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The Function of the Tyrosine Kinase, Itk, in CD4+ T Cell Differentiation and Death: a Dissertation

Andrew Todd Miller
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THE FUNCTION OF THE TYROSINE KINASE, ITK, IN CD4+ T CELL DIFFERENTIATION AND DEATH

A Dissertation Presented

By

Andrew Todd Miller

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

JULY 31st, 2003

IMMUNOLOGY AND VIROLOGY PROGRAM
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THE FUNCTION OF THE TYROSINE KINASE, ITK, IN CD4+ T LYMPHOCYTE DIFFERENTIATION AND DEATH

A Dissertation Presented

By

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Abstract

The Tec family tyrosine kinase, Itk, plays an important role in signal transduction following T cell receptor engagement. Several prior studies have established the importance of Itk in immune system processes, such as T cell development and T cell activation. Additional biochemical studies have found that Itk specifically functions within a multi-molecular signalosome complex, which ultimately functions to provide a platform by which Itk can phosphorylate and activate PLC-γ1, a crucial step in T cell activation. To further study how Itk regulates distinct immune outcomes via T cell effector processes within the peripheral immune system, and to further understand how Itk functions in T cells in response to a physiological ligand-receptor interaction, I crossed Itk-deficient mice to mice transgenic for a TCR specific for a moth cytochrome C peptide. My studies have established a unique role for Itk in several important aspects of T cell function. Following T cell activation, I identified an imperative role for Itk in activation-induced cell death via FasL, a mechanism of immune homeostasis. Furthermore, I found Itk plays a unique role in the process of T cell differentiation, where Itk positively regulates the induction of cytokine genes, such as IL-4, while negatively regulating the induction of T-bet, a transcription factor important for Th1 differentiation. Lastly, following T cell differentiation, I found that Itk mRNA and protein are up-regulated during Th2 differentiation, while Rlk, a related Tec kinase, disappears rapidly from Th2 cells, indicating a critical role for Itk in Th2 cell function. Collectively, my thesis work has more clearly defined an important function for Itk not only in TCR signaling, but also in immune processes such as T cell differentiation and activation-induced cell death that are required for proper immune function.
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Attributions

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Julie Lambert and Dr. Michael Brehm assisted me in the SEB experiments described in Figure 7.

Chapter III.

The dendritic cell analysis in Figure 6 and Figure 7 was a collaborative effort between myself and Dr. Morgan Wallace.

Chapter IV.

The optimization of the quantitative PCR reactions for Itk and Rlk used to analyze expression levels in Figure 5 was a collaborative effort between myself and Martin Felices. The protein gels and immunoblotting in Figure 5 and Figure 6 were performed by Heather Wilcox.
This thesis is dedicated to the
loving memory of my grandparents,

Gerald and Pearl Salk
CHAPTER I.

INTRODUCTION
CD4⁺ T lymphocyte function within the mammalian immune system

Cells of the mammalian adaptive immune system serve several functions in the response to pathogens, as well as in the regulation of allergic responses, autoimmunity, transplant rejection, and tumor surveillance. While the immune system is composed of many different cell types that encompass many different effector capabilities, T lymphocytes are one of the most crucial elements of the adaptive immune system, and often critically influence the outcome of any given response. During development in the thymus, T lymphocytes acquire lineage-specific cell surface receptors, such as the T cell receptor (TCR), several different classes of intracellular signaling molecules, as well as transcription factors, which ultimately give the T cell its unique characteristics and ability to respond and perform specific functions during an immune response. Following development, non-self-reactive T lymphocytes are exported out of the thymus to the peripheral immune system as mature naïve T cells ready for the surveillance of invading organisms in the body.

Upon encounter with an invading organism, antigen-presenting cells (APCs) resident in the host pick up proteins and/or particles of these organisms, either from the extracellular environment or from intracellular compartments, and process these proteins into peptides. Following this breakdown process, antigenic peptides become associated with the major histocompatibility complex (MHC), which is exported to the cell surface as an MHC:peptide complex. The APCs from infected tissues proceed to migrate to the draining lymph nodes, a specialized lymphoid compartment that allows circulating T cells to survey the resident APCs for foreign MHC:peptide complexes. When a T lymphocyte specifically recognizes a foreign complex, it is quickly poised to alert the
immune system and direct an immune response via the secretion of cytokines and the expression of specific soluble and membrane-bound molecules [1]. The multitude of biological processes that occurs through the life of a T lymphocyte will be discussed in further detail below.

**T Cell Activation and Interleukin-2**

Depending on the stage of development of the T cell and its antigen recognition history, a variety of different cellular responses can be induced, making this one of the most fascinating aspects of T cell biology. Typically during an immune response, antigen-specific TCR stimulation initiates a complex series of signaling cascades within the naïve T cell. Among other events, the activation of specific signaling cascades leads to one of the hallmark events in T cells, which is the transcription and production of the T cell growth factor, Interleukin-2 (IL-2) [2]. Regulated primarily at the transcriptional level, the IL-2 promoter possesses binding sites for several families of transcription factors, such as NFAT, AP-1, and NF-κB, which are differentially regulated by upstream biochemical signals initiated at the TCR. This will be discussed in more detail later. Ultimately, upon binding to its receptor, IL-2 initiates cell cycle progression and allows antigen-reactive T cells to undergo a dramatic expansion within the lymphoid compartment, a crucial component of effective immune responses.

**T Cell Differentiation**

Some of the earliest studies examining the immune response to infectious pathogens observed an inclination to generate either an antibody-mediated or a cell-
mediated response, but never both. Further studies elucidated that antibody-mediated responses were important in the defense against extracellular pathogens, such as helminthes and most bacteria, presumably due to the fact that abundant amounts of pathogen-specific immunoglobulins functioned to neutralize the invading pathogen. Contrastingly, the observed cell-mediated responses appeared to be crucial for the defense against intracellular organisms, such as viruses, certain bacteria, as well as protozoans. This cellular-mediated form of immunity was characterized by a cytolytic capability of certain cells in addition to the production of soluble mediators, known as cytokines.

In 1986, after several years of dissecting pathogen-specific responses, Mossman and Coffman were able to identify mouse CD4+ T cell clones that had distinct functions as well as the competence to secrete unique cytokines. These cytokines were in turn found to be associated with either inflammatory immune responses, or with B cell help [3-5]. These distinct T cell clones were termed T helper 1 (Th1) or T helper 2 (Th2) effector cells, respectively. Th1 cells were found to preferentially produce IFN-γ and TNF-α, cytokines that could mediate delayed-type hypersensitivity [6]. Th2 effector cells, however, were characterized by the production of IL-4, IL-5, and IL-10, cytokines that were able to generate IgE and IgG1 responses from B cells [7]. The physiological relevance of these T cell clones was confirmed by Locksley and colleagues who demonstrated that Th1 and Th2 cells in fact exist in vivo during the immune response to *Leishmania major*. Additionally, Sadick et al demonstrated that resistance to this infection was correlated to the capacity to generate a Th1 response [8, 9].
In healthy individuals, following T cell development in the thymus, all CD4\(^+\) T lymphocytes that are exported to the periphery are naïve and must be given the appropriate activation signals to acquire the capability of performing effector functions, such as mediating inflammatory or humoral responses. The ability of a naïve CD4\(^+\) T cell to differentiate into a Th1 or Th2 effector cell is almost completely influenced by the cytokine milieu present during the initial activation. Several *in vitro* and *in vivo* studies have concluded that the time it takes for a naïve CD4\(^+\) T cell to commit to either the Th1 or Th2 lineage is only a few days [10]. Therefore, during an *in vivo* response, upon T cell encounter with an antigen, appropriate cytokines have to be present within hours to wield any effect on T cell priming. While the initial source of these cytokines *in vivo* is still unclear, different cell types within the immune system, including T cells themselves, are potential sources. For instance, NK cells are potent producers of IFN-\(\gamma\), a cytokine that aids in the development of Th1 cells, whereas basophils and mast cells are known to secrete IL-4, a Th2-driving factor. Needless to say, this is just a small illustration of the complexity of an immune response, which in most cases involves the orchestration of many different cell types that possess many different functions.

As opposed to what occurs in healthy individuals, disregulation of CD4\(^+\) T cells can have catastrophic consequences. For instance, a disproportionate amount of Th1 cytokines is often the cause of tissue destruction in many autoimmune diseases. Overall Th1 processes have been strongly associated with several autoimmune diseases such as inflammatory bowel diseases [11], multiple sclerosis [12], and diabetes [13]. Similarly, excess Th2 cytokines have been associated with allergic asthma and atopic diseases [14].
In many circumstances, cytokines from an opposing subset can offer protective effects by counter-acting factors that induce pathology.

Over the past decade, tremendous progress has been made in the understanding of the mechanisms involved in the development and differentiation of CD4+ T cells into either Th1 or Th2 cells. Furthermore, the TCR-induced signal transduction pathways that are activated following T cell stimulation that culminate in the transcription of genes involved in naïve CD4+ T cell differentiation are becoming increasingly clear. The dissection of these signaling pathways, which is the focus of a portion of my thesis work, will be described in greater detail later. The understanding of all of these events is imperative in efforts to control disease. Whether a host will succumb to or clear the invading pathogen is dependent on the ability of the host immune system to generate the proper response. The elicitation of an improper response can often lead to severe immunopathology, and in certain circumstances, death. One of the greatest challenges posed to immunologists today is the ability to understand the etiology of disease, but more importantly, to find methods to specifically control it and elicit protective immune responses.

T Cell Death

The immune system, akin to other organs in the mammalian system, has limited space to function. Therefore, the immune system must maintain a balance between cell proliferation and cell death, which is often termed homeostasis. As touched upon earlier, the numbers of white blood cells, such as lymphocytes and neutrophils, significantly expands following infection. This expansion allows immune cells to differentiate into
effector cells, kill infected cells, produce antibodies to neutralize invading bacteria or viruses, as well as perform many other functions. During this process, many harmful or toxic cells are generated, such as cytotoxic and potentially autoreactive lymphocytes. Furthermore, improper control of this process can lead to deleterious effects, such as splenomegaly, which can often lead to red blood cell destruction, anemia, and thrombocytopenia. Therefore, cell death mechanisms have evolved to allow the immune system to contract cell numbers following an immune response, while concomitantly maintaining repertoire diversity to ultimately maintain homeostasis [15, 16].

T lymphocytes can undergo apoptosis, or programmed cell death, through a variety of different mechanisms. One mechanism, termed cytokine withdrawal, is a passive form of apoptosis, where the deprivation of cytokines can induce cells to die. While this process does require new protein synthesis, it can be strongly inhibited by the membrane expression of Bcl-2 and Bcl-X. However, the process of mitochondrial breakdown and apoptosis following cytokine withdrawal is not clear, and is still an active area of research.

Another mechanism of T cell death, termed activated-induced cell death (AICD) occurs following repeated antigenic stimulation, which leads to the TCR-induced upregulation of the death effector molecules, FasL and TNF. These death effector molecules exert their function upon binding to their cognate receptors, Fas and TNF receptors (type 1 and 2), respectively, where molecules known as caspases are recruited to the cytoplasmic death domains of these receptors, thereby initiating the cell death process. The importance of this mechanism of apoptosis is exemplified in mice possessing mutations in either Fas (lpr mice) or FasL (gld mice), which exhibit defects in
T cell apoptosis, lymphoproliferation, as well as autoimmunity. Moreover, abnormalities in the Fas gene in humans manifests as a severe autoimmune disorder [17-19].

Apoptosis can occur throughout the life of a T cell, whether it be resting or activated. Nevertheless, cell death is extremely important following antigenic challenge to maintain tolerance and homeostasis in the immune system. Akin to T cell differentiation, the understanding of the molecular basis of the TCR signaling pathway which leads to T cell apoptosis is crucial for understanding disease processes. This aspect of T cell biology will be discussed in more detail later, as it is also a major focus of my thesis work.

**Tyrosine kinases**

Over the past twenty years, significant progress has been made in elucidating signaling pathways triggered by the engagement of immune cell receptors for antigen, such as the TCR, B cell receptor (BCR), and Fce receptor. In T lymphocytes, several protein tyrosine kinases are immediately phosphorylated and consequently activated following TCR engagement. Tyrosine kinases are often regulated by phosphorylation and in turn function to either positively or negatively regulate specific substrates by the transfer of a phosphate group from adenosine triphosphate (ATP) to a tyrosine residue on the substrate. These phosphorylation events can also function to generate binding sites for other proteins. For instance, a protein containing an Src homology 2 (SH2) domain can bind to a phosphorylated tyrosine, depending on the context. As touched upon earlier, signaling pathways that are initiated by these tyrosine kinases can lead to a variety of
cellular outcomes, including cytoskeletal rearrangement, cytokine production, clonal expansion, cell differentiation, and apoptosis.

**Src and Syk family kinases**

Based on specific motifs or sequence homology, kinases have been grouped into families. Two of the initial cytoplasmic kinases that were discovered and characterized in lymphocytes, Lck and Fyn, were revealed to be members of the largest family of non-receptor protein tyrosine kinases, the Src family. Following TCR activation, Lck and Fyn are rapidly activated and function to phosphorylate specific tyrosine residues on the cytoplasmic domains of TCR components known as CD3 molecules. These phosphorylation events occur within motifs known as immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3 molecules. Several reports have demonstrated that the phosphorylation of the ITAM motifs is crucial for signaling through antigen receptors [20-23]. Another TCR proximal kinase is ZAP-70, a member of the Syk family of tyrosine kinases. Following the phosphorylation of the ITAM motifs on CD3-ζ, ZAP-70 is recruited to this motif where it is phosphorylated by Lck, and in turn activated [20, 24, 25]. In an effort to dissect TCR-initiated biochemical signaling events and connect them to functional outcomes, many researchers make use of the immortalized Jurkat T cell line. In the absence of the Src or Syk family kinases, Lck or ZAP-70, respectively, Jurkat cells fail to mobilize calcium, an important biochemical signaling event following T cell stimulation. Additionally cells lacking either kinase fail to produce IL-2 following activation [26, 27]. The physiological consequence of mutations in one or more Src or Syk family kinases is exhibited in mice, where either severe defects or blocks in T cell
development and function are observed. Furthermore, human patients possessing mutations in ZAP-70 also exhibit severe functional defects in peripheral T cells, which result in a severe combined immunodeficiency (SCID) condition [28]. Taken together, both genetic and biochemical data exemplify the importance of TCR proximal Src and Syk family tyrosine kinases during thymic development as well as T lymphocyte activation in the periphery.

**PLC-γ and Adaptor Proteins**

While many of the events that immediately occur following TCR ligation are now well-established, up until a few years ago, less was known about the substrates of these TCR proximal molecules, and how these substrates affect more distal events such as T cell proliferation, differentiation, and death. One crucial event during T cell activation is the mobilization of intracellular calcium stored in the lumen of the endoplasmic reticulum (ER). This process is known to activate several different families of signaling molecules and, ultimately, transcription factors that will activate genes essential in T cell activation, namely IL-2. Therefore, many researchers have dedicated their efforts towards elucidating the early TCR signaling events that are coupled to calcium mobilization.

Following TCR ligation, PLC-γ cleaves phosphatidyl inositol bisphosphate (PIP$_2$) into two byproducts, inositol trisphosphate (IP$_3$) and diacylglycerol (DAG). The released IP$_3$ binds to IP$_3$ receptors in the ER membrane, which in turn triggers the release of stored calcium. DAG, on the other hand, is thought to activate members of the PKC family as well as Ras-GRP, thereby activating the Ras/Raf/MAP kinase pathway (see Figure 4). Interestingly, the pharmacological agents, Ionomycin and PMA, which are often used to
stimulate T cells in vitro, function by bypassing proximal TCR signaling molecules that lead to PLC-γ activation, by mimicking IP₃ and DAG production, respectively. These agents can fully activate T cells to induce cytokine secretion and express activation markers, clearly exemplifying the importance of PLC-γ activation in lymphocytes as a crucial event for T cell activation [29].

Several years ago, Finco et al found that Jurkat cells deficient in LAT (linker of activated T cells), a integral membrane protein with no enzymatic activity, possessed defects in calcium flux, Ras activation, and ultimately IL-2 production [30]. While LAT was found to be a substrate of ZAP-70, it was further discovered that LAT associates with PLC-γ following TCR ligation. In addition, this step was found to be dependent on PI3-kinase, a kinase that converts PIP₂ to phosphatidyl inositol bisphosphate (PIP₃), thereby providing a binding site for the PH-domain of PLC-γ. PLC-γ is phosphorylated on multiple tyrosines following receptor engagement, and several pieces of evidence would suggest that Syk family kinases play an important role in this event [31]. However, an emergence of data over the past five years provide strong evidence that another family of tyrosine kinases, the Tec family, is directly involved in the activation of PLC-γ.

**Tec family kinases**

In 1990, almost twelve years following the discovery of the Src kinase, a new type of non-receptor protein tyrosine kinase, termed Tec (tyrosine kinase expressed in hepatocellular carcinoma), was discovered in an effort to identify tyrosine kinases involved in hepatocarcinogenesis [32]. Shortly thereafter, several other related proteins were discovered and grouped into what is now recognized as the second largest family of
non-receptor tyrosine kinases, the Tec family. The importance of Tec family kinases was
highlighted in 1993 by the discovery that point mutations or disruptions in the gene
encoding one family member, Btk, leads to immunodeficiency diseases in both humans
and mice (X-linked agammaglobulinemia (XLA) and X-linked immunodeficiency (xid),
respectively) [33-35].

To date, the Tec family consists of eight members, only five of which are
expressed in mammals: Bmx/Etk, Btk/Atk, Itk/Tsk/Emt, Tec, and Rlk/Txk. In general,
Tec family kinases are expressed in cells of the hematopoietic lineage, but are not
restricted to a particular cell type. For instance, Tec is expressed in all hematopoietic
cells, while Itk and Rlk are restricted to T lymphocytes, NK cells, and mast cells. Btk is
expressed in all hematopoietic cells, with the exception of T lymphocytes and plasma
cells. Interestingly, akin to the other tyrosine kinase families, some Tec kinases are
expressed in cells outside of the hematopoietic lineage; however, I will only focus on
those expressed in the immune system.

**Tec family structure and localization**

The Tec, Syk, and Src family members have similar and distinctive structures.
While each Src family kinase contains an SH1 (kinase domain), SH2, and SH3 domain,
members of the Syk family possess an SH1 domain, followed by two SH2 domains. The
Tec family, however, possess additional domains with distinctive functions. In addition to
possessing an SH1, SH2, and SH3 domain, that is more similar to Src family kinases,
most Tec family members also possess a Tec homology (TH) domain, which contains a
Zn\(^{2+}\)-binding Btk motif, followed by one or two proline-rich region(s) (PRR).
Furthermore, Tec family kinases lack the C-terminal negative regulatory tyrosine found in Src kinases, suggesting a distinct mode of regulation for these proteins (see Figure 1). With the exception of Rlk, all Tec kinases have an N-terminal Pleckstrin homology (PH) domain, which associates with PIP₃ and plays a role in targeting the proteins to the membrane.

An important step in the activation of Tec family members following TCR engagement is localization to the membrane. Recent work has demonstrated that Tec kinases, via the PH domain, are targeted to lipid rafts or glycolipid-enriched membranes (GEMs), which are membrane microdomains that are thought to facilitate the assembly of signaling molecules upon receptor engagement [36, 37]. This event is dependent on the action of PI3-kinase, which converts PIP₂ in the membrane to PIP₃, providing a binding site for PH domain-containing proteins. The atypical Tec family kinase, Rlk, which lacks the N-terminal PH domain, instead possesses N-terminal cysteine repeats, a post-translational modification signal for palmitoylation. This palmitoylation event functions to target Rlk to GEMs [38]. Furthermore, as a result of an internal translation initiation site, a shorter form of Rlk which is also expressed, translocates to the nucleus upon activation, suggesting that Rlk may have distinctive functions in the nucleus and cytosol [39]. In fact, Takeba et al discovered that Rlk can function as a transcriptional activator of the IFN-γ gene, as will be discussed later [40]. While a nuclear function for Tec family members in T cells appeared to be restricted to Rlk, very recent work from Perez-Villar et al found that Itk can also translocate to the nucleus in Jurkat cells via its association with a nuclear importin Rch1α/karyopherinα [41]. Future experiments will be
required to elucidate the more precise role(s) of Tec family kinases in the nucleus and to determine more specifically if and how they may modulate gene expression.
Figure 1: Domains of the Tec, Syk, and Src Family Kinases

Tec Domains: Similar to Src and Syk kinases, Tec kinases possess a C-terminal kinase domain, followed by an SH2 domain. This is typically followed by an SH3 domain in the Src and Tec family kinases, however, the Syk family lacks this domain and instead has another SH2 domain. The unique characteristics of Tec family kinase structure are in the N-terminal region of the protein. Btk and Tec both possess two proline-rich regions (PRRs), while Itk and Rlk only possess one. A Btk homology (BH) domain, the function of which is still not clear, and a Pleckstrin homology (PH) domain are N-terminal to the PRR of Btk, Tec, and Itk. Rlk is the only known Tec family member that does not possess an N-terminal BH and PH domains. Instead, Rlk possesses an N-terminal cysteine-rich region, which, following palmitoylation, targets Rlk to the membrane. At the C-terminus, Tec family kinases lack the regulatory tyrosine found in Src family members.
Figure 1: Domains of Tec, Syk, and Src Family Kinases

Expression pattern
Btk: B & Mast cells
Tec: T, B & Myeloid cells
Itk: T & Mast cells
Rlk: T, Mast, & NK cells
Bmx: Granulocytes & Myeloid cells
Src
Syk
Positive and Negative Regulation of Itk Following TCR Stimulation

Positive Regulation

The mechanisms involved in the complex signals required for Tec kinase activation, or positive regulation, have been elucidated by several groups over the past several years. Much of this information was largely generated from several biochemical experiments performed in cell lines or inferred from experiments performed in cells lacking specific signaling molecules. Based on all of this information, a mechanism of Tec family regulation has been generated. The currently accepted mechanism of Itk activation and de-activation following TCR engagement is discussed below.

Immediately following TCR stimulation, Src and Syk family kinases are activated, leading to the phosphorylation of two adaptor molecules, LAT, which is constitutively associated with lipid rafts, and SLP-76, a cytoplasmic adaptor protein. Concurrently, stimulation leads to a PI3-kinase-dependent increase in PIP3 levels within the cell membrane. The enrichment of PIP3 at the site of the activated receptor recruits Itk to the membrane in a PH domain-dependent manner. Following this recruitment, a multi-molecular signalosome complex consisting of Itk, PLC-γ1, SLP-76, Gads, and Grb-2 form around LAT within lipid rafts. Itk is then transphosphorylated by a Src kinase, followed by Itk autophosphorylation [42], resulting in complete activation of the kinase. Therefore, two events are critical for the proper activation and function of Itk and Tec kinases, in general: localization to the membrane and tyrosine phosphorylation (Figure 2)(reviewed in [43-46]).

