become activated. To further compound the issue, it has also recently been shown that disruption of Itk activity in Jurkat cells by the overexpression of dominant negative form of this kinase leads to reduced phosphorylation of LAT and as a consequence the association of Vav with this complex is reduced (184). Thus, it is clear that the relationship between Vav and Tec kinases is both extensive and complex. In the future, it should be interesting to determine whether or not the role for Vav in activating PLC-γ is completely Tec kinase dependent or whether Vav may directly contribute to PLC-γ activation through non-Tec family kinase mechanisms. If this is the case, it might explain why Tec family kinases are not absolutely critical for the development and activation of T cells.

Despite the fact that gross analysis of the thymus and secondary lymphoid organs, based on total cellularity and T cells subsets, suggested that T cell development in the absence of Itk was relatively normal, the observation that T cell selection in two Itk-

deficient TCR transgenic models was virtually absent inspired us to look more closely at development in Itk-deficient mice (39). Rationally, we began our studies by looking at the ability of other TCRs to be selected in the absence of Itk. It was believed that the TCR transgenes used in the earlier studies had low avidity for their selecting ligands in the thymus due to the low efficiency of positive selection in these transgenic models. Therefore, we began by crossing the Itk-deficient mice to TCR transgenic mice that had been shown previously in our lab to vary in their positive selection efficiencies and therefore were believed to have varying avidities for their selecting ligands (120). These led to the studies in Chapter 2, which demonstrated that selection in the absence of Itk was affected by the avidity of the TCR present on the DP thymocyte. Thus the development of T cells in the absence of Itk required a higher avidity TCR.

Logically, we reasoned that if higher avidity interactions were needed for positive selection in the absence of Itk, that some of the high avidity interactions that normally lead to negative selection might instead result in positive selection. We did not find this to be the case in any of the class II negative selection models we tested, although deletion seemed to happen at a later stage in development, presumably due to a requirement for higher TCR levels to induce deletion in the absence of Itk. Thus, appears that the selection "window" proposed by the avidity model of thymocyte selection is reduced in the absence of Itk, rather than shifted. This suggests that although the strength of signal does influence the outcome of both processes, the type of TCR signal required for each process may differ in a way that can be affected by the activity of Itk. For instance, positive selection requires low sustained signals, especially for the development of CD4

cells. Thus, in the absence of Itk, which is required for sustained Ca^{2+} signals (66) the overall ability of these signals to be maintained throughout the selection/differentiation process may be impaired. On the other hand, it appears that negative selection just requires a signal to reach a certain threshold to induce programmed cell death, and since the signal may be received throughout development, it may be more likely to occur prior to the completion of development even in the absence of Tec kinases.

Some positive selection of a class I restricted TCR transgenic in a system where it is normally deleted, may occur in Itk-deficient mice, but definitely occurs in Itk/Rlk double-deficient mice. One possibility for this discrepancy is that the negative selection models we used were too efficient to detect any positive selection in the absence of Itk. I believe this is unlikely since in the 5C.C7/HEL-cyt transgenic model, the development of "self-reactive" cells in the periphery occurs to a small extent in wild-type mice in the absence of the metallothionein promoter activation. In the Itk-deficient 5C.C7/HEL-cyt mice, we anticipated that the development of the "self-reactive" peripheral cells would be increased due to less efficient negative selection. However, we actually observed reduced development of these cells (data not shown). I think the most likely explanation is that any defect in negative selection of class II restricted cells in the absence of Itk is masked by the impaired differentiation of CD4 lineage cells in *itk*$^{-/-}$ mice. Thus, the selection "window" is differentially affected in the CD4 versus CD8 lineage. As stated above, the selection window is reduced for class II restricted DP thymocytes, but for class I restricted DP thymocytes it might actually be shifted as was originally predicted.