As briefly mentioned earlier, successful T cell activation requires not only signals emanating from the TCR, but also signals from costimulatory molecules, such as CD28
and ICOS. Data supporting a function for Tec family members downstream from CD28 is obscure. Early studies examining costimulatory function concluded that CD28 signals are required for efficient IL-2 production and the prevention of anergy. In addition, it was soon discovered that there were tyrosine phosphorylation events that occurred following CD28 ligation. In an attempt to identify molecules that signal downstream from CD28, August and colleagues reported that Itk associated with the intracellular tail of CD28 and was rapidly phosphorylated following CD28 cross-linking [47]. Additional biochemical data from Gibson et al identified tyrosines in CD28 that were required for the activation of Itk but not PI3-kinase [48]. However, the integration of biochemical signals that emanated from the TCR versus CD28 remained elusive. To further understand the distinct signals from both the TCR and CD28 to determine where they intersected, Michel and colleagues found that CD28 signals amplified PLC-γ1 and calcium responses. Furthermore, their data suggested that CD28 may act to provide activated Itk to the TCR-induced signalosome complex, thereby contributing to the amplification of the calcium response [49]. These data support a mechanism by which CD28 provides a quantitative contribution to T cell activation. However, preliminary data in our laboratory would suggest that Itk is not required for CD28 signaling (C.M. Li and L.J. Berg, unpublished data).

Negative Regulation

One mechanism that likely contributes to the down-regulation of Itk activity is through the action of the phosphatase PTEN, which converts the PH domain substrate, PIP₃, back to PIP₂. This event functions to prevent further recruitment of Itk molecules.
to the site of the activated receptor. However, until recently, little was known about other mechanisms that may be involved in actually down-regulating Itk kinase activity. Recent biochemical and structural studies of Itk by Brazin and colleagues have demonstrated that Itk kinase activity is inhibited by a direct interaction between the Itk SH2 domain and the peptidyl-prolyl isomerase, cyclophilin A (CypA). Consistent with this, treatment of Jurkat T cells with Cyclosporin A, an immunosuppressive drug that binds CypA and inhibits calcineurin activity, disrupts this interaction and results in increased Itk phosphorylation and activation of downstream targets (e.g., PLC-γ). Using NMR structural analysis, this study also showed that the Itk SH2 domain could undergo a proline-dependent conformational switch via a single prolyl imide bond. Furthermore, this cis/trans isomerization within Itk, which is thought to be catalyzed by CypA, directly alters Itk binding specificity for substrates [50]. These intriguing findings suggest a novel mechanism of Itk regulation by CypA. Interestingly, the proline residue involved in this isomerization reaction is not found in any of the other Tec kinase family members, suggesting that this mode of regulation may be unique to Itk (Figure 3).
Figure 2: Positive Mechanisms Regulating Itk Function

Following the engagement of the T cell receptor (TCR), Src family kinases, such as Lck, are activated. Lck phosphorylates the ITAM motifs within the CD3ζ chains, which in turn recruits the Syk family kinase ZAP-70, leading to ZAP-70 phosphorylation and activation by Lck. Activated ZAP-70 proceeds to phosphorylate the adaptor molecules SLP-76 and LAT. Concurrently, PI3-kinase is activated, which catalyzes the conversion of membrane-associated PIP$_2$ to PIP$_3$, the substrate of PH domain-containing proteins such as Itk and PLC-γ1. Cyclophilin A (CypA) has been shown to be constitutively associated with inactive Itk via Itk’s SH2 domain. However, the mechanism by which CypA becomes dissociated is not known. Following Itk’s recruitment to the membrane via its PH domain, a number of complex interactions ensue. While SLP-76 binds to the SH2 and SH3 domains of Itk, the interaction of SLP-76 with Gads leads to an interaction with LAT. Grb-2 can bind to the free PRR of Itk, which can also bind to LAT. Furthermore, PLC-γ1 recruitment to the membrane via its PH domain allows the N-terminal SH2 domain of PLC-γ1 to bind to a phosphotyrosine on LAT. These interactions result in what is termed a signalosome complex. Lastly, following the phosphorylation of Itk by a Src kinase, Itk can in turn autophosphorylate, then proceed to phosphorylate and activate PLC-γ1.
Figure 2: Positive Mechanisms Regulating Itk Function

TCR

PIP2

PI3-K

Src

ZAP-70

PIP

Recruitment of signalosome complex and phosphorylation of Itk by a Src kinase

SLP-76

Itk is recruited to membrane via PIP3.

LAT

ZAP-70 phosphorylates SLP-76 and LAT.

Active Itk phosphorylates PLC-γ

Itk autophosphorylates

Active Itk phosphorylates PLC-γ

Key

Grb-2  gads

Cyclophilin A
Figure 3: Mechanisms Down-Regulating the Activity of Itk

In T cells, following activation of PLC-γ1 by Itk, CD148, a membrane-associated phosphatase, is thought to either indirectly or directly dephosphorylate members of the signalosome complex, such as LAT and SLP-76, thereby terminating signaling. Furthermore, PTEN is also thought to play a crucial role in the down-regulation of Itk, by converting the PH domain substrate, PIP3, back to PIP2, likely preventing the further recruitment of Itk molecules. The mechanism by which CypA becomes re-associated with Itk, and the mechanism by which Itk folds back into its inactive conformation are not known.
Figure 3: Mechanisms down-regulating the activity of Itk
Downstream Effects of Tec Kinase Activation in T Lymphocytes

Several of the initial biochemical and genetic studies utilizing either cell lines or lymphocytes from mice lacking a Tec family member have demonstrated that the absence of Tec family kinases is associated with reduced calcium flux, reduced MAP kinase activation and transcriptional activation, as well as cytoskeletal rearrangements following receptor engagement. A summary of these findings will be discussed shortly, however, an emphasis will be placed on Tec members expressed in T lymphocytes.

Btk, the most well-characterized kinase of all the Tec family members due to its role in the diseases XLA in humans and xid in mice, is expressed in B lymphocytes [51, 52]. The role of Btk in proximal BCR signaling became apparent following the observation that treatment of Btk⁻/⁻ cells with the pharmacological agents, PMA and Ionomycin, bypassed the signaling defect, suggesting that Btk functioned upstream of calcium and PKC activation [53, 54]. Biochemical analyses from Btk-deficient chicken DT40 B cells revealed that the phosphorylation and activation of PLC-γ2 following BCR stimulation was severely reduced [55, 56]. These findings led to the more recent identification of the four Btk-dependent tyrosine phosphorylation sites in PLC-γ2, which are all required for full BCR-coupled calcium mobilization [57].

Upon the identification and characterization of Btk function in B lymphocytes, it became apparent that the T lymphocyte-restricted kinase Itk possessed functional characteristics that closely resembled that of Btk [58]. Liu and colleagues, one of the first groups to generate Itk-deficient mice, established that Itk is important for the phosphorylation and activation of PLC-γ1 in T cells [59, 60]. Wilcox and colleagues more clearly defined the role of Itk in PLC-γ1 activation biochemically, and
demonstrated that, in fact, Itk directly phosphorylates PLC-\(\gamma\)1 [42, 61]. The function of Rlk in TCR signaling is less clear. While Rlk appears to play an important role in the phosphorylation of PLC-\(\gamma\)1 by targeting the adaptor molecule SLP-76, the absence of Rlk in T lymphocytes does not impair IL-2 production and proliferative responses. However, the combined loss of Itk and Rlk results in exacerbated defects in TCR signaling, such as PLC-\(\gamma\)1 activation and calcium mobilization, compared to the absence of Itk alone [62-64]. This will be discussed in more detail later.

As mentioned earlier, the phosphorylation and complete activation of PLC-\(\gamma\)1, is a crucial step in T cell activation [29]. Upon activation, PLC-\(\gamma\)1 hydrolyzes PIP\(_2\) into two byproducts, IP\(_3\) and DAG, which lead to calcium mobilization and activation of the PKC/Ras/Raf pathways, respectively. More specifically, following IP\(_3\) production, IP\(_3\) receptors in the ER membrane induce the release of stored calcium into the cytosol, which in turn leads to the activation of calcium release activated calcium (CRAC) channels. This event allows the influx of calcium from the extracellular environment into the cytosol. In lymphocytes, several cytosolic calcium-sensitive transcription factors, such as NFAT family members, NF-\(\kappa\)B, and JNK, are activated. These factors promote the expression of several genes essential for lymphocyte activation, such as IL-2. In fact, the nature of the calcium signal, whether sustained or transient, can have differential effects on transcription factor activation. For instance, the NFAT family typically requires a continuous rise in calcium to translocate to and remain localized in the nucleus [65, 66]. The production of DAG can activate members of the Protein Kinase C (PKC) family. Activated PKCs are known to activate transcription factors such as NF-\(\kappa\)B, and components of the AP-1 complex, c-Fos and c-Jun [67, 68]. DAG is also involved in
activating Ras-GRP, which ultimately leads to the activation of the Ras/Raf/MAP kinase pathway. Certain transcription factors, such as NFAT and AP-1 components, can interact cooperatively to properly drive transcription of specific effector genes [69, 70]. NF-κB activation is dependent not only on signals emanating from the TCR, but also CD28-initiated signals (see Figure 4).
Signal transduction events initiated by the TCR and costimulatory molecules commences with an early wave of activity by protein tyrosine kinases, such as Lck and ZAP-70 as well as Tec family kinases, such as Itk and Rlk. One of the crucial events in T cell activation following the activation of these kinases is the activation of PLC-γ1. In order for this reaction to occur efficiently, the adaptor molecules SLP-76 and LAT are required. Activated PLC-γ1 cleaves membrane PIP₂ into IP₃ and DAG. Calcium activates the calcineurin-dependent dephosphorylation of cytoplasmic NFAT, allowing NFAT to translocate to the nucleus. DAG functions to activate distinct PKC isoforms and the Ras pathway via Ras-GRP. Activated Ras recruits Raf, which consequently activates multiple downstream mitogen-activated protein (MAP) kinases, such as ERK, JNK, and p38. The MAP kinase pathways play an essential role in the phosphorylation and activation of the transcription factors fos and jun, which dimerize to compose the AP-1 transcription complex. In the nucleus, NFAT and AP-1 factors bind cooperatively to cytokine and effector gene promoters. Another factor important for T cell function is NF-κB, which is dependent on TCR and CD28 activation. Following the dissociation of IκB from the IκB/NF-κB complex, NF-κB can translocate to the nucleus and activate T cell effector genes. Proper activation of all of these signaling pathways are required for an effective T cell responses and immunity.
Figure 4: TCR signaling downstream from Itk

TCR → ITK

CD28

PLC-γ1

LAT

PI3K

ITK SLP-76

Ras/Raf

NFAT

NF-κB

cytoskeletal reorganization

cytoplasmic reorganization

Effector and cytokine genes

Ca++

IP₃

PIP₂

DAG

PKC

ERK

JNK

p38

AP-1
Impairments in PLC-γ activation, and ultimately in the production of IP₃ and DAG, can affect several downstream events in T lymphocytes. As mentioned above, both Btk-deficient and Itk-deficient cells possess impairments in the activation of PLC-γ₁. Upon examination of signaling events downstream of PLC-γ₁ in Itk-deficient cells, calcium mobilization, the activation of NFAT family members, MAP kinases, as well as AP-1 components, were all impaired following TCR activation, as would be predicted based on our knowledge of PLC-γ [59, 60, 71-74]. All of these events were strikingly similar to what was found to occur in Btk-deficient B cells [57]. However, Tec- and Rlk-deficient cells failed to exhibit any significant defects in any of the T cell signaling events that were impaired in Itk⁻/⁻ cells [59, 75]. This observation suggested the possibility of functional redundancy between these Tec family kinases. Therefore, Schaeffer et al took the additional task of generating Rlk⁻/⁻Itk⁻/⁻ mice. The combinatorial mutations of Itk and Rlk in CD4⁺ T lymphocytes exacerbated the defects observed in Itk-deficient cells [59, 64, 72]. Interestingly, upon comparison of Itk, Rlk, and Tec expression levels in CD4⁺ T lymphocytes, we found a hierarchical pattern of expression, where Itk was 2-fold greater than Rlk and 100-fold greater than Tec. This finding strongly correlates with the severity of the phenotypes observed in each of the Tec family-deficient mice (Figure 5).
Figure 5: Relative Expression Levels of Tec Family Members in CD4⁺ T Cells

Quantitative PCR analysis of mRNA levels of the Tec family kinases in CD4⁺ T lymphocytes revealed that Itk is expressed at levels that are 2-fold greater than that of Rlk and 100-fold greater than that of Tec. This information strongly correlated with the severity of the phenotypes that are observed in mice that lack one or more Tec family kinases, where the most striking phenotype is observed in mice lacking both Itk and Rlk, and the least striking in mice lacking Tec. Phenotype severity is based on several observations, including impairments in T cell development, T cell function, and the activation of specific biochemical pathways.
Figure 5

Relative expression levels of Tec family members in CD4\(^+\) T cells

Phenotype severity

Itk\(^{-/-}\)Rlk\(^{-/-}\) > Itk\(^{-/-}\) > Rlk\(^{-/-}\) > Tec\(^{-/-}\)
Ultimately, all of the impaired events discussed above have significant and numerous effects on transcriptional events and effector functions. Reduced NFAT activation in Itk-deficient cells contributes to an impairment in the transcription of several effector genes [60, 72-74]. One of the initial observations of T cell function in the absence of Itk was an impaired proliferative capacity as a result of reduced IL-2 production [60, 76]. Studies of Itk$^{-/-}$ and Rlk$^{-/-}$/Itk$^{-/-}$ mice have also revealed additional roles for Tec kinases in regulating T cell effector functions. For instance, after TCR stimulation \textit{in vitro}, Rlk$^{-/-}$/Itk$^{-/-}$ and Itk$^{-/-}$ CD4$^{+}$ cells are defective in their ability to secrete both Th1- and Th2-type cytokines, a finding that correlates with reduced activation of NFAT and AP-1 transcription factors [72]. As will be discussed later, the majority of my thesis project involves a more careful examination of how Itk regulates distinct T cell effector responses.

In the absence of the more TCR proximal tyrosine kinases, Lck and ZAP-70, calcium mobilization and MAP kinase activation are completely abolished, demonstrating their necessity in TCR signaling [28, 77]. However, in the absence of Tec kinases, as discussed above, all of the signals downstream from the TCR are merely reduced in intensity and duration, suggesting that Tec kinases play more of a modulatory role in receptor signaling. At this point, it is not clear whether this is a result of redundancy of Tec kinase function or whether there are clearly distinct functions. In order to better define Tec family kinases in TCR signal transduction, the generation of mice deficient in all three T cell restricted Tec members (Itk, Rlk, and Tec) will be required.
Other pathways involving Tec kinases

During T cell activation, antigen receptor signaling is highly dependent on actin cytoskeleton reorganization. This process allows numerous signaling molecules to converge at the membrane to ultimately form complexes that are required for proper signal transduction to the nucleus [78]. In T lymphocytes, TCR signaling relies greatly upon the rearrangement of actin filaments, which assist in the formation of the immunological synapse, a structure that is crucial for prolonged TCR signals [79]. Other cellular events following T cell activation, such as cell adhesion, are also highly dependent on actin cytoskeleton integrity.

The first suggestion that Tec family kinases may contribute to actin cytoskeletal reorganization in lymphocytes came from the finding that both Itk and Btk can bind to WASP (Wiskott-Aldrich syndrome protein), a protein involved in the nucleation of new actin filaments [80, 81]. Data from Woods et al. demonstrated that after anti-CD3 antibody stimulation of Jurkat T cells, Itk can activate β1 integrins and β-actin polymerization following its membrane translocation [82]. Previous data have also supported a role for Itk in cytoskeletal reorganization where Itk-deficient cells possess reduced cap formation following TCR stimulation (reviewed in [83]). Defective reorganization of cytoskeletal structures may contribute to defects in calcium mobilization in Itk-deficient cells.
Functional Roles of Tec Kinases in Biological Processes:

T cell Development

The discovery that mutations in Btk lead to defects in pre-B cell development due to blocks in BCR-mediated proliferative responses was the first indication that Tec family kinases are required for developmental processes. Following the generation of Itk-deficient mice, one of the first detectable phenotypes was the striking reduction in the total numbers of peripheral CD4^+ cells, while CD8^+ numbers were unaffected. This indicated possible defects in thymic selection. While data from our laboratory suggest that Itk^-/- mice exhibit selective defects in the positive selection of CD4^+ cells, and not negative selection, further evidence from Schaeffer et al showed that both Itk^-/- and Rlk^-/- Itk^-/- thymocytes possess defects in both positive and negative selection, in addition to exhibiting altered CD4:CD8 ratios, due to fewer mature CD4^+ thymocytes and increased numbers of CD8^+ thymocytes [43-45, 59, 64, 84]. Consistent with a model in which diminished TCR signals in the absence of Tec kinases affects thymocyte fate, defects in thymic selection correlate with reduced TCR signaling, resulting in reduced calcium mobilization and reduced ERK-MAPK activation in T cells from Itk^-/- and Rlk^-/-Itk^-/- mice [64]. Interestingly, in spite of the reduced ERK-MAPK signaling and the altered CD4:CD8 ratio, detailed analysis of several TCR transgenic lines on an Itk^-/- background did not reveal any alterations in CD4:CD8 lineage commitment in these mice [84]. The possibility that there are redundant and unique functions of Tec kinases in TCR signaling was discussed earlier. The generation of Rlk^-/- mice has revealed a seemingly insignificant role for Rlk in T cell development and function. However, overexpression
of Rlk in Itk<sup>−/−</sup> mice led to a partial rescue of positive selection and calcium mobilization, supporting the notion that Tec members may have redundant functions in T cells [62].

**Effector Development and Immune Responses**

**Responses to viruses**

The first study assessing the ability of Tec kinase-deficient cells to respond *in vivo*, examined antiviral immune responses to three different viruses, vesicular stomatitis virus (VSV), vaccinia virus (VV), and lymphocytic choriomeningitis virus (LCMV) in Itk<sup>−/−</sup> mice [85]. Several different effector mechanisms are required for clearance of these particular viruses. Therefore, this study allowed the assessment of CD8<sup>+</sup> and CD4<sup>+</sup> T cell function, in addition to B cell function, in the absence of Itk. For instance, LCMV is almost exclusively cleared by CD8<sup>+</sup> cytotoxic T lymphocytes (CTL), whereas the clearance of VV is more dependent on both CD4<sup>+</sup> and CD8<sup>+</sup> cells [86-90]. Contrastingly, clearance of VSV, which is exclusively eliminated by antibodies, requires a strong early T cell-independent IgM response, followed by a more prolonged T cell-dependent IgG response [91-93].

This study by Bachmann and colleagues found that CTL responses were reduced 2- to 6-fold against all three viruses, indicating a role for Itk in CD8 T cell function. However, while LCMV was completely cleared by day 8 post-infection, the response to VV was cleared with delayed kinetics. The difference between these two responses in Itk-deficient mice may be a reflection of different mechanisms used by the host to eliminate these viruses. Lastly, upon infection with VSV, Itk-deficient mice possess normal T cell-independent and T-dependent B cell responses, and possessed no symptoms of
encephalitis [85]. In summary, this study revealed an important, though not essential, role for Itk in CTL responses to viruses and antiviral immunity.

Intracellular and Extracellular Parasitic Responses and Allergic Responses

Prior studies performed by both Fowell et al. and Schaeffer et al have shown that the biochemical defects observed in Tec kinase-deficient T cells translate in vivo to impaired immune responses upon infection with various pathogens [72, 94]. These defects are observed in responses to pathogens requiring both Th1 and Th2 effector functions for clearance (Table 1). For instance, mice deficient in Rlk, Itk, or both, possess graded defects upon infection with Toxoplasma gondii, an intracellular protozoal pathogen that normally induces a protective Th1 immune response in wild type mice. These data indicate that mice deficient in one or more Tec kinases are incapable of mounting a protective Th1-type CD4+ T cell response to this pathogen.

In contrast, a second study found that Itk−/− mice on both the C57Bl/6 and Balb/c background were able to clear an infection of Leishmania major, an intramacrophage parasite that also requires a Th1 response for protective immunity [94]. This latter finding is particularly interesting as L. major normally elicits a protective Th1 response in C57Bl/6 mice and a non-protective Th2 response in Balb/c mice. While at face value the responses to T. gondii and L. major seem totally incompatible, it is possible that the discrepancy may result from the different requirements for protective immunity to these two pathogens. For instance, T. gondii infects many cell types, proliferates and spreads rapidly in the host, and requires a fast and robust IFN-γ response to prevent lethality. In contrast, L. major infects primarily macrophages and dendritic cells, spreads slowly in
the host, and can be cleared by a slower and less robust Th1 response [95]. Thus, it is possible that reduced kinetics, reduced efficiency, and a reduction in the overall magnitude of the response in Itk\(^{-/-}\) mice compared to wild type mice might explain the different outcomes in response to these two different pathogens.

The responses of Itk\(^{-/-}\) mice to pathogens that require a Th2 effector response for protective immunity are more consistent. In response to the nematode, *Nippostrongylus brasiliensis*, Balb/c mice make a protective Th2 response, however, Itk\(^{-/-}\) Balb/c mice fail to clear the nematode and show a significant reduction in the number of cells making IL-4 compared to wild type mice [94]. Following this discovery, Schaeffer et al reported that Itk\(^{-/-}\) mice on the mixed 129 x C57Bl/6 background were also unable to mount a sufficient Th2-type response to the helminth, *Schistosoma mansoni* [72]. Consistent with the overall reduction in the response to this pathogen, cells isolated from the lymph nodes of infected Itk\(^{-/-}\) mice produced less of the Th2 cytokines, IL-4, IL-5, and IL-10, and more of the Th1 cytokine IFN-\(\gamma\), compared to cells from infected wild type mice. Paradoxically, however, this study also showed that Rlk\(^{-/-}\)Itk\(^{-/-}\) mice, which are more defective in signaling and effector function *in vitro*, were able to elicit a normal Th2 response to *S. mansoni*, similar to that elicited in wild type mice infected with this parasite. Thus, while the Rlk\(^{-/-}\)Itk\(^{-/-}\) situation is unclear, Itk\(^{-/-}\) mice seem to have a significant defect in eliciting Th2 responses, presumably as a consequence of the CD4\(^+\) T cells having an impaired ability to activate NFATc1 and AP-1, and ultimately, to produce IL-4.

As discussed earlier, an immune response is an orchestration of events involving many different cell types. Therefore, how T cells respond *in vitro* may not necessarily
reflect how they function \textit{in vivo}. While these studies have clearly established that Tec family kinases are critical components of signal transduction pathways that control T cell differentiation, there are several remaining questions regarding the mechanisms by which Tec kinases act on pathways affecting genes important in T cell differentiation. Part of my thesis work entailed further clarification of the function of Itk during T cell effector development or differentiation.
Table 1. T helper responses in Itk\(^{-}\) and Rlk\(^{-}\)Itk\(^{-}\) mice

<table>
<thead>
<tr>
<th>Pathogen (Ref)</th>
<th>Protective Wild Type Response</th>
<th>Response of Itk(^{-}) and/or Rlk(^{-})Itk(^{-})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. major</em> [74]</td>
<td>Th1 (B6)</td>
<td>Itk(^{-}) (B6/129) = resistant Itk(^{-}) (Balb/c) = resistant</td>
</tr>
<tr>
<td><em>T. gondii</em> [59]</td>
<td>Th1</td>
<td>Itk(^{-}) (B6) = susceptible (MST = 69 days) Rlk(^{-})Itk(^{-}) (B6) = susceptible (MST = 41 days)</td>
</tr>
<tr>
<td><em>N. brasilensis</em> [74]</td>
<td>Th2 (Balb/c)</td>
<td>Itk(^{-}) (Balb/c) = susceptible</td>
</tr>
<tr>
<td><em>S. mansoni</em> [72]</td>
<td>Th2</td>
<td>Itk(^{-}) (B6) = susceptible Rlk(^{-})Itk(^{-}) (B6) = resistant</td>
</tr>
</tbody>
</table>

MST is mean survival time.
Work presented in this thesis

The primary focus of my thesis work sought to provide a better understanding of how Itk functions in signaling pathways that regulate distinct immune outcomes. The work presented here has provided new insights into the biology and function of Tec kinases in several important aspects of CD4+ T cell effector function, such as AICD and T cell differentiation.

Presented in chapter two, the first issue I focused on was the function of Itk in the process of activation-induced cell death (AICD) of CD4+ cells, an important mechanism of immune homeostasis. Based on information known about the control of the FasL promoter and its similarity to the IL-2 promoter, in addition to the striking increase in the percentage of CD4+ cells with a memory/activated phenotype in Itk−/− mice, we had hypothesized that Itk may play an important role in the signaling pathway that leads to FasL expression. Using several cellular and molecular approaches, I found that in the absence of Itk, CD4+ cells possess an impairment in apoptosis via the Fas-FasL pathway due to a substantial reduction in surface FasL expression by activated Itk−/− CD4+ T cells. Mechanistic analysis underlying this FasL defect was revealed to be the failure of Itk−/− T cells to efficiently upregulate Egr2, Egr3, and consequently, FasL transcription after TCR stimulation. Furthermore, I was able to demonstrate the physiological consequence of these defects in vivo upon immunization with the superantigen staphylococcal enterotoxin B (SEB), where CD4+ T cells from Itk−/− mice were incapable of undergoing AICD efficiently. [71]. This study defined an important role for Itk in TCR signaling, leading to cytokine gene expression and activation-induced cell death.
The experiments presented in chapter three proceed to further define a role for Itk in other CD4+ T cell effector functions. Due to the observation that Itk−/− mice exhibit a two- to three-fold increase in the percentage of CD4+ cells with an activated/memory phenotype, we sought to examine the behavior and differentiation status of these cells in order to gain clues as to how Itk may be regulating additional T cell effector functions. While memory cells from Itk-deficient mice in fact behave like memory cells, as determined by their ability to rapidly produce abundant amounts of IL-2, following the examination of the Th1 and Th2 effector cytokines IFN-γ and IL-4, respectively, we found a striking increase in the numbers of memory cells that can produce either cytokine in the spleen of Itk−/− mice, compared to wild type. This observation suggested that Itk may function to regulate a balance between Th1 and Th2 effector cytokine production during differentiation. To better understand the function of Itk in CD4+ T cell differentiation, we examined the ability of Itk-deficient cells bearing a transgenic TCR to differentiate upon stimulation with the cognate antigen. From these experiments, a great deal of variation was observed upon activation and differentiation of Itk−/− CD4+ T cells as a result of the presence of previously-activated cells that expressed both the transgenic TCR and endogenous alpha chains. These cells had been differentiated in vivo into either the Th1 or Th2 lineage, thus greatly impacting the differentiation of naïve cells in vitro. As a result, we were unable to draw any conclusions about the function of Itk in the differentiation of naïve CD4+ T cells. Chapter four more clearly defines an important function for Itk in T cell differentiation.

As opposed to chapter three, chapter four examines the function of Itk in CD4+ T cell differentiation in a more highly controlled in vitro system. The experiments
presented in this chapter sought to rectify the involvement of Itk in TCR signaling pathways that lead to the activation of specific genes important in controlling T cell differentiation. To understand the function of Itk during T cell differentiation following the activation of one specific TCR with a cognate antigen, and avoid the complication of endogenous alpha chains and memory cells that obscured my experiments in chapter three, we generated Itk-deficient mice that possessed the 5C.C7 TCR on the Rag2- background. Since variation in TCR signal strength is known to generate different outcomes in T cell differentiation, I utilized a controlled in vitro approach, which lacks exogenous skewing cytokines, to ask whether Itk regulates the strength of the TCR signal in a manner that would impact T cell differentiation. This method required varying the dose of peptide that the T cells are initially exposed to, or using altered peptide ligands that are recognized with a weaker avidity. These experiments demonstrated that Itk was crucial for differentiation into the Th2 lineage. In an effort to identify the underlying mechanism, I was able to make a previously unrecognized connection between Itk and its negative regulatory role on the expression of T-bet, the alleged master switch of Th1 differentiation. Using exogenous cytokines to skew the T cells into either the Th1 or Th2 lineage, I discovered that Itk plays an important role in the function of both lineages. However, I found Itk to be more highly expressed in Th2 cells, compared to Th1, an important event for optimal PLC-γ1 activation in Th2 cells. These data support a model in which Itk is not only critically required for Th2 differentiation, but essential for proper Th2 cell function.

Together, the data presented in this thesis demonstrate that the Tec family kinases not only regulate signaling pathways affecting T cell activation and differentiation, but
also pathways involved in effector mechanisms that lead to apoptosis. While it was previously thought that Itk functions in a signaling pathway that activates cytokine and effector genes, such as IL-2, IL-4, and FasL, my work has identified a novel connection between Itk and genes that are involved in the transcriptional control of T cell differentiation, such as T-bet. Overall, these studies have highlighted the complex interactions between Itk and cellular processes, such as T lymphocyte activation, differentiation, effector functions, homeostasis, and apoptotic pathways.
CHAPTER II.
THE FUNCTION OF ITK IN
ACTIVATION-INDUCED
CELL DEATH
Introduction

Stimulation of the T cell antigen receptor (TCR) induces a variety of different cellular responses, depending on the stage of development of the T cell and its antigen recognition history. In mature T lymphocytes, stimulation of the TCR can induce cytokine production, proliferation, anergy, or programmed cell death. In recent years, it has become increasingly clear that apoptotic cell death following a strong immune response is a major mechanism responsible for maintaining homeostasis in the immune system. For instance, after prolonged activation, T cells undergo a process termed activation-induced cell death (AICD), which is mediated by the induced expression of factors such as tumor necrosis factor (TNF) and Fas ligand (FasL) (reviewed in [15]). Activation of T cells via their TCR induces the synthesis of Fas (CD95), and its ligand, FasL (CD95L). The binding of Fas to FasL initiates a cascade of intracellular events in the Fas-expressing cell that ultimately results in the apoptotic death of that cell (reviewed in [16]). The importance of this mechanism of AICD in the immune system is exemplified by the consequences of mutations in either the Fas or the FasL genes, which result in uncontrolled lymphoproliferation and autoimmunity in both humans and mice [17-19].

The signaling pathways that lead to the activation of the FasL gene in T cells have been a major focus of investigation in recent years. However, this pathway is still not well understood. Several lines of evidence have suggested that specific TCR signaling pathways induce the expression of FasL. For instance, Lck and ZAP-70, members of the Src and Syk family of non-receptor protein tyrosine kinases, respectively, have been shown to be critical for TCR-mediated FasL expression; in contrast, FasL expression is
not dependent on Fyn, another proximal tyrosine kinase [96, 97]. Furthermore, both calcineurin and members of the MAP kinase (MAPK) ERK and JNK families have been recognized as important members of the signaling pathway leading to the induction of FasL expression [98-102]. In this study, we focused on the involvement of a Tec family kinase, Itk, in the TCR-mediated upregulation of FasL, and the subsequent induction of AICD.

The importance of Tec family kinases in the immune system is exemplified by Btk, a relative of Itk that is expressed in B cells and mast cells. Mutations in Btk have been linked to x-linked agammaglobulinemia (XLA) in humans and x-linked immunodeficiency (xid) in mice [33-35]. Biochemical studies have indicated a similar role for Itk and Btk in antigen receptor signal transduction in T cells and B cells, respectively [44]. Specifically, mice deficient in Itk exhibit defects in T cell development and function. This is manifested as reduced numbers of peripheral CD4+ cells, indicating a defect in thymic positive selection, as well as reduced cytokine production by peripheral Itk−/− T cells [60, 76]. Biochemical studies have shown that Itk-deficient CD4+ T lymphocytes are defective in proximal TCR-initiated signaling events, such as the activation of PLCγ1 and calcium mobilization [60]. Furthermore, Fowell et al demonstrated that Itk-deficient CD4+ T cells possess defects in the nuclear translocation of NFATc following TCR ligation, which consequently results in the inability to produce IL-4 and to elicit Th2-type responses in vivo [94]. Since NFAT proteins are known to be essential transcription factors for many effector genes such as IL-2, FasL, CD40L, in addition to IL-4, these findings suggested that in the absence of Itk, other T cell effector
functions that are dependent on signals downstream of PLC\(_\gamma 1/Ca^{++}/NFAT\) may be affected.

To further characterize the role of Itk in CD4\(^+\) T cell effector function, we crossed Itk\(^{-/-}\) mice to 5C.C7 TCR transgenic mice [103], expressing a TCR specific for a moth cytochrome c peptide (MCC\(_{93-103}\)) bound to the MHC class II molecule, IE\(^k\) [104]. These mice have provided a system for examining T cell signaling events and effector function in response to the natural receptor-ligand interaction. In this report, we show that in addition to calcium defects, Itk\(^{-/-}\) CD4\(^+\) T cells are defective in the activation of the ERK/MAPK and JNK pathways, the expression of Egr3 and Egr2, and consequently FasL expression. Moreover, we demonstrate the physiological consequence of these defects \textit{in vivo}, where T cells in Itk\(^{-/-}\) mice are unable to undergo efficient AICD in response to a superantigen, SEB.
Results

Naïve Itk−/− CD4+ T cells have defects in IL-2 production and proliferation in response to MHC/peptide stimulation.

Numerous studies over the past few years have indicated that the recruitment and activation of specific signaling pathways in T lymphocytes is determined by the nature of the TCR:peptide:MHC interaction (reviewed in [105]). Nonetheless, prior in vitro studies that have focused on elucidating the role of Itk in T cell signaling and effector function have largely utilized antibodies to T cell surface receptors, such as CD3 and/or CD28, to trigger TCR/costimulation signaling events. Moreover, while initial studies demonstrated that Itk-deficient CD4+ cells have functional defects in response to anti-CD3 stimulation [60, 76], Itk has also been implicated as a negative regulator of CD28 costimulation [106]. Therefore, we were interested in examining the role of Itk in TCR-mediated signaling events in response to the physiological receptor/ligand interaction. To accomplish this, we crossed Itk−/− mice to mice transgenic for the 5C.C7 TCR, which is specific for a moth cytochrome C peptide, MCC103 bound to MHC II IEκ [104, 107].

To first determine the functional responses of TCR transgenic Itk−/− T cells, purified CD4+ T cells from 5C.C7 Itk+/+ or 5C.C7 Itk−/− mice were stimulated in vitro with MCC93-103 peptide plus antigen-presenting-cells (APCs: IEκ and B7.1-expressing CHO cells). As shown in Figure 6A, we found a modest (~two-fold) decrease in the antigen-induced proliferative responses of Itk−/− CD4+ T cells compared to control T cells at all peptide concentrations tested. In contrast, stimulation with a phorbol ester, PMA (P), plus a calcium ionophore, ionomycin (I), induced comparable levels of proliferation, confirming previous data indicating that these pharmacological agents bypass the Itk−/−
defect by directly activating the PKC/Ras and calcium pathways, respectively [76]. Previous studies had also indicated a defect in αCD3 antibody-induced IL-2 secretion by Itk<sup>−/−</sup> T cells. To re-examine this issue with primary TCR transgenic T cells, we determined the extent of IL-2 production by 5C.C7 Itk<sup>−/−</sup> or 5C.C7 Itk<sup>−/−</sup> cells after stimulation with MCC<sub>93-103</sub> peptide and APCs. For these studies, we used intracellular staining of permeabilized cells with an anti-IL-2 antibody. Figures 6B and 6C show the percent of cells making detectable IL-2 following a 16-hour <i>in vitro</i> stimulation. Overall, we observed a 50% reduction in the responses of Itk<sup>−/−</sup> CD4<sup>+</sup> T cells compared to control T cells. Interestingly, these rather modest differences in the percent of responding cells, as measured by intracellular cytokine staining, correlate with much greater differences in IL-2 secretion as measured by ELISA (data not shown). Thus, these data are in close accordance with previously published experiments, utilizing anti-CD3 or anti-CD3 plus anti-CD28 antibodies, showing decreased IL-2 production by naïve Itk<sup>−/−</sup> T cells [60, 76].
Figure 6: Naïve Itk⁺⁻ CD4⁺ T cells have defects in IL-2 production and proliferation in response to MHC/peptide stimulation

(A) Purified CD4⁺ T cells were stimulated in vitro with APCs and the indicated concentrations of MCC93-103 peptide or with PMA and Ionomycin (P+I), and proliferative responses were measured. The data displayed are the averages of triplicate wells. Standard deviations for the triplicates are shown as error bars along the y-axis.

(B) and (C) Purified 5C.C7 Itk⁺⁻ and 5C.C7 Itk⁻⁻ CD4⁺ T cells were stimulated for 16 hours with APCs and MCC93-103, or PMA and Ionomycin. Cells were then stained with anti-CD4-Cy and anti-Vα11-FITC, fixed, permeabilized, and stained intracellularly with anti-IL-2-PE. Ten thousand CD4⁺ Vα11⁺ events were collected on a flow cytometer. An example of raw data is shown in (B), and a summary of one representative experiment out of three is shown in (C).
Figure 6

A

![Graph showing H-Thymidine Incorporation (cpm X 10^3) for 5C.C7 Itk+/- and 5C.C7 Itk-/- under different concentrations of [MCC93-103] nM.](image)

B

![Flow cytometry plots for IL-2 and CD4 expression in 5C.C7 Itk+/- and 5C.C7 Itk-/- under different peptide concentrations.](image)

C

![Histogram showing the percentage of CD4+ cells producing IL-2 under different peptide concentrations.](image)
Itk$^{−/−}$ CD4$^{+}$ T cells proliferate more vigorously than control T cells upon secondary stimulation

Prior studies that have investigated the role of Itk in T cell signaling pathways have focused primarily on the responses of naïve T cells that lack Itk. Therefore, we were interested in determining how Itk$^{−/−}$ cells respond upon secondary stimulation. To accomplish this, 5C.C7 Itk$^{+/−}$ and 5C.C7 Itk$^{−/−}$ CD4$^{+}$ T cells were stimulated in vitro with 2μM MCC93-103 peptide plus APCs, and expanded in medium containing IL-2 until restimulation. In addition, to ensure a homogeneous population of activated T cells, these stimulations were performed in Th1-skewing conditions (IL-12 plus anti-IL-4 antibody). On day 14 after the initial stimulation, cells were restimulated with a range of concentrations of MCC93-103 peptide plus APCs, and T cell proliferation was assessed. Interestingly, Itk$^{−/−}$ T cells proliferated far more vigorously than control (Itk$^{+/−}$) T cells upon secondary stimulation, indicating that the control T cells had either failed to proliferate or had undergone AICD (Figure 7A). To ensure that these previously-activated Itk$^{−/−}$ T cells still possessed defects in IL-2 production in a secondary response, we performed intracellular IL-2 staining. These assays indicated that previously-activated Itk$^{−/−}$ T cells consistently produced reduced levels of IL-2 over a wide range of peptide concentrations (Figure 7B). These data support the notion that Itk$^{−/−}$ CD4$^{+}$ T cells retain a TCR signaling defect after secondary in vitro stimulation.
Figure 7: Itk−/− CD4+ T cells proliferate more vigorously than control T cells upon secondary stimulation

(A) and (B) 5C.C7 Itk+/− and 5C.C7 Itk−/− CD4+ T cells that were initially stimulated with 2μM of MCC93−103 were restimulated on day 14, and proliferative responses were measured. In (B), cells were restimulated on day 12 for 6 hours, and analyzed for IL-2 production by intracellular staining. (C) Proliferative responses of 5C.C7 Itk+/− and 5C.C7 Itk−/− CD4+ T cells that were restimulated in the presence of anti-FasL antibody or an isotype control Ig (5μg/mL). Antibodies were added at time 0 and again at 20 hours.
Figure 7

A

3H-Thymidine Incorporation (cpm x 10^3)

No Peptide 0.000001 0.0001 0.001 0.01 0.1 1 10 100 1000 10000 P + 1

SC.C7 Itk+/- SC.C7 Itk- 70 60 50 30 20 10 0

B

%CD4+ cells producing IL-2

No Peptide 0.001 0.01 0.1 1 P + 1

SC.C7 Itk+/- (Ctrl Ig) SC.C7 Itk- (Ctrl Ig)

C

3H-Thymidine Incorporation (cpm x 10^3)

No Peptide 0.000001 0.0001 0.1 100

SC.C7 Itk+/- (anti-FasL) SC.C7 Itk-/- (anti-FasL)
FasL upregulation is defective in Itk−/− CD4+ T cells

FasL transcription is regulated by a number of factors, including NF-κB, NFAT, AP-1 (fos/jun), and Egr family members, all of which are activated in response to TCR stimulation [108-113]. A previous study has demonstrated that Itk−/− CD4+ T cells are impaired in their ability to efficiently translocate cytoplasmic NFAT to the nucleus upon TCR stimulation [94]. Together with our observation that Itk−/− T cells proliferate more vigorously upon secondary stimulation compared to Itk+/+ T cells, this finding suggested that Itk−/− T cells may be impaired in the expression of FasL. As an initial effort to assess whether proliferative differences between control and Itk−/− T cells were due to differences in Fas/FasL-mediated AICD, we repeated the secondary in vitro proliferation assays in the presence of a neutralizing anti-FasL antibody (Figure 7C). These experiments indicated that the presence of anti-FasL antibody, but not an isotype control antibody, blocked AICD and restored the proliferative capacity of control (Itk+/+) T cells. In contrast, the anti-FasL antibody had no effect on the proliferative responses of Itk−/− T cells. Interestingly, at high peptide concentrations (100nM), both wild type and Itk−/− T cells undergo AICD in the presence of anti-FasL antibody. This is likely due to the upregulation of FasL in the Itk−/− T cells in response to very strong TCR signaling (100nM peptide versus 10^-6 or 10^-1 nM peptide), which may be more difficult to block with the concentrations of anti-FasL antibody used. Nonetheless, at lower concentrations of peptide, it appears that Itk−/− T cells fail to upregulate FasL after stimulation.

As an additional measure of FasL upregulation, previously-activated T cells were restimulated with peptide and APCs for 9 hours, stained for surface Fas and FasL, and analyzed by flow cytometry. As shown in Figure 8, we observed induced surface
expression of FasL on Itk⁺ T cells at all peptide concentrations, with maximal levels at the highest peptide concentration tested (100nM). In contrast, Itk⁻ T cells failed to detectably upregulate FasL, except perhaps at the highest peptide concentration where a slight shift in FasL staining can be seen. Both Itk⁺ and Itk⁻ T cells show no difference in the expression of Fas upon stimulation, indicating that differences in AICD between control and Itk⁻ T cells are not due to differences in surface expression of Fas.
Figure 8: FasL upregulation is defective in Itk<sup>−/−</sup> CD4<sup>+</sup> T cells

Previously-activated 5C.C7 Itk<sup>+/+</sup> and 5C.C7 Itk<sup>−/−</sup> CD4<sup>+</sup> T cells were restimulated on day 14 with APCs and the indicated concentrations of MCC<sub>93-103</sub> peptide. After 9 hours, cells were stained for CD4, Vα11, Fas, and FasL, and analyzed by flow cytometry. Ten thousand CD4<sup>+</sup> Vα11<sup>+</sup> events were collected. In each panel the dotted line represents non-stimulated cells, and the bold line shows staining of stimulated cells. These data are representative of three independent experiments.
Figure 8

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IpmM

100 pM

10 nM

FasL ———> Fas
To confirm that the decreased proliferative responses of Itk$^{+/}$ T cells and the increased induction of FasL expression correlated with increased apoptosis, wild type and Itk$^{-/}$ T cells were stained with Annexin V and propidium iodide following stimulation. As shown in Figure 9A and 9B, a substantial degree of apoptosis is induced in control T cells following peptide stimulation, whereas Itk$^{-/}$ T cells require stimulation with $10^3$-10$^5$-fold higher concentrations of peptide to induce a comparable degree of apoptosis. The presence of a neutralizing anti-FasL antibody was able to increase cell viability, on average, by 2.4-fold in stimulations of control T cells. In contrast, cell viability was only increased by 1.3-fold when anti-FasL antibody was included in cultures of Itk$^{-/}$ T cells. This observation further supports the conclusion that greater levels of functional FasL are expressed on Itk$^{+/}$ compared to Itk$^{-/}$ previously-activated CD4$^+$ T cells. These data are also consistent with a previous finding that thymocytes from Itk$^{-/}$ mice are defective in activation-induced cell death in response to anti-CD3 antibody stimulation[64].