As these studies were ongoing, the quantitative model of lineage commitment, was beginning to take shape. We were extremely intrigued by this model since proteins both upstream and downstream of Itk were implicated in CD4/CD8 lineage choice. In addition, the *itk*$^{-/-}$ mice had increased numbers of CD8^{+} T cells in the thymus, as would be predicted since lower TCR signals had been shown to support the switching of class II restricted cells into the CD8^{+} T cell lineage. However, in five class II TCR transgenic models we never observed an increase in the development of TCR transgenic CD8^{+} T cells (Chapter 4). Since these TCR transgenics covered a larger range of TCR avidities, we believed that we could reasonably conclude that lineage choice/commitment was not altered in the absence of Itk. However, in the course of this work, we discovered that the CD8^{+} T cells that were accumulating had a distinctive phenotype. These phenotype of these cells was similar "previously-activated", or as we would later find out, homeostatically expanded cells. We determined that these cells were not only phenotypically, but were also functionally similar to "previously-activated" T cells, indicating that these cells had undergone the differentiation process that occurs when cells are activated, induced to homeostatically expand, or are exposed to γ_c cytokines. However, the origin of these cells and the mechanism by which they underwent this differentiation remained elusive for a long time.

There was one experimental result and two main developments being reported in the literature that began to shape our current view of CD8 lineage differentiation and allowed us to make sense of all the rest of the data we were accumulating. The experimental result was the discovery that Itk/IL-15 double deficient mice lacked

peripheral $CD8^+$ T cells, but still had $CD8^+$ SP thymocytes with the previously activated phenotype (Figure 4.7). This suggested to us, that the development of these cells was occurring in the thymus during T cell selection and lineage differentiation. Next, evidence for the kinetic model of lineage commitment was accumulating due to the increasingly substantial evidence implicating a role for cytokines in $CD8^+$ T cell differentiation, along with evidence that the duration of the TCR signal definitely influenced the outcome of CD4/CD8 lineage differentiation (38). The second thing that emerged in the literature was descriptions of mice that had a similar $CD8^+$ phenotype as that seen in the thymus of *itk*$^{-/-}$ mice. These mice all had mutations that made them hyperresponsive to cytokine signaling (152, 162, 165). Taken together, these pieces of evidence have led us to hypothesize in the absence of Itk, cytokine signaling is somehow altered. While the evidence for this hypothesis remains circumstantial, work is ongoing to try and determine if this hypothesis is correct and the specific role of Itk in cytokine signaling at the DP to SP transition.

The ability to determine if cytokine responses are hyperactive in the absence of Itk have been difficult since the majority of the $CD8^+$ cells in these mice have already differentiated into the phenotype that exposure to cytokines would induce. However, we are in the process of regenerating OT-1 Itk deficient mice, since the phenotype of the OT-1 $CD8^+$ cells is comparable in the presence and absence of Itk. These experiment should resolve whether or not Itk is directly involved in cytokine signaling and if it is, further work will be done to determine what aspects of the cytokine signaling pathway is altered in an attempt to uncover the function of Itk. However, if Itk affects cytokine