One previous study has indicated that another Tec kinase family member, Btk, functions as an inhibitor of signaling through Fas in B cells[114]. Therefore, to determine if signaling through Fas is altered in Itk$^{+/}$ T cells, cells were treated with a Fas agonist, soluble FasL (sFasL). As shown in Figure 9B, stimulation with sFasL led to comparable levels of apoptosis in both Itk$^{+/}$ and Itk$^{-/}$ T cells. These data indicate that signaling through Fas is unperturbed in Itk$^{-/}$ T cells.
Figure 9: Decreased apoptosis correlates with defective FasL expression in
Itk\(^{-}\) CD4\(^{+}\) T cells

(A) and (B) Previously-activated 5C.C7 Itk\(^{+/\,-}\) and 5C.C7 Itk\(^{-/-}\) CD4\(^{+}\) T cells were
stimulated for 16 hours with MCC\(_{93-103}\) peptide and APCs, in the presence of anti-FasL or
an isotype control Ig. Following stimulation, cells were stained with Annexin V and
Propidium Iodide (PI) and analyzed by flow cytometry. Panel (A) shows an example of
dot-plots of Annexin V versus PI fluorescence, and the numbers in the lower left
quadrant indicate the percentage of live cells present at the time of analysis. Panel (B)
shows a summary of data from all stimulation conditions. As a control, cells were
stimulated with soluble FasL (sFasL) alone to ensure comparable susceptibility to Fas-
mediated apoptosis of the Itk\(^{+/\,-}\) and Itk\(^{-/-}\) T cells. In each case, the percentage of live cells
was determined by calculating the ratio of live cells in the treated wells to live cells in the
wells incubated in the absence of MCC peptide.
Figure 9

A

5C.C7 Itk+/-

5C.C7 Itk-/-

No Peptide

10 pM

Annexin V

PI

87

49

94

72

B

% Live cells

5C.C7 Itk+/-

5C.C7 Itk-/-

No peptide

0.01

0.01

10

10

1000

1000

w/ Ctrl Ig

w/ anti-FasL

w/ anti-FasL

[MCC93-103] nM

No peptide + sFasL

No peptide
Although IL-2 is commonly recognized as a growth-promoting cytokine that triggers survival and proliferative signals upon binding its receptor, IL-2 can also potentiate AICD by inducing maximal FasL expression [115, 116]. This is thought to occur through the action of IL-2 receptor (IL-2R) -mediated transcription factors such as SP-1 [117]. Furthermore, IL-2R signals have also been shown to down-regulate FLICE-like inhibitory protein (FLIP), an anti-apoptotic molecule [118]. Thus, signals through the IL-2R can cooperate with TCR signals to provide a feedback mechanism that renders activated T cells more susceptible to apoptotic death. In light of these data, we were interested to determine if the reduced ability of Itk$^+$ T cells to undergo AICD was due, in part, to decreased levels of IL-2 production (Figure 7B). To address this issue, exogenous IL-2 was added to cultures during restimulation. We found that addition of exogenous IL-2 did not enhance FasL-induced cell death or FasL surface expression on Itk$^-$ T cells (data not shown), indicating that the defect in FasL expression is not secondary to the decreased levels of IL-2 production seen in Itk$^-$ CD4$^+$ T cells. Furthermore, as both Itk$^+$ and Itk$^-$ T cells are cultured in an excess of exogenous IL-2 during the primary stimulation, differences in IL-2R signaling are unlikely to account for differential expression of FasL or responsiveness to AICD during the subsequent in vitro stimulations.
Calcium, ERK, and JNK pathways are defective in previously-activated Itk\(^{-}\) CD4\(^{+}\) T cells

Stimulation of the TCR leads to the activation of signaling pathways that ultimately result in the generation of active transcription factors leading to new gene expression [119]. Previous biochemical studies have demonstrated that Itk plays a role in the phosphorylation and activation of PLC\(\gamma\)I following stimulation of the TCR [60, 61]. Activated PLC\(\gamma\)I then converts the membrane phospholipid, PIP\(_2\), into IP\(_3\), an activator of calcium release channels in the endoplasmic reticulum, and DAG, an activator of the Ras and Protein Kinase C (PKC) pathways. A sustained increase in intracellular calcium concentrations following TCR stimulation leads to the calcineurin-dependent dephosphorylation of cytoplasmic NF A T, resulting in NF A T translocation to the nucleus [120-122]. To confirm that previously-activated Itk\(^{-}\) CD4\(^{+}\) T cells retain the biochemical defects characterized in primary resting Itk\(^{-}\) CD4\(^{+}\) T cells [60], intracellular calcium mobilization was measured upon restimulation of 5C.C7 Itk\(^{-}\) T cells initially stimulated with 2\(\mu\)M of MCC\(_{93-103}\) plus APCs and cultured for ten days in exogenous IL-2. As demonstrated in Figure 10A, Itk\(^{-}\) T cells show a significant defect in calcium mobilization compared to Itk\(^{+}\) T cells following stimulation through the TCR. These data indicate that previously-activated Itk\(^{-}\) T cells exhibit a comparable deficit in signaling compared to freshly-isolated ex vivo Itk\(^{-}\) T cells.

Recently, several reports focusing on Itk biochemistry have demonstrated that Itk is recruited to the LAT/SLP-76 complex in response to TCR signaling, thereby providing a scaffold for Itk to activate PLC\(\gamma\)I, potentially by direct phosphorylation. These data place Itk intermediate between proximal TCR signaling events and downstream events
such as the activation of the Ras pathway [36, 123-125] (reviewed in [44]). Activated Ras is known to activate the Raf/Mek/ERK pathway, which subsequently leads to the transcription of fos proteins, and ultimately to the formation and activation of AP-1 complexes (reviewed in [126]). In addition, the ERK proteins, ERK1 and ERK2, have recently been shown to play a role in AICD by inducing FasL transcription [99]. Consistent with these findings, the FasL promoter was also found to possess target sites for AP-1 transcription complexes [112]. In light of these data, we were interested in determining whether the reduced ability of Itk−/− CD4+ T cells to upregulate FasL in response to TCR signaling was due, in part, to defective activation of the Raf/Mek/ERK pathway. As shown in Figure 10B, following anti-CD3 stimulation, Itk−/− T cells failed to achieve maximal levels of phosphorylated ERK proteins over the course of a ten-minute stimulation. In contrast, treatment with PMA and ionomycin, which bypass the proximal signaling events, induced comparable levels of phosphorylated ERK in both Itk+/− and Itk−/− T cells, demonstrating that there is no intrinsic defect in the ability of the Ras pathway to activate ERK in the absence of Itk. These data are consistent with a previous report by Schaeffer et al who demonstrated that Rlk−/− Itk−/− T cells and thymocytes show a marked reduction in ERK phosphorylation following TCR stimulation [59, 64].

Activated Ras also plays a role in the activation of the MEKK-1/JNK pathway, which is essential for transcriptional activation of the FasL promoter via the activation of c-Jun [98, 127]. To further assess the role of Itk in the activation of the Ras pathway and its downstream effectors, we examined the level of JNK/SAPK phosphorylation in Itk−/− cells upon TCR stimulation. As demonstrated in Figure 10B, the activation of the JNK/SAPK pathway is also impaired in Itk−/− CD4+ T cells. Similar to ERK
phosphorylation, treatment with PMA and ionomycin induced comparable levels of SAPK/JNK phosphorylation in Itk\(^{+/−}\) and Itk\(^{-/−}\) cells. Collectively, these biochemical data strongly suggest that Itk\(^{-/−}\) CD4\(^{+}\) T cells are unlikely to accumulate normal levels of active c-Fos and c-Jun, and thus, are likely to have reduced levels of AP-1 complexes after TCR stimulation.
Figure 10: Calcium, ERK, and JNK pathways are defective in previously-activated Itk<sup>+</sup> CD4<sup>+</sup> T cells

(A) 5C.C7 Itk<sup>+</sup>- and 5C.C7 Itk<sup>-/-</sup> CD4<sup>+</sup> T cells were loaded with Fluo-3 and Fura-Red on day 10 post-initial stimulation with 2μM of MCC<sub>93-103</sub> peptide. Intracellular calcium was then measured in response to anti-CD3 antibody cross-linking, followed by ionomycin stimulation. Data are displayed as the ratio of Fluo-3 to Fura-Red fluorescence.

(B) Previously-activated 5C.C7 Itk<sup>+</sup>- and 5C.C7 Itk<sup>-/-</sup> CD4<sup>+</sup> T cells were restimulated on day 14 by anti-CD3 antibody cross-linking for 0, 2, 5, or 10 minutes. As a control, cells were treated with PMA and ionomycin for 15 minutes. Total lysates were immunoblotted with an anti-phospho-ERK (p-ERK1&2) or an anti-phospho-SAPK/JNK (p-SAPK/JNK) antibody. The membrane was stripped and reprobed for ERK or SAPK/JNK protein, respectively.
Figure 10

A

700
600
500
400
300
200
100
0

Intracellular Calcium

0 200 400 600 800 1000
Time (seconds)

anti-CD3ε
S.Av

5C.C7 Itk+/-

5C.C7 Itk-/-

B

5C.C7 Itk+/-

Anti-CD3ε

5C.C7 Itk-/-

Anti-CD3ε

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Reduced Egr2, Egr3, and FasL transcription after TCR stimulation of Itk^- T cells

Several transcription factors such NFAT, NFκB, Egr2, as well as Egr3, have been implicated in the TCR-mediated activation of the FasL promoter. In fact, the FasL promoter contains consensus sequences for NFAT, NFκB, Egr, as well as the AP-1 factors, c-Fos and c-Jun [109, 111, 128, 129]. The Egr2 and Egr3 genes are normally expressed at low basal levels in resting T cells, and are transcriptionally induced following TCR stimulation [130]. Furthermore, the Egr2 and Egr3 promoters themselves are targets of NFAT proteins, and in turn function as strong transactivators of the FasL promoter [113]. Consistent with these findings, the over-expression of either Egr2 or Egr3 in T cell hybridomas or HeLa cells induces FasL transcription [109, 128]. Additional evidence also indicates that the induction of Egr2 and Egr3 transcription, and consequently FasL expression, are inhibited by the calcineurin inhibitor, cyclosporin A (CsA)[109, 128]. In spite of these compelling data, there have been conflicting results regarding which factor, Egr2 or Egr3, is more critical for FasL transcription. Nonetheless, the bulk of the evidence indicate that NFAT and Egr factors act synergistically in the activation of the FasL promoter.

Recent studies have demonstrated that Itk^- CD4^+ T cells are defective in the nuclear translocation of NFAT upon TCR stimulation [94]; furthermore, dominant-negative Itk can inhibit TCR-induced NFAT-dependent transcription [36]. Given these observations, and the fact that the Egr2 and Egr3 promoters are regulated by NFAT, we reasoned that Egr2 and Egr3 transcription might be defective in Itk^- T cells, resulting in impaired FasL transcription. To test this idea, we utilized real-time quantitative PCR
analysis (Q-PCR) to determine the mRNA levels of Egr2, Egr3, FasL, and β-actin in resting and stimulated T cells.

Since Fas-dependent death can occur at low levels in naïve CD4$^+$ T cells upon strong stimulation of the TCR within the first 18 hours [131], we first sought to determine the expression levels of the Egr2, Egr3, and FasL transcripts in naïve 5C.C7 Itk$^{+/−}$ or Itk$^{−/−}$ CD4$^+$ T cells that were stimulated with anti-CD3 antibody for 0, 6, 18, or 48 hours. For these experiments, CD4$^+$ CD44$^+$ T cells were sorted from mice of each genotype, to prevent ambiguities caused by the presence of activated/memory T cells in the unsorted populations. For the analysis of previously-activated T cells, 5C.C7 Itk$^{+/−}$ and 5C.C7 Itk$^{−/−}$ CD4$^+$ T cells were stimulated with 2μM of MCC$_{93-103}$ peptide plus APCs in Th1 skewing conditions, and then expanded in IL-2. These cells were then restimulated on day 14 with anti-CD3 antibody for 0, 1, 2, 4, and 6 hours.

Figure 11A shows an example of raw data obtained from this analysis. The amount of PCR product present, as measured by fluorescence intensity, is indicated for each PCR cycle. As can be seen, the β-actin curves for both samples (Itk$^{+/−}$ and Itk$^{−/−}$) are virtually superimposable, indicating nearly identical amounts of cDNA in these samples. In contrast, the FasL curves do not superimpose, indicating a difference in the copy number of FasL transcripts between the stimulated Itk$^{+/−}$ and Itk$^{−/−}$ T cells. By interpolation of these data to a standard curve, absolute values for FasL copy numbers can be obtained for each sample. To normalize for the amount of cDNA present in each sample, a ratio of the average copy numbers of Egr2, Egr3, and FasL to β-actin copy numbers was calculated for each data point. The data from a representative experiment of each type are shown in Figure 11B. This analysis demonstrated decreased levels of
Egr2, Egr3, and FasL transcripts in primary ex vivo Itk⁻/ᵃ CD4⁺ T cells after stimulation, although the magnitude of this reduction is modest. A more striking deficit was seen in the analysis of previously-activated Itk⁻/ᵃ T cells, where we observed, on average, a 5- and 6-fold decrease in Egr2 and Egr3 levels, respectively, and a 3-fold decrease in levels of FasL transcripts compared to the levels in the Itk⁺/ᵃ T cells at the peak of the response. Interestingly, these data also clearly demonstrate the dramatic enhancement in TCR-mediated Egr3 and FasL transcription in previously-activated compared to naïve CD4⁺ T cells. For example, the peak of Egr3 expression in previously-activated Itk⁺/ᵃ T cells is nearly 40-fold higher than in naïve T cells, as is the case for FasL as well. In contrast, peak Egr2 levels only increase by approximately two-fold in previously-activated compared to naïve CD4⁺ T cells. These data are consistent with Egr3 playing a more important role in FasL transcription than Egr2. Together these data clearly indicate that signaling through Itk plays an important role in the TCR-induced upregulation of Egr factors, and that impaired expression of Egr2 and Egr3 in Itk⁻/ᵃ T cells correlates with impaired FasL expression.
Figure 11: Reduced Egr2, Egr3, and FasL transcription after TCR stimulation of Itk-/- T cells

2 X 10^6 purified naïve (CD44lo) 5C.C7 Itk+/- or 5C.C7 Itk-/- CD4+ T cells were stimulated with anti-CD3 antibody for 0, 6, 18, and 48 hours (primary). For secondary cells, 2 X 10^6 5C.C7 Itk+/- and 5C.C7 Itk-/- CD4+ were initially stimulated with 2µM MCC peptide plus APCs on day 0, and restimulated with anti-CD3 antibody on day 14 for 0, 1, 2, 4, and 6 hours. Following stimulation, RNA was isolated and 1µg was reverse transcribed into cDNA and subjected to real-time quantitative PCR analysis for β-actin, FasL, Egr2, and Egr3.

(A) An example of raw data obtained from real-time quantitative PCR analysis. The dashed line indicates the cycle threshold value at which individual samples were compared.

(B) Egr2, Egr3, and FasL transcript levels were determined for naïve (primary) and secondary cells. Each sample was run in triplicate and the average template copy number was determined by interpolating the cycle threshold (Ct) value on a standard curve. These values were normalized to β-actin transcript values determined for each respective data point. The Y-axis represents the ratio of Egr2, Egr3, or FasL to β-actin in the sample. The error bars are the standard deviation of the Egr2, Egr3, or FasL values obtained from triplicate reactions. These data are representative of three independent experiments.
Figure 11

A

B

PRIMARY

SECONDARY

Egr2

Egr3

FasL

Hours of stimulation

Hours of stimulation

72
CD4+ T cells in Itk−/− mice are defective in AICD in vivo

It has been well documented that mice deficient in the expression of Fas (lpr) or FasL (gld) possess profound defects in the peripheral deletion of activated lymphocytes, and develop severe autoimmune disorders as a result of a failure to maintain peripheral T cell tolerance (reviewed in [16]). Furthermore, CD4+ T cells from mice bearing the lpr or gld mutations are resistant to TCR-mediated apoptosis upon anti-CD3 or superantigen stimulation [132-135]. When injected into mice, staphylococcal enterotoxin B (SEB), a bacterial superantigen, selectively activates Vβ8+ T cells. This leads first to the expansion of Vβ8+ T cells, followed by a steep decline in the percentage of these cells as a result of Fas-mediated cell death [136, 137]. Thus, we were interested in determining whether Itk-deficient T cells would possess defects in AICD in vivo, as a result of the defective FasL expression we observed in vitro. To assess this, we injected Itk+/− and Itk−/− mice with SEB and examined peripheral blood T cells on days 3, 7, 11, and 15 post-injection. Figure 12 shows the percentage of CD4+ cells bearing Vβ8, or Vβ6 as a control, over the course of the response to SEB. Interestingly, Itk+/− and Itk−/− mice initially responded similarly to SEB, as seen by the equivalent increases in Vβ8+ CD4+ T cells on day 3 after injection. Following the expansion phase of the response, Itk+/− Vβ8+ T cells underwent deletion, as has previously been reported. In contrast, Itk−/− Vβ8+ T cells survived to a much greater degree than control cells, indicative of reduced AICD. Figure 12B demonstrates that both Itk+/− and Itk−/− mice had comparable percentages of the control Vβ6+ CD4+ T cells, which are not reactive to SEB. Overall, these data suggest that Itk−/− CD4+ T cells are inefficient at undergoing AICD, most likely as a result of reduced FasL expression. Consistent with this conclusion, we routinely
observe a two-fold increase in the proportion of CD4$^+$ T cells with an activated/memory phenotype in Itk$^{-/-}$ compared to Itk$^{+/+}$ control mice (data not shown). However, we cannot rule out the possibility that the levels of IL-2 in vivo may be decreased in SEB-injected Itk$^{-/-}$ mice, thereby rendering Itk$^{-/-}$ CD4$^+$ T cells less susceptible to FasL-mediated death.
Figure 12: CD4⁺ T cells in Itk⁻/⁻ mice are defective in AICD *in vivo*

Itk⁺/⁻ and Itk⁻/⁻ mice received intravenous injections of SEB on day 0. Peripheral blood was drawn from the tail vein on day -1, 3, 7, 11, and 15. Following RBC lysis, cells were stained with anti-Vβ8.1/8.2-FITC or anti-Vβ6-FITC, and anti-CD4-Cy. The percentages of Vβ8⁺ and Vβ6⁺ populations of the CD4⁺ subset were determined by FACS analysis. The data plotted are means ± standard deviations of independent mice analyzed, n=2 Itk⁺/⁻, n=5 Itk⁻/⁻. These data are representative of two independent experiments.
Figure 12

A

Days

B

Days

%Vb8+ CD4+

%Vb6+ CD4+

Itk+/-

Itk-/-
Discussion

Aberrant regulation of the Fas/FasL system has detrimental effects on the health of an organism. Mutations in the Fas or FasL genes result in autoimmunity and lymphadenopathy in mice, and autoimmune lymphoproliferative syndrome (ALPS) in humans [17-19]. Conversely, increased expression of FasL in HIV-1-infected T cells has been found to be a factor in the T cell depletion that ultimately causes AIDS [138, 139]. Currently, the signaling requirements for proper FasL expression are not completely understood. Therefore, investigation of the molecular mechanisms regulating this pathway is warranted. In our efforts to elucidate the role of a Tec-family kinase, Itk, in CD4$^+$ T cell effector function, we discovered that Itk plays a crucial role in the signaling pathway that induces FasL expression.

Previous studies by several groups have established that Itk is important for T cell effector functions, including cytokine production as well as the development of protective immunity to pathogen infections [59, 60, 76, 94]. The data presented in this manuscript address the role of Itk in T cell homeostasis, as opposed to effector function, and demonstrate that Itk is required for efficient apoptosis induced by TCR stimulation. These experiments further strengthen the notion that Itk is a crucial component of the TCR signaling cascade required for the transcription of genes important for proper immune function. The physiological relevance of these findings is reflected in the altered response of Itk$^{-/-}$ mice to the superantigen, SEB.

These results also support the notion that Itk may be involved in setting the threshold for TCR signaling [64]. In the absence of Itk, the efficiency of TCR signaling is reduced, as assessed by a variety of biochemical and functional readouts, including
reduced PLC-γ1 activation, calcium mobilization, MAP-kinase activation, and cytokine production. This reduced signaling lowers the overall effector response of the cell. Given that the ability of CD4+ T cells to upregulate FasL is dependent on the integration of TCR signals, Itk-/ T cells may require more receptor stimulation to attain a threshold of signals great enough to induce FasL upregulation. This is consistent with our observation that, at high peptide concentrations, Itk-/ T cells do upregulate low levels of FasL, and can undergo AICD.

These experiments led to the surprising finding that, during the initial phase of the response, Itk-/ T cells expand comparably to Itk+ T cells in response to SEB injection in vivo. As previous studies have documented both reduced IL-2 production and proliferation of Itk+ T cells in vitro, these findings were somewhat unexpected. One interesting explanation for this discrepancy is the possibility that SEB produces such a strong activation signal in naïve T cells that FasL expression may be induced early during the activation process. Thus, the net expansion of Vβ8+ T cells in the control mice may reflect the combined effects of proliferation being offset by some apoptosis. Consistent with this possibility, upon infection of lpr/lpr mice with LCMV, there is a notable increase in the rate of expansion of anti-viral CTLs during the initial phase of the response compared to what is typically observed in wild type mice [140]. Thus, T cells in Itk-/ mice may proliferate more poorly, but may also undergo less apoptosis, yielding the same net outcome of Vβ8+ T cell numbers at the peak of the response as are found in Itk+ mice.

While our data directly demonstrate reduced transcription of FasL in stimulated Itk-/ T cells in vitro, the interpretation of the in vivo experiments is clearly more complex.
Activation-induced cell death can involve the action of other molecules in addition to Fas/FasL, such as TNF-α and Bcl-2 family members. We have not fully ruled out the possibility that Itk−/− T cells are defective in the expression of one or more of these additional molecules, and that such differences might also contribute to the decreased AICD of Itk−/− cells in vitro and in vivo. Studies addressing this possibility are currently underway. In addition, it is also possible that Itk−/− T cells express greater amounts of the Flice-like inhibitory protein (FLIP), an inhibitor of the Fas pathway. Since IL-2 receptor signaling inhibits FLIP transcription [118], it is possible that reduced production of IL-2 by Itk−/− T cells in vivo may result in increased FLIP, thereby rendering Itk−/− T cells less susceptible to Fas-mediated apoptosis. While differences in FLIP expression in vivo between control and Itk−/− T cells will be interesting to examine in the future, this concern is unlikely to be relevant to our in vitro studies, as stimulated T cells were all cultured in an excess of exogenous IL-2. Another concern is the observation by Bonfoco et al that non-lymphoid FasL is essential for the deletion of SEB-reactive T cells [141]. These investigators also demonstrated that T cell activation was necessary for the induction of non-lymphoid FasL transcription. Therefore, we cannot fully rule out the possibility that Itk−/− mice may be defective in the expression of cytokine(s) or effector molecule(s) necessary to induce the upregulation of non-lymphoid FasL. Again, this possibility applies only to the in vivo studies, and is not relevant to our in vitro studies using purified T cells.

Finally, we also considered the possibility that reduced AICD in vivo by Itk−/− CD4+ T cells may reflect reduced activation and/or preferential differentiation of Itk−/− T cells into Th2 effectors that express FasL poorly [142, 143]. These possibilities were
tested by examining Vβ8+ CD4+ T cells at the peak of the response (day 3) after SEB injection. When examined by flow cytometry for a panel of activation markers, both Itk+ and Itk− T cells showed comparable percentages of activated T cells. Furthermore, in vitro stimulation of these cells followed by intracellular staining for IL-2, IFN-γ, and IL-4 indicated no increased proportion of IL-4 producing cells among Itk+ T cells compared to controls (data not shown). Thus we find it unlikely that the reduced AICD we observe in vivo in Itk− mice is due to a skewed differentiation of Itk− T cells into Th2 effectors.

Similar to the FasL gene, the Fas gene is transcriptionally regulated by factors such as c-Fos and c-Jun. A report by Li et al demonstrated that, although PKC and JNK are involved in the activation of the Fas gene upon TCR stimulation, PI3-kinase, calcineurin, and ERK kinases play no role in the signaling pathway leading to Fas transcription [144, 145]. These findings suggest that the TCR signaling pathways leading to Fas and FasL transcription are distinct. Our data support the notion that Itk does not play a role in the TCR-mediated upregulation of Fas. Another recent study indicated that Btk, a Tec kinase family member, acts as an inhibitor of the Fas signaling pathway in B cells [114]. Based on our observations that Itk− T cells are equally susceptible to apoptosis following treatment with soluble FasL, the possibility that Itk plays a comparable role in the Fas signaling pathway in T cells seems unlikely.

Based on the data presented here, we speculate that Itk− mice may have altered susceptibility to autoimmune disease. For instance, experimental autoimmune encephalomyelitis (EAE), which can be induced in mice upon adoptive transfer of myelin-specific Th1 cells, is a result of Th1 CD4+ T cells initiating tissue damage to the
central nervous system [146, 147]. Interestingly, mice possessing the *lpr* or *gld* mutations are resistant to the induction of EAE [148]. These findings suggest that FasL-expressing T cells may mediate apoptosis within a target tissue, thereby contributing to the pathology of this disease. Given these observations, it is possible that *Itk*<sup>−/−</sup> mice may also show decreased susceptibility to EAE. Alternatively, since the Fas/FasL pathway has been shown to be crucial for the removal of autoreactive T cells in the periphery [149], it is also possible that *Itk*<sup>−/−</sup> mice may be more susceptible to other types of autoimmune diseases. For instance, FasL-expressing CD4<sup>+</sup> T cells have been shown to be important in the deletion of autoreactive B cells [150], raising the intriguing possibility that *Itk*<sup>−/−</sup> mice might have increased susceptibility to autoantibody-mediated autoimmune diseases.
Figure 13: A model for the role of Itk in TCR-mediated FasL expression

Upon repeated activation of the TCR, the activation of Itk results in the activation of several downstream pathways, which result in the efficient activation of the FasL gene. Itk functions to activate PLC-γ1, which in turn activates NFAT transcription factors via the calcium pathway, as well as components of the AP-1 transcription complex via the activation of the ERK/MAP kinase pathway. NFAT serves several functions once in the nucleus. In addition to directly transactivating the FasL gene, NFAT transactivates the promoters of two transcription factors, Egr2 and Egr3, also important in FasL transcription. Following transcription and translation of Egr2 and Egr3, these proteins further drive the expression of FasL upon binding to the FasL promoter. Lastly, CD28 signals, in addition to TCR signals through Itk, activate NF-κB, another important factor in FasL transcription.
Figure 13:
A model for the role of Itk in TCR-mediated FasL expression
CHAPTER III.

ABERRATIONS IN THE

PHENOTYPE OF CD4⁺ T

LYMPHOCYTES FROM

ITK-DEFICIENT MICE
**Introduction**

CD4+ lymphocytes play a critical role in the defense against many invading organisms. Following the engagement of TCR/CD28 molecules on a naïve T cell with specific MHC/peptide/B7 complexes on antigen-presenting cells (APCs), an intricate series of signaling cascades are activated which culminate in the transcription of genes that are involved in differentiating a naïve CD4+ cell into an effector cell. Over the past few years numerous studies have focused on the biochemical events that occur following TCR stimulation and the consequence of the activation of specific signaling pathways on T cell differentiation. The Tec family of nonreceptor tyrosine kinases is now recognized as an important player in TCR proximal signaling events.

The role of Tec family kinases in T cell effector function was initially discovered by Liao et al, who demonstrated that in the absence of Itk, CD4+ T cells were defective in IL-2 production and consequently proliferation [76]. Liu et al extended these findings by demonstrating biochemically that, analogous to Btk in BCR signaling, Itk is involved in proximal TCR-mediated signaling events, as Itk-deficient cells were specifically impaired in the phosphorylation of PLC-γ1 and as a result, in IP₃ production and the mobilization of intracellular calcium [60]. Infectious disease studies in Itk-deficient mice have revealed a general failure to elicit responses to several different types of pathogens, especially those that require a protective Th2 response, such as *N. brasiliensis* and *S. Mansoni*. Furthermore, upon skewing of CD4+ cells into the Th2 lineage *in vitro*, Itk-deficient cells exhibit severe defects in the ability to produce IL-4. This defect was observed in both the C57Bl/6 genetic background as well as the IL-4 permissive strain, Balb/c [72, 74]. These data support a role for Itk in the process of Th2 differentiation.
In striking contrast to the aforementioned impaired Th2 immune responses observed in Itk\(^{-/-}\) mice following infection, several studies have reported other phenotypic abnormalities in unimmunized Itk-deficient mice that are suggestive of a bias toward, rather than away from, Th2 differentiation. For instance, Schaeffer et al reported that although Itk-deficient mice fail to elicit a Th2 response to *S. Mansoni*, unimmunized Itk\(^{-/-}\) mice exhibit a 5-fold increase in the basal levels of IgE, an antibody subclass dependent on IL-4. Other antibody subclasses were unaffected [72]. Consistent with this observation, Mueller and August observed that while Itk\(^{-/-}\) mice are incapable of eliciting an allergic asthmatic response to an OVA allergen, they exhibit increased OVA-specific IgE responses, in addition to excessive levels of total IgE [151]. Additional data from our laboratory correlate with the observation that unimmunized Itk\(^{-/-}\) mice exhibit a Th2 bias. Karen Liu, a former graduate student in the laboratory, observed germinal center hyperplasia in the spleens and mild eosinophilia in the lymph nodes of Itk\(^{-/-}\) mice, both of which are dependent on type 2 cytokines [73]. While the etiologies of these abnormalities are unknown, they suggest Itk\(^{-/-}\) mice exhibit excess Th2-type cytokine production.

Over the past few years, several studies have suggested that the recruitment and activation of specific signaling pathways in T lymphocytes are determined by the nature of the TCR:peptide:MHC interaction (reviewed in [105]). Furthermore, up to this point, all prior studies examining T cell function and signaling events in the absence of Itk have utilized antibodies to CD3 and/or CD28 to trigger signaling events in T cells. In this chapter we sought to understand the function of Itk both *in vivo* and *in vitro* by characterizing the phenotype of T cells from Itk-deficient mice and T cell function in the
absence of Itk. More specifically, we examined the activation/memory status of CD4\(^+\) T cells in Itk\(^{-/-}\) mice compared to wild type. Furthermore, we examined the ability of wild type and Itk\(^{-/-}\) CD4\(^+\) T cells to produce the effector cytokines IFN-\(\gamma\) and IL-4 immediately \textit{ex vivo}. Lastly, in order to understand how Itk may function in T cell receptor signaling events that affect the process of CD4\(^+\) T cell differentiation in response to a natural ligand:receptor interaction, we examined differentiation \textit{in vitro} following the stimulation of T cells bearing the transgenic 5C.C7 TCR with the natural ligand, MCC\(_{93-103}\) in the context of MHC II IE\(^k\).

The data presented in this chapter identify additional phenotypic abnormalities in Itk-deficient mice in an attempt to understand further processes that Itk may be regulating in CD4\(^+\) T cells. Although a mechanistic explanation for these observations is lacking, the data indicate that Itk contributes to several T cell effector processes \textit{in vivo}. In correlation with data presented in chapter two, Itk-deficient mice possess an increase in the percentage and numbers of CD4\(^+\) T cells that exhibit an activated/memory phenotype, indicating a role for Itk in homeostasis \textit{in vivo}. Although Itk is not expressed in dendritic cells (DCs), DC populations and activation status were unaltered in Itk\(^{-/-}\) mice, compared to wild type. Interestingly, Itk-deficient mice exhibit an increase in the number of memory CD4\(^+\) cells that can immediately produce either Th1 or Th2 effector cytokines. Nonetheless, even in a TCR transgenic situation, where the T cell repertoire is greatly restricted, the presence of previously-activated cells greatly affects the differentiation of naïve cells upon stimulation and \textit{in vitro} culturing. Collectively these data indicate a role for Itk in regulating CD4\(^+\) T cell differentiation. Furthermore, in agreement with findings made by other groups, these data emphasize the discrepancy between the selective defect
in Th2 immune responses and the abnormal Th2-type phenotype in unimmunized Itk-deficient mice.
Results

Itk-deficient mice exhibit a 2- to 3-fold increase in the percentage and numbers of CD4+ memory cells

In order to understand the function of Itk in other T cell processes, we were interested in examining the phenotype of CD4+ T cells in unimmunized Itk−/− mice compared to wild type. To accomplish this, we purified CD4+ T cells from both wild type and Itk−/− mice and stained these cells for a series of activation markers, such as CD44, CD62L, CD25, and CD69. As shown in Figure 14, analysis of these activation markers revealed that the CD4+ T cell population in Itk−/− mice have a 2- to 3-fold increase in the percentage of cells that possess a memory/activated phenotype. These data strongly correlate with the failure to undergo AICD as discussed in chapter two.

To determine whether Itk may affect memory cell function and whether Itk-deficient memory-like cells in fact behave like activated/memory cells, we stimulated CD4+ T cells purified from the spleen of wild type and Itk−/− mice (C57Bl/6 background) with PMA (P) and Ionomycin (I) for 6 hours. Treatment of T cells with PMA and Ionomycin bypasses proximal TCR signaling events, including Itk, and stimulates the PKC and calcium pathways, respectively. Following stimulation, we stained cells for CD44 and measured the ability of the CD44hi population to produce IL-2 by intracellular IL-2 staining and analysis by flow cytometry. Since memory cells (CD44hi) respond rapidly and produce high levels of IL-2 compared to naïve cells, we expected to observe increased IL-2 production in the memory population. As demonstrated in Figure 15A, cells with an activated/memory phenotype from both wild type and Itk−/− mice produce more IL-2 than naïve cells (CD44lo) as seen by the increased mean fluorescent intensity.
in the CD44hi population. In light of the fact that Itk-deficient mice typically have less peripheral CD4+ cells, as a result of a defect in positive selection, we calculated the absolute number of IL-2 producing memory cells. As represented in Figure 15B, Itk-deficient mice exhibit a two-fold increase in the numbers of memory cells producing IL-2 compared to wild type. Similar observations were made in Itk-deficient mice on the Balb/c genetic background, indicating that Itk likely plays a similar role, in terms of T cell homeostasis, on both genetic backgrounds (data not shown). These data indicate that in addition to possessing an increase in the percentage of CD4+ cells with a memory/activated phenotype, Itk−/− mice also have more activated/memory cells than wild type mice. These memory cells in Itk−/− mice behave like memory cells by producing more IL-2. The cause of T cell activation in vivo remains elusive. Collectively, however, our data support a role for Itk in T cell homeostasis and apoptotic processes.
Figure 14: Itk-deficient mice exhibit a 2- to 3-fold increase in the percentage and numbers of CD4$^+$ memory cells

Splenocytes from Itk$^{+/}$ and Itk$^{-/-}$ mice were stained with antibodies to CD4, CD44, CD62L, CD69, and CD25. Histograms were gated on the CD4$^+$ cells and plotted for the indicated activation marker. Data are representative from a combination of experiments performed on different days.
Figure 14

Itk+/-  

CD44  

12%  

CD62L  

21%  

CD69  

11%  

CD25  

15%

Itk-/-  

38%  

45%  

20%  

22%
Figure 15: Increase in the number of CD44^{hi} cells producing IL-2 in Itk^{-/-} spleen

(A) 1 X 10^6 purified CD4^{+} cells from Itk^{+/+} and Itk^{-/-} mice were stimulated for 6 hours with PMA and Ionomycin. Golgi blocking reagents were added for the last 2 hours. Following stimulation, cells were stained with anti-CD4-Cyochrome and anti-CD44-FITC, fixed, permeabilized, and stained intracellularly with anti-IL-2-PE. Cells were analyzed immediately by flow cytometry. (B) The total number of memory (CD44^{hi}) cells producing IL-2 represented in a bar graph.
**Figure 15**

**A**

- **Itk+/-.**

- **Itk-**

  - No stimulation
  - PMA + Iono.

**B**

- 

  - # of CD44hi cells making IL-2 in spleen

  - Itk+/-
  - Itk-
Itk may regulate CD4\(^+\) T cell differentiation \textit{in vivo}

In an effort to determine whether Itk functions in the regulation of T cell differentiation, we examined \textit{in vitro} the differentiation status of the memory/activated cells that are observed \textit{in vivo} in Itk-deficient mice compared to wild type. To accomplish this, we stimulated purified CD4\(^+\) T cells from wild type and Itk\(^{-/-}\) mice (C57Bl/6 background) as before with P + I for 6 hours. Following stimulation, cells were stained for CD44, as above, followed by intracellular staining for IFN-\(\gamma\) and IL-4. As demonstrated in Figure 16A, following the \textit{ex vivo} stimulation with PMA and Ionomycin, both wild type and Itk-deficient memory (CD44\(^{hi}\)) cells rapidly produce IFN-\(\gamma\). Interestingly, upon analysis of IL-4 production by memory cells in Figure 16B, a significant percentage of Itk-deficient cells rapidly produced IL-4, whereas virtually no IL-4-producers were detected in wild type mice. The memory populations that produce IFN-\(\gamma\) or IL-4 are in fact distinct populations as demonstrated in Figure 16C, where IFN-\(\gamma\) versus IL-4 is shown on a CD44\(^{hi}\) gated plot.
Figure 16: Th1/Th2 cytokine imbalance in Itk-deficient memory cells

Following the purification of splenic CD4+ T cells from both wild type and Itk-/- mice (C57Bl/6), 1 x 10^6 cells were left untreated or stimulated with PMA (2.5ng/ml) and Ionomycin (375ng/ml) for 6 hours in a 96-well plate. After 4 hours, golgi-blocking reagents were added to prevent cytokine secretion. The cells were then surface stained with anti-CD4-Cy and anti-CD44-FITC, fixed, permeabilized, and stained intracellularly with anti-IL-4-PE and anti-IFN-γ-APC. Cells were then immediately analyzed by flow cytometry. CD4+ cells plotted for CD44 versus IFN-γ in (A) or versus IL-4 in (B). Panel (C) represents CD4+CD44hi gated cells plotted for IFN-γ versus IL-4 to demonstrate that these are distinct populations.
Figure 16

A

Itk+/+

Itk-/-

No stimulation

PMA + Iono.

B

No stimulation

PMA + Iono.

C

CD44 hi gated

I tk+/+

I tk-/-

PMA + Iono.

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Mice from the C57BL/10 and Balb/c genetic backgrounds differ in their response to certain pathogens. Experiments from several groups have revealed that while CD4+ T cells from C57BL/10 mice are more prone to producing IFN-γ and differentiating into Th1 cells following activation, CD4+ T cells from Balb/c mice have a greater tendency to produce IL-4 and differentiate into Th2 cells. Studies by Bix and colleagues identified two genetic loci in Balb/c mice that could influence the intrinsic ability of a CD4+ T cell to produce IL-4. These identified loci were determined to influence IL-4 production prior to signals mediated by the IL-4 receptor [152]. Therefore, we were interested in determining whether Itk affects T cell differentiation similarly on both the C57BL/10 and Balb/c genetic backgrounds. To address this, we examined the ability of memory (CD44hi) cells in both wild type and Itk−/− mice on the C57BL/10 (Figure 17A) and Balb/c (Figure 17B) genetic backgrounds to produce IFN-γ and IL-4 immediately ex vivo following stimulation with P + I. A comparison of total numbers of memory cells producing effector cytokines is shown in Figure 17. Memory cells from wild type mice on both genetic backgrounds produced IFN-γ, for reasons that are not clear. However, Itk-deficient mice on both genetic backgrounds exhibited a striking increase in the numbers of cells producing IFN-γ and the numbers of cells producing IL-4, compared to their wild type counterparts. This data indicates that Itk may be playing a similar role in CD4+ T cell effector function on both genetic backgrounds.
Figure 17: Increased numbers of IFN-γ and IL-4 producers in Itk−/− spleen in both the C57BL/10 and Balb/c backgrounds

Bar graph representation of the numbers of CD44hi cells in the spleen producing either IFN-γ or IL-4 from wild type and Itk−/− mice on the (A) C57BL/10 or (B) Balb/c genetic backgrounds.
Figure 17

A

C57BL/10

# of CD44hi cells in spleen producing cytokine

- IL-4
- IFN-γ

Itk+/+

Itk−/−

B

Balb/c

# of CD44hi cells in spleen producing cytokine

- IL-4
- IFN-γ

Itk+/+

Itk−/−
Altered T cell differentiation *in vitro* in the absence of Itk

In our initial attempts to determine how Itk functions in the process of T cell differentiation, we examined the ability of wild type and Itk$^{-/-}$ T cells bearing the 5C.C7 transgenic TCR to differentiate *in vitro* following stimulation with the cognate antigen, MCC$_{93-103}$ in the context of MHC class II IE$^k$. In these experiments, purified CD4$^+$ T cells from 5C.C7 TCR transgenic Itk$^{+/+}$ and Itk$^{-/-}$ mice were stimulated *in vitro* with the MCC$_{93-103}$ peptide plus CHO cells transfected with MHC class II IE$^k$ and B7.1 (APCs) in conditions that lacked any exogenous skewing cytokine. Therefore, the ability of the cells to differentiate into either a Th1 or a Th2 cell was solely based on the cytokines the cells themselves produced. Following 4-7 days in culture we assessed T cell differentiation by restimulating the cultured cells with APCs and MCC$_{93-103}$ peptide and stained them intracellularly for IFN-γ and IL-4. The data presented in Figure 18A are results from four separate experiments. While wild type cells in each experiment uniformly differentiated into Th1 cells, or IFN-γ producers, variable results were obtained from Itk-deficient cells. In several experiments Itk-deficient cells differentiated into Th1 cells, in others Th2 cells, and in many instances differentiated into a combination of both Th1 and Th2 cells. Surprisingly, upon further analysis of T cells from 5C.C7 Itk$^{-/-}$ mice, the CD4$^+$ T cells continued to exhibit an increase in the percentage of cells with a memory phenotype. As shown in Figure 18B, in addition to a 3-fold increase in the percentage of Itk-deficient CD4$^+$ T cells possessing a memory phenotype, we also consistently observed that the bulk of the CD44$^{hi}$ population expressed low levels of the transgenic TCR, Vα11 (Figure 18B). Together, these findings strongly suggest that T cells expressing endogenous TCR α chains become activated in the 5C.C7 Itk$^{-/-}$ mice, and
differentiate \textit{in vivo} into either IFN-\textgamma- or IL-4-producing cells. Consequently, these previously-activated cells likely affect the differentiation of naïve cells in vitro when total purified CD4$^+$ cells are stimulated in Figure 18A.
Figure 18: 5C.C7 Itk<sup>−/−</sup> CD4<sup>+</sup> T cells differentiate into both Th1 and Th2 subsets under non-skewing conditions

CD4<sup>+</sup> T cells were purified from the spleens and lymph nodes of 5C.C7 Itk<sup>+/−</sup> and 5C.C7 Itk<sup>−/−</sup> littermates. CD4<sup>+</sup> T cells (1 x 10<sup>6</sup>) were cultured in 24-well plates with 1μM of MCC<sub>93-103</sub> peptide plus 1 x 10<sup>6</sup> mitomycin C-treated CH27 cells (APCs). After 24 hours, the cells were expanded in fresh medium containing IL-2. Following a 4- to 7-day culture period, cells were restimulated with varying amounts of the MCC<sub>93-103</sub> peptide and APCs for 6 hours. The final 2 hours included Golgi-blocking reagents. Cells were then surface stained with anti-Vα11-FITC and anti-CD4-Cy, fixed, permeabilized, and stained intracellularly with anti-IL-4-PE and anti-IFN-γ-APC. Cells were immediately analyzed by flow cytometry. Panel (A) depicts data from four independent experiments where cells had been restimulated with 1μM of MCC<sub>93-103</sub>. Plots are gated on CD4<sup>+</sup> and Vα11<sup>+</sup> cells. In panel (B), purified CD4<sup>+</sup> cells from 5C.C7 Itk<sup>+/−</sup> and 5C.C7 Itk<sup>−/−</sup> mice were stained with anti-Vα11-FITC and anti-CD44-Cy. The indicated FACS plot demonstrates Vα11 versus CD44.
Figure 18
A  CD4⁺ Vα11⁺ gated

B  CD4⁺ gated
Normal dendritic cell populations in Itk⁻/⁻ mice

Dendritic cells (DCs) are thought to be the main antigen-presenting cell in the priming of T cells. The triggering by pathogens induces the maturation of DCs and the expression of high amounts of MHC and costimulatory molecules. In the mouse, DCs of the spleen can be classified into two major subsets, either the lymphoid-derived (CD11c⁺ CD8α⁺ CD11b⁻) or myeloid-derived (CD11c⁺ CD11b⁺). The myeloid-derived DCs can further be broken down into either CD4⁺ or CD4⁺CD8α⁻. Interestingly, these different populations of dendritic cells are have been shown to selectively determine different types of T cell-mediated immune responses (reviewed in [153, 154]). For instance, the subcutaneous injection of CD8α⁺ DCs loaded with antigen are capable of priming Th1 responses, whereas CD8α⁻ DCs are capable of priming Th2 responses [155]. The direction of T cell responses by CD8α⁻ and CD8α⁺ DCs directly corresponds to the cytokines produced by these subsets [154]. CD8α⁺ DCs are thought to directly induce Th1 responses by secreting copious amounts of IL-12 [155]. Furthermore, CD8α⁺ dendritic cells are thought to be involved in the deletion of CD4⁺ cells via a Fas-FasL mechanism [156]. Clearly, throughout an immune response, DCs and T lymphocytes are constantly interacting.

In order to determine whether alterations in T cell function, as a result of the absence of Itk, contributes to any alterations in dendritic cell populations in vivo in Itk⁻/⁻ deficient mice, we isolated the low-density fraction of cells from the spleen and analyzed this fraction for DC markers by FACS analysis. This fraction contains the bulk of splenic dendritic cells (30% is CD11c⁺). As demonstrated in Figure 19A, Itk⁻/⁻ mice possess normal percentages and numbers of both myeloid- and lymphoid-derived DCs.
Furthermore, as shown in Figure 19B, there are also normal percentages and numbers of myeloid-derived subsets. These data suggest that the disregulation of T cells in the absence of Itk does not affect dendritic cells on a population basis.
Figure 19: Normal dendritic cell populations in Itk$^{-/-}$ mice

The low-density fraction of spleen cells, which is typically 30% CD11c$^+$, was isolated from Itk$^{+/+}$ and Itk$^{-/-}$ mice. Cells were stained for a panel of dendritic cell markers. In panel (A), live cells were gated and plotted for CD11c versus CD11b or CD8a. In panel (B), CD11c$^+$ cells were gated and plotted for CD4 versus CD8$\alpha$ or CD8$\beta$ versus CD8$\alpha$. The percentage of the different dendritic cell populations are shown on the FACS plot.
Figure 19

A

Itk+/-

Itk-/

CD11b

CD8α

CD11c

B CD11c+ gated

Itk+/-

Itk-/

CD8α

CD4

CD8α

CD8β
Normal activation markers on dendritic cells from Itk<sup>−/−</sup> mice

Immature DCs primarily function in antigen uptake and processing into MHC:peptide complexes. Upon their maturation into terminally differentiated DCs, which are capable of rapidly activating T cells, DCs upregulate numerous activation markers on their cell surface. These molecules permit additional communication between DCs and T cells. For instance, the upregulation of CD40L on recently activated T cells binds to CD40 on dendritic cells, thereby inducing greater expression levels of the molecules B7.1 and B7.2. The upregulation of B7.1 and B7.2 provides the T lymphocyte with the necessary costimulatory signals required for complete activation [157]. Hence, the communication between T lymphocytes and DCs is a dialogue where DCs can also respond to T lymphocytes.