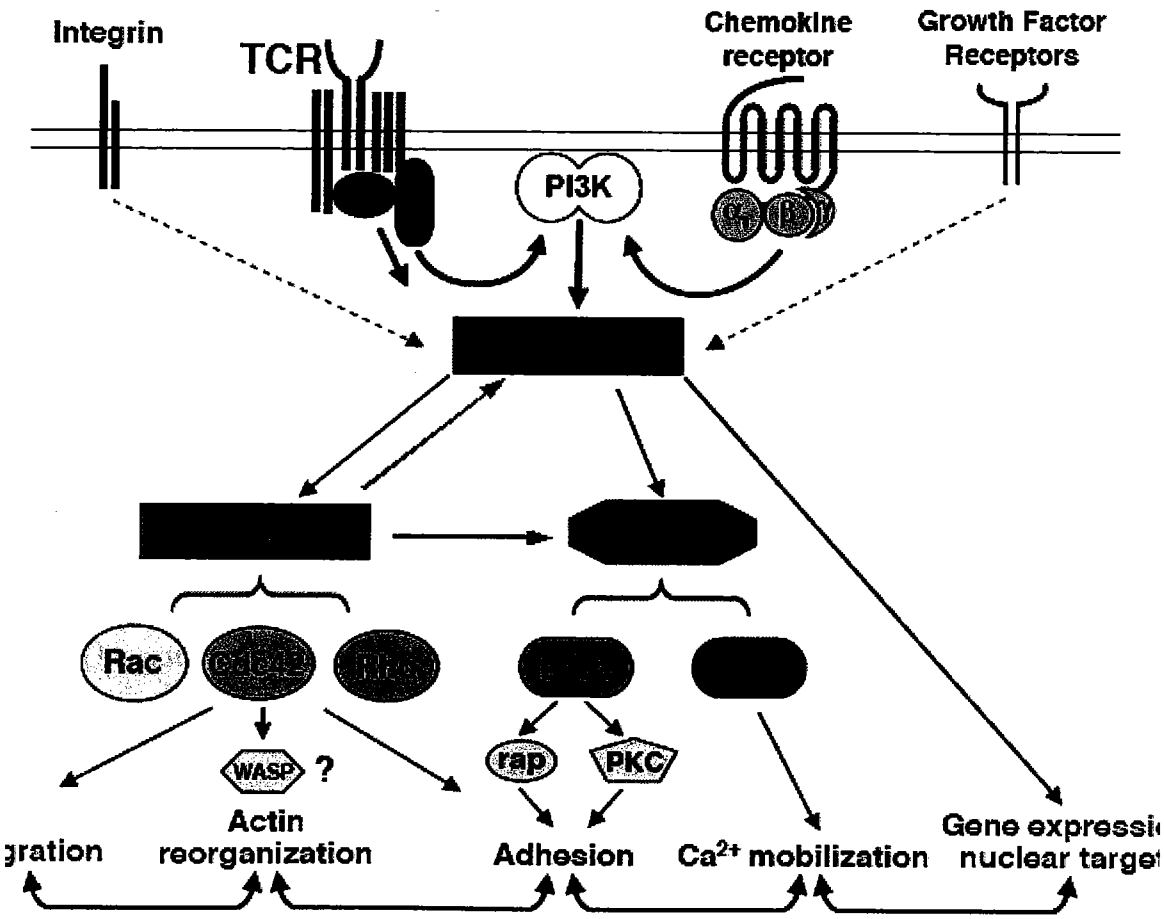
responsiveness by regulating a gene during positive selection, we may not observe any differences in response to cytokine stimulation since the development of OT-1 *itk*$^{-/-}$ CD8^{+} T cells appears to be normal. To further investigate this possibility it might be possible to examine the gene expression patterns between wild-type and Itk deficient OT-1 TCR transgenic thymocytes that develop in mice that do not express the selecting ligand for OT-1. These DP cells will not have received any selection signals, and therefore we can make use of altered peptide ligands to send different signals into the OT-1 thymocytes. Using the approach we may be able to identify cytokine signaling genes with altered expression due to the weaker positive selection signals in the absence of Itk.

An alternative version of our hypothesis is that Itk does not play a role in cytokine signaling or does not affect the expression of cytokine signaling molecules, but instead become differentiated by cytokines in the thymus because the *itk*$^{-/-}$ CD8^{+} thymocytes are exposed to them longer than wild-type cells due to impaired emigration from the thymus. Indeed, Itk has recently been shown to affect responses to chemokines (170, 171). As an attempt to determine if this hypothesis is likely we are crossing Itk deficient mice to Rag-GFP transgenic mice. These transgenic mice express GFP under the control of the Rag promoter and due to the stability of the GFP protein, recent thymic emigrants can be identified in the periphery by residual GFP present in these cells (185). We have reasoned if CD8^{+} SP emigration is delayed then we may see a reduction or loss of GFP^{+} cells in the periphery of Itk-deficient mice compared to wild-type mice. Depending on the outcome of the experiments described, investigation the role of Itk in chemokine or cytokine signaling in thymocytes will offer exciting new direction to the role of Tec

kinases in thymocyte development downstream of receptors besides the TCR. Figure 5.1 depicts known and proposed roles for Tec family kinases downstream of multiple cell surface receptors in T cells.

Figure 5.1 Tec family kinases are involved in signaling downstream of multiple cell surface receptors [slightly adapted from (50)]

In B and T cells, Tec-kinases are activated downstream of antigen receptors (TCR and surface immunoglobulin) and G protein-coupled chemokine receptors through the actions of PI3K and Src family kinases. In other cell types Tec-kinases are also activated by integrins and growth factor/cytokine receptors. Following stimulation of these rececptors, Tec kinases regulate multiple cellular functions including activation of PLC-γ and actin cytoskeleton reorganization, which in turn help regulate Ca^{2+} mobilization, activation of PKC, the MAPK pathway, and transcription factors, as well as cell adhesion and migration.