To determine whether DCs from Itk<sup>−/−</sup> mice express greater levels of activation markers that would correlate to the observed increase in the percentage of CD4<sup>+</sup> with an activated/memory phenotype, both myeloid (CD11c<sup>+</sup> CD11b<sup>+</sup>) and lymphoid (CD11c<sup>+</sup> CD8α<sup>+</sup>) DCs were stained with antibodies recognizing a series of activation markers. As shown in Figure 20, there does not appear to be any striking difference in the expression levels of B7.1, B7.2, CD40, CD25, and Fas between wild type and Itk<sup>−/−</sup> myeloid and lymphoid dendritic cells. These data suggest that in vivo, aberrations in T cell function in the absence of Itk, such as T cell differentiation and cell death, do not correlate with DC activation markers.
Figure 20: Normal activation markers on dendritic cells from Itk−/− mice

As in Figure 19, the low-density fraction of spleen cells isolated from wild type and Itk−/− mice were stained for the dendritic cell markers CD11c and CD11b, or CD11c and CD8α+, in addition to the activation markers B7.1, B7.2, CD40, CD25, or Fas. Histograms of these activation markers were gated on either CD11c+/CD11b+ or CD11c+/CD8α+ dendritic cells.
Figure 20

CD11c+/CD11b+ gated

CD11c+/CD8α+ gated

B7.1

B7.2

CD40

CD25

Fas

Itk+/−

Itk−/−
Discussion

In this chapter, we sought to further clarify the role of Itk in additional CD4$^+$ T cell effector functions, such as T cell differentiation. In our efforts to elucidate Itk function, we found that Itk-deficient mice exhibit a two- to three-fold increase in the percentage and numbers of CD4$^+$ T cells with an activated or memory phenotype, based on the activation markers CD44, CD62L, CD25, and CD69. Cells exhibiting a memory phenotype from Itk-deficient mice behaved like memory cells as characterized by the production of high levels of IL-2. These data strongly correlate with data presented in chapter two, where a defect in FasL-mediated activation-induced cell death was impaired in Itk-deficient cells.

As discussed earlier, reports from Schaeffer et al and Fowell et al suggested that Itk regulates T cell differentiation [72, 74]. In order to gain a better understanding of whether Itk may regulate Th1 and Th2 cytokine production in vivo, we examined effector cytokine production from memory/activated CD4$^+$ T cells immediately ex vivo. We found that the bulk of memory cells in Itk-deficient mice were Th1 cells, or IFN-γ producers, compared to wild type memory cells. Strikingly, however, a significant number of Th2 cells, or IL-4 producers, were also detected in Itk-deficient mice, while none were detected in wild type mice. This was observed in both Itk-deficient mice on the C57Bl/6 and Balb/c genetic backgrounds. Lastly, this observation is in agreement with data from Karen Liu, who observed by RNAse protection that upon stimulation of CD4$^+$ T cells directly ex vivo with anti-CD3 and anti-CD28 for 8 hours, that IL-4 was expressed at higher levels in Itk$^{-/-}$ cells, compared to wild type [73].
In an effort to gain a better understanding of how Itk may function in the T cell differentiation process \textit{in vitro}, we examined the ability of wild type and Itk$^{-/-}$ CD4$^+$ T cells expressing the transgenic receptor, 5C.C7, to differentiate following stimulation with the cognate antigen. Unfortunately, our ability to assess Itk's function in the process of T cell differentiation was greatly hindered by the presence of previously-activated cells that had likely been skewed \textit{in vivo} to either the Th1 or Th2 lineage, ultimately yielding tremendous variability in these experiments. Therefore, we re-examined the function of Itk in CD4$^+$ T cell differentiation in a more highly controlled \textit{in vitro} system in Chapter IV.

The origin and history of the IL-4 producing CD4$^+$ T cells in the Itk$^{-/-}$ mice, at this stage, remains a mystery. One possible explanation is that the repertoire of T cells selected in the thymus of Itk$^{-/-}$ mice are either auto-reactive and become activated by self-antigen, or alternatively, are reactive to an environmental antigen that induces differentiation into the Th2 lineage. The explanation for why this occurs in Itk-deficient mice, but not in wild type mice, has not been determined, but may relate to defects in thymic selection or to an altered peripheral environment due to changes in the non-T cell populations that express Itk. Nonetheless, several lines of evidence from Schaeffer et al and our own laboratory support the hypothesis that there is some type of Th2-biased immune response, autoimmune or otherwise, occurring \textit{in vivo}. First, Schaeffer et al as well as Mueller et al reported that Itk$^{-/-}$ mice have a 5-fold increase in serum IgE levels, an IL-4-dependent antibody subclass [72, 151]. Second, we have observed a significant level of eosinophilia in the lymph nodes of Itk$^{-/-}$ mice, which is likely the result of Th2 effector cells \textit{in vivo} [73]. Third, the phenotype observed in Tec family kinase-deficient
mice is strikingly similar to that seen in mice possessing mutations in signaling molecules such as LAT, JNK1, and certain NFATs, all of which have all been implicated in the same pathway(s) as Itk. This will be discussed in much greater detail in Chapter VI.

Lastly, in order to determine if the absence of Itk results in any dendritic cell abnormalities, we examined dendritic cell populations and dendritic cell activation markers in the spleens of wild type and Itk<sup>−/−</sup> mice. We found that the absence of Itk does not disrupt any dendritic cell populations <i>in vivo</i>. Furthermore, while Itk-deficient CD4<sup>+</sup> T cells exhibit an increase in the percentage of cells with an activated phenotype, as discussed earlier, dendritic cells from Itk<sup>−/−</sup> mice do not exhibit any incongruencies in terms of activation markers.

In addition to signaling defects revealed <i>in vitro</i>, mice lacking Itk exhibit other phenotypic abnormalities that remain to be clarified. As touched upon earlier, in correlation with the observed defect in FasL expression in Itk-deficient cells, CD4<sup>+</sup> T cells from Itk-deficient mice possess an increase in the percentage of CD4<sup>+</sup> T cells with an activated/memory phenotype, according to CD44. While the data indicate that Itk may regulate an important aspect of T cell differentiation, we have not ruled out the possibility that these phenotypic abnormalities are a consequence of other cell types, such as mast cells, which may be disregulated in the absence of Itk.
CHAPTER IV.

THE FUNCTION OF ITK IN CD4⁺ LYMPHOCYTE DIFFERENTIATION
Introduction

Triggering of the T cell antigen receptor (TCR) is one of the hallmarks of the adaptive arm of the immune system during responses to pathogenic infection. Following this highly specific interaction, a complex series of biochemical events takes place within the T cell, involving the action of many signaling molecules including tyrosine kinases, phosphatases, adaptor molecules, and others. Ultimately, these biochemical reactions culminate in the activation of specific genes, whose products will function in the generation of a robust immune response to combat the invading organism. Over the past several years, there has been an increasing amount of evidence, both in vitro and in vivo, demonstrating the importance of the Tec family tyrosine kinase, Itk, in the generation of critical CD4+ T cell effector functions.

Expressed predominantly in T cells and mast cells, Itk is activated in response to antigen receptor stimulation. Biochemical studies have indicated that signaling through the TCR leads to Itk recruitment to a multimolecular complex that includes SLP-76, LAT, Gads, Grb2, and PLC-γ1, providing a platform for Itk to interact with and phosphorylate PLC-γ1 [46, 158]. In support of this model, TCR stimulation of Itk−/− CD4+ T cell results in substantially impaired PLC-γ1tyrosine phosphorylation and activation, intracellular calcium mobilization, MAP kinase activation, as well as NFATc nuclear translocation [59, 60, 72, 94]. As a consequence, the transcription of numerous cytokine and effector genes, such as IL-2, IL-4, IFN-γ, and FasL are all reduced in stimulated Itk−/− CD4+ T cells [59, 60, 71, 72, 94]. Overall, these data have indicated that TCR signaling in the absence of Itk is greatly diminished, but not entirely abolished.
Interest in the role of Itk in CD4⁺ T cell effector function was heightened by intriguing observations demonstrating that Itk⁻/⁺ mice have impaired responses to pathogenic infection. For instance, in response to T. gondii, a pathogen that normally elicits a protective Th1 response, Itk-deficient mice succumb to the infection with a mean survival time of 69 days [59]. In contrast, in response to L. major, a pathogen that normally elicits a protective Th1 response in C57BL/6 mice and a non-protective Th2 response in Balb/c mice, Itk-deficient mice on both genetic backgrounds mounted a protective Th1 response [94]. However, in response to the nematode, N. brasiliensis, which elicits a protective Th2 response in Balb/c mice, Itk⁻/⁺ Balb/c mice were unable to clear the infection. Further in vitro data demonstrated that an inability to translocate NFATc to the nucleus following TCR activation contributed to the observed impairment in IL-4 gene expression in Itk⁻/⁺ cells, as well as to the failure of these cells to differentiate into Th2 effector cells in vivo [94]. Data from Schaeffer et al further supported these observations by showing that, upon challenge with the helminth, S. mansoni, Itk⁻/⁻ mice generate a Th1 effector response to this pathogen instead of the Th2 response normally observed in wild type mice [72]. Finally, recent work from Mueller and August examined an allergic asthma response after priming with the antigen ovalbumin, and found that Itk⁻/⁻ mice did not generate this allergic response [151]. Collectively, these data demonstrate that mice lacking Itk exhibit a selective and profound impairment in generating Th2-polarized CD4⁺ T cell responses.

The ability of naïve CD4⁺ T cells to differentiate into Th1 or Th2 effector cells is regulated by a number of factors, the most important of which is the cytokine milieu during T cell activation. Although many cell types, such as dendritic cells, natural killer
cells, and mast cells, can serve as a source of effector cytokines that influence T helper cell differentiation, the initial source of these cytokines *in vivo* following pathogenic infection is not always apparent. One hypothesis is that the T cells themselves produce cytokines that affect their own fate. In this regard, *in vitro* studies have demonstrated that varying the strength of TCR signaling by altering antigen concentration or potency can strongly influence T helper cell polarization [159-167].

In this study we sought to clarify the role of Itk in T helper cell differentiation. As described, all of the *in vivo* data indicate that T cells in Itk-deficient mice are unable to mount a protective Th2 response. However, whether this reflects a defect in T helper cell differentiation per se, a selective defect in Th2 cytokine production, or a defect in cytokine production by cells of the innate immune system in Itk−/− mice could not be discerned from the previous studies [72, 94]. The most straightforward possibility is that impaired IL-4 production by Itk−/− CD4+ T cells is responsible for this defect. Yet mice lacking IL-4R or Stat6 can still elicit Th2 responses [168, 169], suggesting that this simple explanation may not be sufficient to account for the Th2-specific deficiency in Itk−/− mice. Additionally, impaired cytokine production by other cell types may contribute to the ineffective Th2 responses in the absence of Itk. This latter possibility arises due to the known expression of Itk in NK T cells [170], NK cells [171], and mast cells [172], each of which can produce cytokines that may influence T helper cell differentiation. For this reason, we investigated Itk−/− CD4+ T cells for intrinsic defects in Th2 cytokine production, transcription factor expression, and T helper cell differentiation.

In this report, we establish a highly controlled *in vitro* system to examine T helper cell activation and differentiation. Using this system, we demonstrate that Itk−/− CD4+ T
cells are fully capable of differentiating into both Th1 and Th2 effector subsets when exposed to exogenous cytokines. However, these Th1 and Th2 differentiated cells exhibit profound defects in both IFN-γ and IL-4 transcription and secretion, respectively. In contrast, in the absence of exogenous cytokines, Itk−/− T cells fail to differentiate into Th2 cells when stimulated in conditions that promote strong Th2 polarization by wild type T cells. These contrasting behaviors of wild type vs. Itk−/− T cells correlated with a dramatic overexpression of T-bet mRNA in Itk−/− T cells within the first 12 hours following TCR stimulation. We also found that differentiating wild type Th2 cells up-regulate Itk mRNA and protein levels, and completely down-regulate expression of the related Tec kinase, Rlk. Therefore, in the absence of Itk, CD4+ T cells possess an intrinsic inability to differentiate into Th2 effector cells, as a result of two factors: aberrantly high expression of T-bet plus a further deficit in TCR signaling due to the loss of Rlk.
Results

Itk−/− CD4+ T cells can efficiently differentiate into polarized Th1 and Th2 cells

Previous in vitro studies examining effector cytokine production by Itk−/− CD4+ T cells have indicated that these cells show dramatic defects in effector cytokine production. Based on ELISA data measuring cytokine secretion following restimulation, Itk−/− CD4+ T cells were found to be severely impaired in IL-4 secretion, with a less profound defect in IFN-γ secretion [72, 94]. However, these studies did not address the extent to which Itk−/− CD4+ T cells are defective in T helper cell differentiation per se versus showing a selective defect in effector cytokine gene expression following TCR restimulation. To examine this issue and determine if Itk−/− CD4+ T cells are able to differentiate into both Th1 and Th2 cells in vitro, we established a highly controlled in vitro assay using homogeneous populations of naïve CD4+ T cells from Itk−/− and Itk+/+ mice. To accomplish this, Itk−/− and Itk+/+ mice were crossed to the 5C.C7 TCR transgenic line on a RAG−/− background. Naïve CD4+ T cells were isolated from these mice, and stimulated in vitro with CH27 B lymphoma cells as antigen-presenting-cells (APCs) plus the cognate antigen, a peptide derived from moth cytochrome c (MCC93-103).

In our initial experiments, T cells were stimulated with APCs and 10nM of MCC93-103 peptide in both Th1-skewing (rIL-12 + anti-IL4 antibody) and Th2-skewing (rIL-4 + anti-IFN-γ antibody) conditions for 3 days, in the presence of excess rIL-2. As opposed to chapter II, 10nM of peptide was used here, as it was found to drive a higher degree of differentiation. Following this 3-day culture period, cells were restimulated with APCs and varying doses of the MCC93-103 peptide for 6 hours, and assayed for IFN-γ and IL-4 by intracellular cytokine staining. Figure 21A shows raw data obtained from
this analysis, indicating that, as expected, cells stimulated in Th1-polarizing conditions produce IFN-γ and not IL-4, whereas cells stimulated in Th2-polarizing conditions produce IL-4 and not IFN-γ. In Figure 21B, these data are converted into a bar graph format in which the percentage of cells that are producing either IFN-γ or IL-4 are shown as a function of the peptide concentration used for the restimulation. From these data, it is clear that both Itk+/− and Itk−/− cells are able to produce the appropriate effector cytokine after stimulation in either Th1 and Th2 polarizing conditions; furthermore, it is interesting to note that in the absence of Itk, there is only a slight decrease in the percentage of cells that have differentiated into effector cells of each lineage. However, upon examination of the mean fluorescence intensity of both IFN-γ and IL-4 staining in this assay, it is evident that, of the cells that are producing cytokine, on a per cell basis Itk−/− T cells produce less of the respective cytokine than wild type T cells (data not shown). A similar observation has been reported for IL-2 production by Itk−/− CD4+ T cells [42]. Overall, these data demonstrate that after stimulation in the presence of skewing cytokines, nearly equivalent percentages of Itk-deficient cells differentiate into both Th1 and Th2 effector cells. We have consistently observed, as others have [173], that upon stimulation of CD4+ T cells in either Th1- or Th2-skewing conditions, a greater proportion of cells differentiate into Th1 effectors than Th2 effectors.

To determine if the amount of effector cytokines produced by Itk-deficient cells is indeed decreased compared to wild type T cells, we restimulated both Th1- and Th2-skewed cells from Itk+/− and Itk−/− cultures for 24 hours with APCs and varying concentrations of the MCC93−103 peptide, and then assayed for IFN-γ and IL-4 in the supernatants by ELISA. As shown in Figure 21C, Itk−/− Th1 cells secrete 1.5- to 200-fold
less IFN-γ than Itk<sup>+/−</sup> T cells. Likewise, Th2 cells lacking Itk secrete 3.5- to 130-fold less IL-4 than control T cells. In addition, we found similar reductions in IL-5 and IL-10 secretion by Itk-deficient Th2 cells (data not shown). These results are comparable to data reported by Fowell et al and Schaeffer et al following stimulation of Itk<sup>−/−</sup> cells by anti-CD3 antibody cross-linking [72, 94]. However, it is apparent from the data presented here that the magnitude of the deficiency observed varies significantly depending on the dose of antigen used to restimulate the cells. This is consistent with many of our observations on Itk<sup>−/−</sup> T cells, that the signaling deficiencies observed are greatly exacerbated by suboptimal TCR stimulation [42, 71]. This observation may account for discrepancies in effector cytokine production profiles of Itk<sup>−/−</sup> T cells reported previously [72, 94].

Thus far our data indicate that Itk<sup>−/−</sup> CD4<sup>+</sup> T cells can differentiate efficiently into both Th1 and Th2 effector cells, but that these differentiated cells are poor producers of their respective effector cytokines. Based on the role of Itk in PLC-γ1 activation downstream of the TCR, it seemed likely that the cytokine production defect resulted from impaired transcriptional activation of the IFN-γ and IL-4 genes, respectively, in differentiated Itk<sup>−/−</sup> T cells. To test for steady-state mRNA levels of IFN-γ and IL-4, we performed real-time quantitative RT-PCR on RNA prepared following a 6-hour restimulation of Th1- and Th2-skewed cells. For these experiments, Itk<sup>+/−</sup> and Itk<sup>−/−</sup> cells were stimulated with APCs and varying concentrations of the MCC<sub>93-103</sub> peptide. As shown in Figure 21D, these data precisely parallel the cytokine secretion data, showing greatly reduced transcripts for both IFN-γ and IL-4 in Itk<sup>−/−</sup> effector cells following TCR stimulation. These findings confirm that reduced cytokine secretion by Itk<sup>−/−</sup> T cells is a
consequence of reduced transcription of these cytokine genes in differentiated Itk\textsuperscript{-/-} effector CD4\textsuperscript{+} T cells.

The data presented above strongly suggest that Itk\textsuperscript{-/-} CD4\textsuperscript{+} T cells have no intrinsic defect in their ability to respond to extrinsic cytokine signals by differentiating into the appropriate lineage of helper T cell. To assess this issue further, we examined mRNA levels for two transcription factors, T-bet and GATA-3, that are the master regulators of Th1 and Th2 differentiation, respectively [174, 175]. Specifically, T-bet has been shown to play a crucial role in chromatin remodeling at the IFN-\(\gamma\) locus, as well as in the induction of IL-12R\(\beta2\) expression, and ultimately controls the IL-12/Stat4/IFN-\(\gamma\) autocrine loop [176-179]. Similarly, GATA-3 functions in several processes during Th2 differentiation, including chromatin remodeling at several Th2 cytokine loci, repression of Stat4, and its own autoactivation [180-184]. As shown in Figure 21E, Itk\textsuperscript{-/-} Th1 and Th2 cells express comparable levels of T-bet and GATA-3, respectively, compared to Itk\textsuperscript{+/+} cells. In addition, we examined the mRNA levels for another transcription factor, c-Maf, that is essential for trans-activation of the IL-4 promoter in Th2 cells [185]. As can be seen in Figure 21E, Itk\textsuperscript{-/-} Th2 cells show no reduction in c-Maf mRNA levels compared to control Th2 cells. Taken together, these data demonstrate that, given exogenous cytokines, Itk-deficient CD4\textsuperscript{+} T cells are fully capable of differentiating into both Th1 and Th2 cells, as evidenced by their normal frequency of effector cytokine producers as well as by their expression of the signature Th1 or Th2 transcription factors. Nonetheless, these cells are impaired in their ability to produce IFN-\(\gamma\) and IL-4 on a per cell basis compared to control T cells. As Itk\textsuperscript{-/-} Th1 cells have wild type levels of T-bet, and Itk\textsuperscript{+/+} Th2 cells have normal levels of GATA-3 and c-Maf, it is likely that their defects
in effector cytokine production reflect reduced activation of additional factors required for the transcription of these cytokine genes.
Figure 21: Itk⁻/⁻ CD4⁺ T cells can efficiently differentiate into polarized Th1 and Th2 cells

Purified CD4⁺ T cells from 5C.C7 Itk⁺/⁻ RAG⁺/⁻ and 5C.C7 Itk⁻/⁻ RAG⁺/⁻ mice were stimulated *in vitro* with APCs and peptide in Th1- or Th2-skewing conditions. (A) Following 3 days of culture, cells were restimulated with fresh APCs and MCC₉₃-₁₀₃ peptide for 6 hours, stained with anti-CD4-Cy and anti-Vα₁₁-FITC, fixed, permeabilized, and stained intracellularly with anti-IL-4-PE and anti-IFN-γ-APC. Ten thousand CD4⁺ Vα₁₁⁺ events were collected on a flow cytometer. An example of raw data is shown. (B) Intracellular cytokine staining data from the experiment shown in (A) in bar graph format, depicting the percentage of cells that are producing IFN-γ or IL-4, in the Th1- or Th2-skewed cultures, respectively, in response to varying doses of MCC₉₃-₁₀₃. (C) Following 7 days in culture, cells were restimulated for 24 hours with fresh APCs and varying doses of MCC₉₃-₁₀₃. Supernatants were analyzed for the production of IFN-γ and IL-4, respectively, by ELISA. Error bars indicate SD of values obtained from stimulations performed in triplicate. (D and E) Cells were restimulated for 6 hours on Day 7 with fresh APCs and varying doses of MCC₉₃-₁₀₃. Following stimulation, RNA was isolated and 1μg was reverse transcribed into cDNA and subjected to real-time quantitative PCR analysis for β-actin, IFN-γ, and IL-4 (D) or T-bet, GATA-3, and c-Maf (E). The y-axis values represent the respective transcript normalized to the β-actin values determined for each sample. Error bars are the SD of transcript values obtained from triplicate reactions.
Figure 21

A

Th1-skewed

No peptide

100 nM

Th2-skewed

No peptide

100 nM

SC_C7 Itk+/−

IL-4

IFN-γ

SC_C7 Itk−/−

B

Th1-skewed

Th2-skewed

% of cells producing IFN-γ

[CC93-103] nM

C

SC_C7 Itk+/−

SC_C7 Itk−/−

[CC93-103] nM

[CC93-103] nM

D

SC_C7 Itk+/−

SC_C7 Itk−/−

[CC93-103] nM

[CC93-103] nM

E

T-bet

GATA-3

c-Maf

SC_C7 Itk+/−

SC_C7 Itk−/−
Low avidity TCR stimulation promotes Th2 differentiation by Itk\(^{+/−}\), but not by Itk\(^{−/−}\), CD4\(^{+}\) T cells

As described above, previous infectious disease studies examining immune responses of Itk\(^{−/−}\) mice to *L. major*, *S. mansoni*, and *N. brasiliensis* had indicated that Itk\(^{−/−}\) T cells were selectively impaired in generating protective Th2 responses. To determine whether these in vivo findings could result from intrinsic defects in Itk\(^{−/−}\) CD4\(^{+}\) T cell responses, we sought to establish in vitro conditions to test this hypothesis. To address this issue, we required a system that preferentially leads to Th2 differentiation by wild type CD4\(^{+}\) T cells in the absence of overt skewing by cytokines and blocking antibodies. Since our Itk\(^{−/−}\) mouse line is on a C57Bl/6 background, we knew that straight-forward in vitro stimulation of naïve CD4\(^{+}\) T cells with MCC peptide plus APCs would lead to almost exclusive Th1 polarization. However, a number of previous studies have demonstrated that stimulation of naïve CD4\(^{+}\) T cells with high versus low avidity TCR engagement can differentially promote Th1 versus Th2 differentiation in the absence of exogenous cytokines. Specifically, high concentrations of agonist peptide that elicit robust activation of proximal TCR signaling molecules, leading to sustained calcium influx and ERK activation, induce the generation of Th1 cells, whereas altered peptide ligands (APLs) or low concentrations of agonist peptide, that induce a transient calcium response and weaker ERK signal, promote the generation of Th2 cells [162, 164, 186-189].

As shown in Figure 22, this phenomenon holds true for naïve T cells purified from Itk\(^{−/−}\) 5C.C7 TCR transgenic RAG\(^{−/−}\) mice. For these experiments, T cells were initially stimulated with APCs plus a high (100nM) versus a low (1nM) concentration of
MCC\textsubscript{93-103}. T cells were also stimulated with high concentrations of two APLs, MCC\textsubscript{T102S} and MCC\textsubscript{A96S}, that have previously been shown to be recognized with lower affinities and to induce weaker responses by 5C.C7\textsuperscript{+} T cells [190, 191]. After three days of culture in the absence of any additional cytokines or blocking antibodies, T cells were restimulated with fresh APCs and varying concentrations of the normal MCC\textsubscript{93-103} peptide for 6 hours. Cells were then stained intracellularly for IFN-\(\gamma\) and IL-4 (as in Figure 21A) and analyzed by flow cytometry. As can be seen in the raw data shown in Figure 22A and summarized in bar graph format in Figures 22B-E, Itk\textsuperscript{+/−} T cells differentiate predominantly into IFN-\(\gamma\)-producing cells following stimulation with a high concentration of MCC\textsubscript{93-103}, but become IL-4-secreting cells when initially stimulated with a low concentration of MCC\textsubscript{93-103}. Both of the APLs induce a mixed response from Itk\textsuperscript{+/−} cells, with cultures displaying proportions of both IFN-\(\gamma\)- and IL-4-producing cells. Interestingly, when 5C.C7 TCR transgenic RAG\textsuperscript{−/−} Itk\textsuperscript{+/−} CD4\textsuperscript{+} T cells were stimulated with these varying peptides and peptide concentrations, and then restimulated to test for T helper cell differentiation, all of the stimulation conditions induced Itk\textsuperscript{−/−} T cells to become IFN-\(\gamma\)-producing cells (Figure 22A-E). Collectively, these data demonstrate that, upon stimulation with conditions that induce wild type T cells to differentiate into Th2 cells, Itk-deficient T cells fail to do so, and instead differentiate into Th1 cells. In some cases, the cultures of Itk-deficient T cells contain a greater percentage of cells that have differentiated, as measured by the total percent of cells producing effector cytokines (see Figures 22C-E); this interesting phenomenon may be a consequence of the fact that Th1 cells are more efficiently generated \textit{in vitro} than Th2 cells (see Figure 21B).
To further assess the differentiation status of cells stimulated in the various conditions, we examined the levels of GATA-3 and T-bet mRNAs 3 days post-stimulation in Th1- and Th2-skewing conditions, as well as in the various non-skewing conditions shown in Figures 22B-D. As shown in Figure 22F, Itk\textsuperscript{+/−} as well as Itk\textsuperscript{−/−} cells stimulated in Th1 or Th2 polarizing conditions show the expected profiles of GATA-3 and T-bet mRNA expression. When the cells were stimulated in the absence of exogenous cytokines and blocking antibodies, control T cells (Itk\textsuperscript{+/−}) exhibited an interesting pattern of T-bet and GATA-3 expression. Three days after stimulation with a high concentration of the agonist peptide, Itk\textsuperscript{+/−} T cells express substantial amounts of T-bet mRNA, but also show significant levels of GATA-3 mRNA. After stimulation with conditions that promote Th2 differentiation these cells express dramatically high levels of GATA-3, but very little T-bet mRNA, even in cultures that contain mixed populations of IL-4- and IFN-γ-producing cells. In contrast, Itk\textsuperscript{−/−} T cells express moderately high levels of T-bet under all stimulation conditions (Figure 22F). It is particularly striking that Itk\textsuperscript{−/−} cells also express substantial levels of GATA-3 mRNA, suggesting that the low avidity TCR signals that promote GATA-3 mRNA up-regulation are still functional in the absence of Itk. This dual expression of T-bet and GATA-3 may also account for the increased proportion of Itk\textsuperscript{+/−} cells that produce both IFN-γ and IL-4 compared to that seen in Itk\textsuperscript{+/−} cultures (Figure 22C, D). Overall, these data indicate that Itk\textsuperscript{−/−} T cells preferentially up-regulate T-bet and differentiate into IFN-γ-producing cells under low avidity TCR stimulation conditions that promote Th2 differentiation in wild type T cells.
Figure 22: Low avidity TCR stimulation promotes Th2 differentiation by Itk^{+/−}, but not by Itk^{−}, CD4^{+} T cells

Purified CD4^{+} T cells from SC.C7 Itk^{+/−} RAG^{−/−} and SC.C7 Itk^{−/−} RAG^{−/−} mice were stimulated in vitro with APCs and either 100nM or 1nM of MCC_{93-103}, 100nM of MCC_{T102S}, or 100nM of MCC_{A96S}. Following 3 days in culture, cells were restimulated for 6 hours and assayed for IFN-γ and IL-4 production by intracellular cytokine stained as described in Figure 21.

(A) An example of raw data showing cytokine staining profiles.

(B, C, D, E) The percentage of cells that produce either IFN-γ, IL-4, or both, are depicted in bar graph formats corresponding to the initial stimulus: 100nM MCC_{93-103} (B), 1nM MCC_{93-103} (C), 100nM MCC_{T102S} (D), or 100nM MCC_{A96S} (E).

(F) RNA was isolated from cells on Day 3, transcribed into cDNA, and subjected to real-time quantitative PCR analysis for β-actin, T-bet, and GATA-3. The y-axis values represent the respective transcript normalized to the β-actin values determined for each sample. Error bars are the SD of transcript values obtained from triplicate reactions.
Figure 22

A Initial stimulus:
Restimulation: No peptide

SC.C7
Itk+/-

500 M MCC
10 nM MCC
100 nM MCC
1000 nM MCC

IFN-γ

500 nM MCC
10 nM MCC
100 nM MCC
1000 nM MCC

SC.C7
Itk-/-

IL-4

B 100 nM MCC

C 1 nM MCC

D 100 nM MCC_T102S

E 100 nM MCC_A96S

F Ratio of GATA-3/β-actin

Ratio of T-bet/β-actin

G1.000 0.003 0.006 0.009 0.012 0.015 0.018 0.021 0.024 0.027 0.030

Th1

Th2

100 nM MCC

1 nM MCC

100 nM MCC_T102S

100 nM MCC_A96S

SC.C7 Itk+-

SC.C7 Itk-/-
T-bet mRNA expression is aberrantly regulated by TCR signals in the absence of Itk

The regulation of T-bet and GATA-3 expression during T helper cell differentiation is complex, involving TCR signals, cytokine signals, and autocrine feedback loops. In naïve CD4⁺ T cells, T-bet is expressed at low levels, and is upregulated during Th1 differentiation by a Stat1-dependent pathway that is independent of IL-12/STAT4 signals; ongoing T-bet expression is then required to maintain the competence of cells to express IFN-γ [176, 178, 179]. Akin to T-bet, GATA-3 is thought to maintain the competence of cells to activate transcription of the IL-4 gene [180, 183, 184, 192, 193]. In addition, GATA-3 also possesses the ability to inhibit Th1 differentiation by repressing Stat4, and ultimately, IFN-γ transcription [180, 182]. While also expressed at low levels in naïve T cells, GATA-3 is activated following TCR stimulation and autoactivates its own transcription [181]. In addition, IL-4R signaling via Stat6 can further increase GATA-3 levels [180]. However, GATA-3 alone is not sufficient for IL-4 transcription, and the action of several other factors such as JunB, NFATc1, and c-Maf are required [185, 194, 195].

The mechanism by which low levels of T-bet and GATA-3 proteins are activated in naïve T cells, as well as how the T-bet and GATA-3 genes are further upregulated following TCR stimulation, has not been fully elucidated. However, our findings suggested that TCR signaling via Itk may be critical for the proper regulation of T-bet and/or GATA-3 levels in stimulated naïve CD4⁺ T cells. To address this possibility, we examined the levels of T-bet and GATA-3 mRNAs, as well as those for IFN-γ and IL-4, in Itk⁺/⁻ and Itk⁻/⁻ cells within the first 24 hours after stimulation. In this analysis we
examined cells that had been stimulated with MCC$_{93-103}$ plus APCs in Th1- and Th2-skewing conditions, as well as cells stimulated with the APCs plus the APL, MCC$_{102S}$, in both non-skewing (no exogenous cytokine) and neutralizing (with anti-IL-4 and anti-IFN-γ antibodies) conditions. The APL stimulation conditions were specifically chosen for their ability to induce divergent responses from Itk$^{+/\cdot}$ and Itk$^{-/-}$ cells, allowing us an opportunity to determine the underlying molecular mechanism for the aberrant T helper cell polarization of Itk$^{-/-}$ cells in these experiments.

As shown in Figure 23A, naïve Itk$^{+/\cdot}$ T cells up-regulate T-bet mRNA levels following strong TCR stimulation in both Th1 and Th2 skewing conditions, although within 12 hours after stimulation there is a ~10-fold excess of T-bet mRNA in the cells cultured in IL-12 plus anti-IL-4 antibody. This difference is further enhanced by 24 hours after stimulation, by which time there is a 70-fold increase in T-bet mRNA in the Th1- versus the Th2-skewed cells. GATA-3 mRNA shows an inverse pattern of expression in Itk$^{+/\cdot}$ cells, with a >10-fold increase seen in Th2-skewed cells versus Th1-skewed cells by 12 hours after stimulation (Figure 23B). These patterns of transcription factor expression correlate nicely with cytokine gene expression, as shown in Figure 23C-D. IFN-γ transcripts are induced at 12 hours following TCR stimulation in both sets of polarizing conditions, but by 24 hours IFN-γ transcripts have fallen in the Th2-skewed cells, and continued to rise in the Th1-skewed cells, resulting in a ~100-fold difference (Figure 23C). Similarly, IL-4 transcripts are just above the level of detection in the Th2-skewed Itk$^{+/\cdot}$ cells at 12 hours, and rise dramatically by 24 hours (Figure 23D). Interestingly, the patterns of GATA-3 and IL-4 gene expression are nearly identical between the Itk$^{+/\cdot}$ and Itk$^{-/-}$ T cells stimulated under these conditions (Figure 23B, D).
contrast to Itk<sup>+/−</sup> T cells, Itk<sup>−/−</sup> T cells show dramatic increases in T-bet mRNA levels at 12 hours after stimulation in both Th1- and Th2-skewing conditions, although the levels of T-bet mRNA do drop substantially by 24 hours in the Th2-skewed cells (Figure 23A). In parallel, Itk<sup>−/−</sup> Th2-skewed T cells also have higher levels of IFN-γ transcripts at the 12 hour timepoint, which also diminish by 24 hours (Figure 23C). These data further support the aberrant regulation of T-bet and/or IFN-γ transcript levels immediately following TCR stimulation of naïve Itk<sup>−/−</sup> T cells. Interestingly, this early Th1 bias in the response of Itk<sup>−/−</sup> T cells is quickly overturned by the strong cytokine signals provided in vitro with Th2-skewing conditions, indicating that Itk<sup>−/−</sup> T cells have normal responses to IL-4 signaling pathways.

In an effort to address the mechanism by which Itk-deficient cells failed to differentiate into Th2 cells in non-skewing conditions, we examined the levels of T-bet, GATA-3, IFN-γ, and IL-4 mRNAs in response to 100nM of MCC<sub>T102S</sub>, a condition that induces a mixture of Th1 and Th2 cells in wild type cultures but only Th1 cells in Itk<sup>+/−</sup> cultures. To exclude the possibility that the initial cytokines produced by the cells could feedback in an autocrine loop and influence gene expression, we performed these stimulations in both non-skewing (no exogenous cytokines) as well as neutralizing conditions (no cytokines plus anti-IL-4 and anti-IFN-γ antibodies). As shown in Figure 23E, following the stimulation of cells in non-skewing conditions with MCC<sub>T102S</sub>, T-bet expression is rapidly induced in Itk-deficient cells, while it is only weakly upregulated in control Itk<sup>+/−</sup> cells. Consistent with the recent finding that IFN-γ-induced STAT1 signals further upregulate T-bet expression [176], the presence of neutralizing anti-IFN-γ antibody significantly reduces the T-bet expression in Itk<sup>−/−</sup> cells by 24 hours. A similar
pattern of expression is observed for IFN-γ (Figure 23G). Conversely, GATA-3 transcript levels are rapidly upregulated in both control and Itk−/− cells at 12 and 24 hours post stimulation, although only the Itk+/− cells produce detectable levels of IL-4 transcripts (Figures 23F, H). These latter data are consistent with a previous report demonstrating that wild type and Itk+ cells regulate GATA-3 comparably [72]. Taken together, these data clearly demonstrate that in response to TCR signals, Itk-deficient cells show a dramatic alteration in the regulation of T-bet mRNA expression, but regulate GATA-3 normally.
Figure 23: T-bet mRNA expression is aberrantly regulated by TCR signals in the absence of Itk

Purified CD4+ T cells from 5C.C7 Itk+/− RAG−/− and 5C.C7 Itk−/− RAG−/− mice were stimulated in vitro with APCs and MCC93.103 in Th1- or Th2-skewing conditions (panels A-D) or stimulated with APCs and 100nM of MCCT1028 (panels E-H) in either non-skewing (no exogenous cytokine) or neutralizing conditions (anti-IL-4 and anti-IFN-γ antibodies). Cells were stimulated for either 0, 12, or 24 hours. Following the indicated time points, RNA was isolated from cells, transcribed into cDNA, and subjected to real-time quantitative PCR analysis for GATA-3 (A, E), T-bet (B, F), IL-4 (C, G), or IFN-γ (D, H). The y-axis values represent the respective transcript normalized to the GAPDH values determined for each sample. Error bars are the SD of transcript values obtained from triplicate reactions. The asterisk (*) next to the horizontal bar indicates the limit of quantitation for the cytokine transcripts. The ratio for the limit of quantitation was calculated by dividing the actual lowest quantifiable copy of IFN-γ or IL-4 cDNA by the average GAPDH value.
Figure 23
Th1/Th2 skewing conditions
Initial Stimulus: 10nM MCC

Non-skewing/Neutralizing conditions
Initial Stimulus: 100nM MCC_T102S

Non-skew: No exogenous cytokine
Neutral: anti-IL-4, anti-IFN-γ
Collectively, the data presented above indicate that, after stimulation by low avidity TCR engagement, Itk\(^{+/−}\) T cells express increased T-bet mRNA and preferentially differentiate into Th1 cells. This is in direct contrast to Itk\(^{+/−}\) T cells, which under these same conditions express very little T-bet mRNA, and differentiate predominantly into IL-4-producing cells. Since high avidity TCR engagement induces Th1 differentiation from both Itk\(^{+/−}\) and Itk\(^{−/−}\) cells, we were interested in determining whether T-bet transcript levels varied depending on the strength of the TCR signal. As shown in Figure 24, 12 hours following stimulation of naïve T cells with a range of MCC\(_{93−103}\) or MCC\(_{1028}\) peptides in the presence of neutralizing antibodies to IL-4 and IFN-γ, Itk\(^{+/−}\) T cells show a relatively uniform induction of T-bet mRNA compared to unstimulated cells. In contrast, Itk\(^{−/−}\) T cells express increased levels of T-bet mRNA as the strength of the TCR signal decreases. These findings suggest, first of all, that the Th2-prone differentiation behavior of wild type T cells upon stimulation with low avidity TCR engagement is not due primarily to a reduced induction of T-bet transcripts, but instead results from increased GATA-3 expression under these conditions. This conclusion is consistent with a previous report demonstrating increased GATA-3 induction in T cells stimulated with low avidity ligands [196] (data not shown). Second, these data suggest that Itk is required for negative regulation of T-bet mRNA expression following low avidity TCR stimulation.
Figure 24: Itk negatively regulates T-bet upon low avidity TCR stimulation

Purified CD4+ T cells from SC.C7 Itk<sup>-/-</sup> RAG<sup>-/-</sup> and SC.C7 Itk<sup>-/-</sup> RAG<sup>+/+</sup> mice were stimulated with the indicated concentrations of either MCC<sub>93-103</sub> or MCC<sub>11028</sub> peptide plus APCs for 0 or 12 hours in neutralizing conditions (with anti-IL-4 and anti-IFN-γ antibodies). Following stimulation, RNA was isolated, transcribed into cDNA, and subjected to real-time quantitative PCR analysis for T-bet and GAPDH. The y-axis represents T-bet normalized to the GAPDH values determined for each sample. Error bars are the SD of transcript values obtained from triplicate reactions.
Figure 24

![Bar graph showing the ratio of T-bet/GAPDH for different concentrations of MCC93-103 and MCC102S in SC.C7 Itk+/+ and SC.C7 Itk-/- cells.]

- **SC.C7 Itk+/+**
  - MCC93-103:
    - 0 μM: 0.00005
    - 1 μM: 0.00010
    - 0.1 μM: 0.00015
    - 0.01 μM: 0.00020
- **SC.C7 Itk-/-**
  - MCC93-103:
    - 0 μM: 0.00005
    - 1 μM: 0.00010
    - 0.1 μM: 0.00015
    - 0.01 μM: 0.00020
  - MCC102S:
    - 1 μM: 0.00015
    - 0.1 μM: 0.00020
Itk is upregulated and Rlk is downregulated during Th2 differentiation

Several years ago, another Tec family tyrosine kinase expressed primarily in T lymphocytes, Rlk/Txk, was identified and was also found to contribute to the activation of PLC-γ, suggesting some functional redundancy within the Tec family [29, 63]. Interestingly, Takeba et al recently demonstrated that Rlk can function as a transcriptional activator of the IFN-γ gene by binding to the IFN-γ promoter [40, 59], suggesting a unique function for Rlk in differentiated Th1 cells [197]. In conjunction with these findings, our own data indicating a role for Itk in negatively-regulating T-bet expression suggested the possibility that Itk as well as Rlk may be differentially expressed following T cell differentiation. To address this, we analyzed Itk and Rlk mRNA and protein levels in wild type CD4+ T cells differentiating in vitro after stimulation in Th1- versus Th2-skewing conditions. As shown in Figure 25A, we found that Rlk mRNA is rapidly downregulated within 6 hours following stimulation of naïve cells in both Th1 and Th2 conditions. However, Rlk transcripts begin to be upregulated again in Th1 cells after 24 hours and remain increased over the following 11 days of culture, but fail to be re-induced in Th2 cells (Figure 25B). In contrast to Rlk, Itk mRNA levels are maintained in both Th1 and Th2 conditions during the first 24 hours following stimulation (Figure 25C); however, around 8 days post-stimulation in Th2 culture conditions, Itk mRNA levels are upregulated (Figure 25D). Interestingly, analysis of the putative promoter regions of Itk and Rlk provide some clues about their differential regulation in Th1 versus Th2 cells. Sequence analysis using the TRANSFAC database (http://www.cbil.upenn.edu/tess) indicated that the promoter region of Itk contains several canonical GATA protein binding motifs, including GATA-3 sites. In contrast,
the Rlk promoter region lacks GATA-3 binding motifs, providing a potential explanation for the lack of re-expression of Rlk mRNA in differentiating Th2 cells.

Analysis of Itk and Rlk protein levels in wild type CD4+ T cells cultured in Th1 or Th2 skewing conditions confirmed the differential expression observed at the mRNA level. As shown in Figure 25E, Rlk protein is detectable in Th1, but not in Th2 cells, whereas Itk protein is present in both cell types, but at a significantly higher level in Th2 cells. Consistent with this latter observation, a recent report found that Itk mRNA is dramatically elevated in T cells from patients afflicted with atopic dermatitis, an inflammatory skin disease in which Th2 cytokines play a major role [198]. Overall these findings are summarized in Figure 25F.
Figure 25: Itk is upregulated and Rlk is downregulated during Th2 differentiation

Purified CD4^+ T cells from 5C.C7 Itk^+/+ RAG^+/+ and 5C.C7 Itk^-/- RAG^-/- mice were stimulated in Th1- or Th2-skewing conditions and harvested at 0, 6, 18, and 24 hours (A and C) as well as at 1, 4, 8, and 11 days post stimulation (B and D). Following the indicated time points, RNA was isolated and subject to real-time quantitative PCR for either Rlk (A and B) or Itk (C and D). Panels A and C represent Rlk and Itk copy numbers, respectively, normalized to cell number equivalents. Panels B and D represent Rlk and Itk copy numbers, respectively, normalized to GAPDH values. The asterisk (*) denotes that the indicated ratio is greater in these particular circumstances as a result of GAPDH values being very low in naive T cells compared to activated and expanding cells in culture.

(E) In the top panel, 3.5 x 10^6 Th1 and Th2 cells were removed from culture at 8 (lane 1,3) and 11 (lane 2,4) days post stimulation. Lysates were blotted for Rlk.

In the bottom panel, lysates from 3.5 x 10^6 Th1 (lane 1), 0.9 x 10^6 Th1 (lane 2), 3.5 x 10^6 Th2 (lane 3), and 0.9 x 10^6 Th2 cells at day 11 post stimulation were blotted for Itk.

As controls, lysates from Rlk^-/- Itk^-/- CD4^+ T cells and from the AE7 Th1 cell line were blotted for Rlk and Itk as indicated.