Integrin
TCR
Chemokine receptor
Growth Factor Receptors
PI3K
Rac
WASP
?
rap
PKC
Actin reorganization
Adhesion
Ca2+ mobilization
Gene expressi
nuclear targe

Nonetheless, there are some remaining questions with the respect to the role of Tec family kinases downstream of the TCR in the DP to SP transition. At this point, it has not been determined if a DP thymocyte expressing any existing TCR transgene can be selected in the absence of both Tec and Rlk. Indeed, results from the two TCR transgenic systems used suggest that positive selection is worse in the absence of both. It may be useful to cross these mice to additional transgenics to get a sense of how defective positive selection is in these mice, especially in light of the fact that they have relatively normal numbers of T cells. It would also be interesting to look at negative selection in the Itk/Rlk double deficient mice in a class II system to try and determine if there are any differences in negative selection (or the final outcome of differentiation) of class II versus class I restricted DP thymocytes especially, since it has been proposed that reduced negative selection may be the reason that there is not a reduction in $CD4^+$ T cell numbers in *itk*$^{-/-}$*rlk*$^{-/-}$ mice as there is in *itk*$^{-/-}$ mice. Additionally, as already suggested it would be extremely interesting to study triple Itk/Rlk/Tec knockout mice to determine the effect of complete Tec kinase deletion on the activation of PLC-γ and the development of T cells. Similarly, it might be worthwhile to intercross Tec family deficient mice with Vav deficient mice to determine if there are any additional thymocyte/T cell knockouts than in Tec family-deficient mice. This may help to determine if the role of Vav in activating PLC-γ is all upstream of Tec kinases, or if it contributes to PLC-γ activation via other mechanisms/pathways.

Despite the anticipated importance of Tec family kinases in pre-TCR signaling and the DN to DP transition, no defects were detected in *itk*$^{-/-}$ mice, and only minor

defects were observed when *itk*$^{-/-}$ progenitors had to develop in competition with wild-type progenitors. No further disruption in the β selection process was observed when both Itk and Rlk were absent from the developing thymocyte. This is in direct contrast to repertoire selection, which is significantly more defective when both Itk and Rlk are absent from developing thymocytes, but similar to CD8^{+} T cell differentiation. Future work in this area, should try and attempt to determine, which specific β selection events are affected during the DN to DP transition in the absence of Itk. Preliminary investigation of a role for Itk in allelic exclusion could be done either by a PCR or FACS based approach in mice expressing an already rearranged β chain; to do this, the *itk*$^{-/-}$ mice should be crossed to 2B4 β chain mice. The role of Itk in survival following pre-TCR signaling could be initially explored by examining the expression patterns of Bcl-2 family members. We have shown that Bcl-2 levels are regulated normally at the DP to SP transition; however, DP thymocytes should have low expression of Bcl-2 and high levels of Bcl-xL. Bcl-2 is induced by cytokine signaling at early stages of DN differentiation, but following pre-TCR signaling Bcl-2 levels decrease and Bcl-xL levels increase. Enforced expression of Bcl-2 or the loss of Bcl-xL both negatively impact the DN to DP transition. Proliferation can also be examined in more detail by more complex BrdU experiments than the ones presented here. Finally, as stated above for the DP to SP transition, analysis of the DN to DP transition should also be examined in Tec family-triple knockout mice or Vav deficient/Tec family deficient mice.

In conclusion, I believe the work in this thesis contributes to general knowledge by providing a better understanding of the role of Itk in T cell development, but more

importantly aids in the discernment of how distinct developmental processes are regulated in the thymus. The biggest effect of Itk deletion on T cell development occurs during positive selection and differentiation of $CD4^+$ T cells, which is the process that requires the longest most sustained signals, thus Itk seems most important for TCR signaling at this level. This is also in agreement with the minor defects at the DN to DP transition, which are also believed to require sustained signaling, but for a shorter period of time. In contrast, lineage choice seems to be unaffected, so perhaps this process occurs quickly at the stage in which DP cells first downregulate $CD8^+$, and this signal happens normally in the absence of Itk. Also further study of $CD8^+$ T cell differentiation in the absence of Itk may lend further support to the kinetic model of lineage differentiation and perhaps contribute to the knowledge of how cytokine signaling is regulated at the stage of development. Clinically, inhibitors of Itk are being developed as therapeutic agents for allergies and asthma, since Th2 responses are extremely defective in the absence of Itk. Thus, better understanding the role of Itk in T cell development and function is important in order to anticipate what other effects these inhibitors may have.

CHAPTER VI

LITERATURE CITED

LITERATURE CITED:

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