(F) A summary of the changes in Rlk and Itk expression levels in differentiating Th1 and Th2 cells. Differences between Th1 and Th2 cells are first observed at 24 hours for Rlk, and at ~day 8 of differentiation for Itk.
Figure 25

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

D

![Graph D](image)

E

![Graph E](image)

F

<table>
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<th>Th1</th>
<th>Th2</th>
<th>Time</th>
<th>Rlk</th>
<th>Itk</th>
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<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>&lt;24hrs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+++</td>
<td>++++</td>
<td>~8 days</td>
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Signaling deficiencies in Th1 versus Th2 cells lacking Itk

A number of studies have indicated that there are differences in proximal TCR-induced signaling pathways between Th1 and Th2 effector subsets [199, 200]. For instance, following TCR stimulation, Th2 cells do not efficiently phosphorylate ZAP-70 or Fyn [201]. Additionally, calcium mobilization is not sustained in Th2 cells following activation, apparently as a result of more rapid calcium clearance from the cytosol [202-204]. The molecular basis for these observed differences is unclear; however, a recent report from Balamuth et al, demonstrated that Th1 and Th2 cells differ in the organization of their plasma membranes and their usage of lipid rafts, possibly leading to the activation of distinct signaling pathways in these two cells types, and ultimately affecting gene transcription [205].

To address whether signaling pathways in Th1 and Th2 cells are affected by the differential expression of Itk and Rlk, we assessed the ability of both wild type and Itk-deficient Th1 and Th2 cells to activate PLC-γ1, as well as the downstream ERK-MAP kinase pathway. Based on our observations that Itk is more highly expressed in Th2 cells and that Rlk is absent from Th2 cells, we hypothesized that Itk−/− Th2 cells would show a greater deficit in the activation of PLC-γ1 and downstream events. To test this, wild type and Itk−/− cells were stimulated and cultured in Th1- and Th2-skewing conditions for 11 days, a timepoint at which Itk is upregulated in Th2 cells and Rlk is absent. On day 11, cells were restimulated by anti-CD3 antibody cross-linking, and lysates prepared for analysis. As shown in Figure 26, both Itk−/− Th1 and Th2 cells show greatly impaired phosphorylation of Tyrosine-783 on PLC-γ1, one of the tyrosines that is crucial for the
regulation of PLC-γ1 [206], compared to the Itk<sup>−/−</sup> control cells. Interestingly, Itk<sup>−/−</sup> Th2 cells show a greater deficiency in PLC-γ1 phosphorylation than the Itk<sup>−/−</sup> Th1 cells.

A similar finding was obtained after analysis of ERK activation in Th1 versus Th2 cells lacking Itk. The ERK pathway is important in inducing the transcription of Fos proteins, and thus, in the formation of AP-1 complexes that are crucial for T helper cell effector function (reviewed in [126]). In accordance with impaired PLC-γ1 activation, the phosphorylation of ERK1/ERK2 is more severely impaired in Itk<sup>−/−</sup> Th2 versus Th1 cells (Figure 26). One potential caveat in this experiment is the observation that Itk<sup>−/−</sup> Th2 cells show slightly reduced ERK activation compared to the control Th1 cells; thus Th2 cells, in general, may have a less potent ERK signaling pathway. Nonetheless, following TCR engagement, Itk<sup>−/−</sup> Th2 cells are more impaired in their ability to activate PLC-γ1 and ERK compared to both control Th1 and Th2 cells, as well as Itk<sup>−/−</sup> Th1 cells. Collectively, these data support a model in which Itk is not only crucial for the differentiation of CD4<sup>+</sup> T cells into Th2 effector cells, but may also be important for the function of differentiated Th2 cells. The upregulation of Itk mRNA and protein in Th2 cells is likely to be critical to compensate for the loss of Rlk from these cells.
Figure 26: Signaling deficiencies in Th1 versus Th2 cells lacking Itk

Purified CD4+ T cells from SC.C7 Itk+/+ RAG+/+ and SC.C7 Itk−/− RAG−/− mice were stimulated in Th1- or Th2-skewing conditions and cultured for 11 days. Cells were then restimulated by anti-CD3 antibody cross-linking for either 0, 1, or 2 minutes. Total lysates were immunoblotted with an anti-phosphospecific antibody to PLC-γ1 (P-PLC-γ1(PY783)) or anti-phospho-ERK (p-ERK1&2). The membrane was stripped and reprobed for total PLC-γ1, ERK, and Itk protein.
Figure 26

Th1

Itk+/-. Itk-/.

Th2

Itk+/-. Itk-/.

Time (min)

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<th>1</th>
<th>2</th>
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P-PLC-γ1(PY783)

PLC-γ1

P-ERK1

P-ERK2

ERK2

Itk
**Discussion**

In this report, we demonstrate that Itk is involved in several aspects of T helper cell differentiation. While previous biochemical studies demonstrated a quantitative deficiency in TCR signaling pathways in the absence of Itk, the data described herein indicate that Itk affects qualitative aspects of the TCR signal as well. We have shown that the activation of Itk following stimulation with low avidity TCR ligands is a critical factor in promoting Th2 differentiation by negatively-regulating T-bet mRNA expression. Thus, these findings have important implications for understanding the mechanism by which different strengths of TCR engagement can lead to distinct fates during T cell differentiation, as well as to the activation of distinct subsets of effector functions.

These data have clarified the mechanism by which Itk influences T helper cell differentiation. Two previous studies established that Itk$^{-/-}$ mice are impaired in their ability to generate a protective Th2 response to pathogens such as *N. brasiliensis* or *S. mansoni* [72, 74]. However, it was not clear whether this resulted from a simple deficit in IL-4 production, or was the result of a more complex impairment in Th2 differentiation by Itk$^{-/-}$ CD4$^+$ T cells. Furthermore, the molecular explanation for the observation that Itk-deficient mice preferably generate a Th1 response to pathogens that normally elicit a Th2-response also remained a mystery [72, 74]. Our data have demonstrated that Itk$^{-/-}$ CD4$^+$ T cells are fully capable of differentiating into either Th1 or Th2 effector subsets when exposed to the appropriate cytokines in their environment. But, as previously reported, the resulting Th1 or Th2 effector cells have significant impairments in their ability to produce each subset of effector cytokines [72, 74]. Interestingly, we find that the magnitude of the impairment in cytokine production varies depending on the dose of
peptide Ag with which the cells are restimulated, providing a potential explanation for discrepancies between previous reports examining IFN-γ and IL-4 production by Itk−/− T cells [72, 94].

Our most significant finding is the demonstration that Itk plays a critical role in determining the fate of T cells differentiating in response to low versus high avidity TCR signals in the absence of exogenous cytokines. These data provide an interesting contrast to several reports demonstrating that weak TCR signaling induced by low antigen dose, or by low affinity TCR ligands (APLs), induces transient calcium mobilization and ERK activation, and promotes differentiation into the Th2 lineage [186, 187, 189]. Based on these reports, one might have predicted that Itk−/− CD4+ T cells would be more prone to differentiate into Th2 cells, as several studies have shown that Itk-deficient CD4+ T cells have defects in calcium mobilization and ERK activation following TCR stimulation [59, 60, 64, 71]. However, the experiments presented here argue that, in the absence of Itk, several aspects of T cell activation and differentiation are altered, the net result of which is to favor Th1 over Th2 differentiation.

The first obvious alteration in Itk−/− CD4+ T cells is their poor production of IL-4, as first described by Fowell and colleagues [74]. Our data show that, in contrast to Itk+/− T cells stimulated with an APL, in which IL-4 transcripts can be detected within 24 hours after stimulation, Itk−/− T cells fail to accumulate IL-4 mRNA under these same conditions. This defect is not a result of impaired GATA-3 expression, as Itk+/− and Itk−/− cells have identical levels of GATA-3 mRNA following stimulation with low avidity TCR signals. Instead, as reported previously, the initial impairment in IL-4 transcription is likely a result of defective NFATc translocation [94], and may be linked to
inappropriate modulation of the ratio of NFATc1 and NFATc2 in the nucleus [207]. Interestingly, the defect in Th2 differentiation observed with Itk−/− T cells stimulated in non-skewing conditions can be overcome by addition of exogenous IL-4, which not only induces GATA-3, thus activating endogenous IL-4 expression, but more importantly, also suppresses Th1 development.

The second, and most striking alteration we observed upon activation of Itk-deficient T cells by low avidity TCR engagement was the rapid induction of T-bet expression. To date, little is known about the regulation of T-bet gene expression in response to TCR stimulation. The data presented here indicate that Itk signaling functions in a pathway that inhibits this expression. A recent study by Hartenstein and colleagues found that T-bet mRNA levels fail to be repressed in JunB−/− T cells that have been skewed towards the Th2 lineage [208], suggesting that JunB activity is required to repress T-bet mRNA induction during Th2 differentiation. Consistent with this observation, analysis of the putative T-bet promoter sequence using the TRANSFAC database indicates the presence of several canonical AP-1 binding sites (http://www.cbil.upenn.edu/tess). In addition, activation of JunB in T cells is mediated by JNK [194], a signaling pathway we have previously shown to be deficient in Itk−/− CD4+ T cells [71]. These conclusions are also consistent with the data of Schwartzberg and colleagues demonstrating impaired AP-1 activation in T cells lacking Itk [72]. Thus, one explanation to account for the aberrant expression of T-bet in Itk−/− CD4+ T cells stimulated by low avidity TCR engagement is that reduced JNK signaling leads to impaired JunB activation and a failure to repress T-bet mRNA induction. Alternatively,
our data do not rule out the possibility of a direct affect of Itk on T-bet function that ultimately feeds back to inhibit T-bet expression.

As opposed to the aberrant regulation of T-bet expression in Itk-deficient cells, GATA-3 induction remained largely intact. Several reports have suggested that NF-κB activation via TCR plus CD28 signaling is a crucial event in the induction of GATA-3 transcription, but has no bearing on T-bet or IFN-γ expression [209, 210]. In accordance with this finding, Fowell et al have previously found that NF-κB activation is unaltered in Itk−/− T cells following TCR/CD28 stimulation [74]. Thus, our data showing a role for Itk signaling in the regulation of T-bet, but not GATA-3, expression is consistent with the known pathways modulating these important transcription factors.

Our data also support a model proposing that the strength of TCR signaling influences Th1 versus Th2 differentiation by differential induction of GATA-3, rather than T-bet. Our own data demonstrate that the levels of T-bet mRNA induced within 12 hours in normal CD4+ T cells stimulated with a wide range of antigen concentrations and affinities are remarkably constant. In contrast, we find that GATA-3 mRNA levels increase as the strength of the TCR signal declines (data not shown), as has previously been reported [196]. Thus, when T cells are stimulated under high avidity conditions, modest levels of T-bet are induced together with low GATA-3 levels. These conditions promote IFN-γ transcription, leading to feedback through the IFN-γ receptor to induce more T-bet, and thus to Th1 differentiation. While T-bet itself is not thought to directly repress Th2 cytokine loci, enhanced T-bet expression ultimately results in a secondary effect of IFN-γ mediated repression of the IL-4 gene, consistent with that described by Elser and colleagues [211]. In contrast, following stimulation with low avidity ligands,
naïve CD4+ T cells induce modest levels of T-bet, but greatly increased levels of GATA-3. Due to the dramatic auto-activation property of GATA-3 [181], plus its ability to suppress Th1 differentiation [180], this early increase in GATA-3 expression may be sufficient to lead to Th2 polarization in the absence of exogenous cytokines. Lastly, the costimulatory molecules CD28 and CTLA-4 have both been found to influence T cell differentiation in the context of strong versus weak TCR signals [212, 213]. Therefore, we have not ruled out the possibility that Itk may play a role downstream from these costimulatory molecules. This possibility is discussed further in figure 27.

While this report provides insight into the function of Itk in CD4+ T cell differentiation, the role of Rlk is less clear. Although T cells from Rlk-/-Itk-/- mice have more severe impairments in TCR signaling than those lacking Itk alone [59], Rlk-/-Itk-/- mice generated a protective Th2 immune response to S. Mansoni, while Itk-/- mice could not, and instead, generated an inappropriate Th1 response [72]. One possible explanation for this discrepancy is that the absence of Rlk in the Rlk-/-Itk-/- T cells leads to a selective defect in IFN-γ transcription, resulting in a bias towards Th2 differentiation. Consistent with this hypothesis, Rlk-/-Itk-/- mice are unable to generate a protective Th1 response to T. gondii infection [59]. However, another possibility to explain the disparity in differentiation observed between Rlk-/-Itk-/- and Itk-/- cells is abnormal regulation of T-bet and/or GATA-3 expression. In fact, CD4+ T cells from Rlk-/-Itk-/- mice showed impaired repression of GATA-3 mRNA 24 hours following anti-CD3 and anti-CD28 antibody cross-linking compared to wild type cells. These findings, together with our own data, suggest a previously unappreciated connection between the Tec family kinases Rlk and Itk, and the factors controlling T helper cell differentiation, GATA-3 and T-bet.
Figure 27: A model for Itk in the process of CD4⁺ T lymphocyte differentiation and effector function

The genes encoding the effector cytokines IFN-γ and IL-4 are normally in a repressed state in naïve CD4⁺ T cells. In order for these genes to be accessible for transcription, the master regulators of T cell differentiation, T-bet and GATA-3, are required for the remodeling of chromatin at the respective locus. Thus, it is currently believed that following the activation of T lymphocytes through the TCR, two crucial events must occur for differentiation to occur: the activation of specific loci, otherwise known as chromatin remodeling, and acute transcriptional effects that induce transcription only at remodeled gene targets. The data presented in this chapter support a model in which the strength of signal through the TCR affects T cell differentiation by the differential regulation of GATA-3 expression, and not T-bet, as well as the activation of specific transcription factors involved in the acute phase. This figure will present models which depict our current view on how Itk regulates not only factors involved in the acute transcription of cytokine genes, but also molecules which participate in the competency to express these genes following different modes of stimulation.

(A) STRONG TCR SIGNAL: Prior to activation, naïve CD4⁺ T lymphocytes express low levels of the master regulators of Th1 and Th2 differentiation, T-bet and GATA-3, respectively. Our data indicate that upon ligation of the TCR with a strong signal, T-bet and GATA-3 are both modestly upregulated in a fashion that is not dependent on Itk. The (*) denotes that other Tec family members may be activated upon a strong TCR signal, which may reduce the requirement for Itk function. Additionally, high avidity signals via Itk activate transcription factors,
such as NFATp, that promote IFN-γ transcription. Upon feedback through its receptor, IFN-γ induces more T-bet, which ultimately will drive Th1 differentiation, and suppress Th2 development.

(B) WEAK TCR SIGNAL: Upon recognition of a low affinity ligand or a weak TCR signal, T-bet and GATA-3 are induced via an Itk-independent mechanism. However, our data indicate, as observed by others, that GATA-3 mRNA levels are increased as the strength of the TCR signal is reduced. Increased GATA-3 leads to Th2 polarization via the maintenance of the IL-4 locus in an open state as well as in its own auto-activation. Furthermore, reduced TCR signaling activates factors, such NFATc, important in IL-4 transactivation. Once secreted, IL-4 feedbacks through its receptor to induce more GATA-3 expression, thus amplifying an autocrine loop. The data presented in this chapter demonstrate that T-bet levels are unaltered in low avidity conditions. Our data support a role for Itk in the active repression of T-bet upon recognition of a weak ligand, which would ultimately assist in driving T cells down the Th2 pathway, and suppress Th1 development.

(C) EFFECTOR FUNCTION: Once differentiation into either the Th1 or Th2 lineage has taken place, Itk functions in signaling pathways that lead to optimal cytokine production in both subsets. Signals through Itk activate PLC-γ1, which are crucial for the activation of downstream molecules such as NFAT and components of the AP-1 transcriptional complex. Our data indicate that the upregulation of Itk in Th2 cells is an important event for proper Th2 function.
Disclaimer:

The above models are one interpretation of the data presented in this chapter. However, we have not ruled out the possibility that Itk may play a role in signaling through costimulatory receptors, such as CD28 and CTLA-4. Both CD28 and CTLA-4 affect T cell differentiation in part by controlling the strength of signal through the TCR. For instance, CD28 signals favor Th2 differentiation, whereas ligation of CTLA-4 promotes Th1 differentiation. These costimulatory molecules can only mediate their effects upon binding to their ligand following TCR engagement [212, 213]. Precisely where CD28 and CTLA-4 signals intersect with TCR signals is not known. One alternative interpretation of the data discussed in this chapter is that CD28 signals via Itk function in a signaling pathway that is important for the proper inhibition of T-bet and upregulation of genes important for Th2 differentiation. Likewise, CTLA-4-mediated signals could be suppressed by Itk, which would also function to promote Th2 differentiation. Future work addressing these possibilities will be very informative.

Alternative Models of Lineage Commitment:

While this study is in agreement with most reports that examined the strength of the TCR signal and its' influence on T cell differentiation, conflicting data has led to the proposal of alternative models of lineage commitment. While the bulk of the data suggest that strong TCR signals induce Th1 differentiation and weak TCR signals induce Th2, Hosken and colleagues proposed a three-tiered mode of regulation, where both strong and weak signals would lead to Th2 differentiation, and intermediate signals would result in Th1 differentiation [162]. The discrepancies between these two models are likely a result
of different in vitro systems. For instance, one of the main variables in these experiments is the type of APC being used, which can greatly impact T cell differentiation as discussed in chapter III with dendritic cells. Thus, it would be interesting to examine the role of Itk in the process of T cell differentiation upon stimulation with different types of APCs.
Figure 27A: Strong TCR signal

Naive CD4+ T cell

Chromatin remodeling at cytokine gene loci

Th1 differentiation

Key:
- = a particular gene
- = transcription/translation event
= positive regulatory event
= negative regulatory event
Figure 27B  Weak TCR signal

Naive CD4⁺ T cell

Chromatin remodeling at cytokine gene loci

Transcription factor access to cytokine gene loci

Th2 differentiation

KEY
- = a particular gene
- - = transcription/translation event
== = positive regulatory event
--- = negative regulatory event
Figure 27C: The role of Itk in Th1 and Th2 effector cells

**Th1 cells**

- CD28
- TCR
- Itk
- NF-κB
- AP-1
- T-bet
- STAT4
- IFN-γ
- GATA-3
- IL-12R

**Th2 cells**

- CD28
- TCR
- Itk
- NF-κB
- AP-1
- NFAT
- GATA-3
- IL-4
- STAT6
- T-bet
- IL-4R

**KEY**

- ➔ = a particular gene
- ➔ = transcription/translation event
- ➔ = positive regulatory event
- ➔ = negative regulatory event

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CHAPTER V.

DISCUSSION AND FUTURE DIRECTIONS
A brief synopsis

The data presented in this thesis have defined an important role for the tyrosine kinase, Itk, in several CD4⁺ T cell effector functions, such as AICD and T cell differentiation, which are required for proper immune function. The knowledge of how both of these processes are regulated at the molecular and cellular levels is imperative in efforts to control disease processes.

Chapter II demonstrates a crucial role for Itk in the signaling pathway that leads to activation-induced cell death via FasL. In this chapter, CD4⁺ T cells from both wild type and Itk⁻/⁻ mice were initially stimulated with a high level of peptide, rendering wild type cells more susceptible to AICD. Following restimulation, wild type cells underwent AICD, whereas Itk-deficient cells failed to. Further characterization of this deficiency revealed that Itk⁻/⁻ cells were impaired in their ability to activate specific signaling pathways, which resulted in the failure to upregulate the transcription of the regulatory factors Egr2 and Egr3, and ultimately FasL. This study more clearly defined the function of Itk in the process of FasL-mediated cell death.

Chapter III attempted to clarify the role of Itk in other CD4⁺ T cell effector functions. Interestingly, in correlation with our findings in chapter II, we found that Itk-deficient mice possessed an increase in the percentage of CD4⁺ T cells with an activated or memory phenotype. In addition, we found there to be a vast increase in the numbers of CD4⁺ memory cells producing both Th1 or Th2 cytokines in Itk⁻/⁻ mice, indicating that Itk may regulate T cell differentiation. Unfortunately, further efforts to address the role of Itk in the process of naïve T cell differentiation in vitro was hindered by the presence
of previously-activated cells which had been skewed \textit{in vivo} to either the Th1 or Th2 lineage. Thus, we utilized a more highly-controlled \textit{in vitro} approach in chapter IV.

Chapter IV further elucidated the role of Itk in the process of CD4\(^{+}\) T cell differentiation. As opposed to chapters II and III, a slightly different \textit{in vitro} approach was utilized. The experiments presented in this chapter revealed that naïve Itk\(^{-/-}\) CD4\(^{+}\) T cells respond normally to cytokine skewing signals, and can differentiate efficiently into either Th1 or Th2 lineage cells. In the absence of skewing cytokines, wild type CD4\(^{+}\) T cells stimulated with low avidity ligands preferentially express GATA-3 mRNA and differentiate into Th2 cells. Under these same stimulation conditions, Itk\(^{-/-}\) T cells produce large amounts of T-bet mRNA, and differentiate into IFN-\(\gamma\)-producing cells. These findings not only provide a molecular explanation for the essential role of Itk in Th2 differentiation, but also provide insight into the mechanisms of how altering the strength of TCR signaling can generate qualitatively different signals leading to altered T cell fates in differentiation.

\textbf{A connection between CD4\(^{+}\) T cell death and differentiation in Itk\(^{-/-}\) mice}

While the aberrations in AICD and T cell differentiation observed \textit{in vitro} in Itk-deficient T cells appear to be distinct, as discussed in chapters II and IV, respectively, we have not ruled out the possibility that there may be a connection between the two defects \textit{in vivo}. This possibility is raised due to the disparity between the experiments presented in chapter IV, where I discovered that in situations in which wild type cells undergo Th2 differentiation, Itk-deficient cells failed to do so, and Chapter III, where I identified an increase in the percentage of CD4\(^{+}\) cells with a memory phenotype in Itk\(^{-/-}\) mice that can immediately produce the Th2 effector cytokine IL-4. This disparity was also observed by
other groups where Itk<sup>-/-</sup> mice are clearly unable to elicit Th2 responses to many different pathogens, yet unimmunized Itk<sup>-/-</sup> mice possess a 5-fold increase in basal levels of IgE, germinal center hyperplasia, and the presence of mild eosinophilia, all of which are suggestive of excess Th2-type cytokine production [72, 73, 151].

While at first glance it is difficult to reconcile, these findings suggest the possibility that mice deficient in signaling molecules of this particular biochemical pathway not only possess defects in T cell function, but also defects in the function of other cell types that express these signaling molecules, such as mast cells. Consequently, alterations in the function of mast cells may contribute to an environment in which T cell activation and differentiation are dramatically affected. In chapter III, which describes an increase in the percentage of CD4<sup>+</sup> cells with a memory phenotype in Itk<sup>-/-</sup> mice, it is not clear how or why these cells are being activated compared to wild type. Nevertheless, the data in chapter IV would argue that following activation, Itk<sup>-/-</sup> cells would preferentially differentiate into Th1 cells, unless exposed to IL-4 produced by another cell type. Furthermore, as discussed in chapter II, defects in AICD would likely result in the accumulation of CD4<sup>+</sup> cells with a memory/activated phenotype that have differentiated into either the Th1 or Th2 lineage. Below, I elaborate on the remarkable similarities of the phenotypes of mice deficient in these signaling proteins and the likelihood that these phenotypes may not necessarily be a result of CD4<sup>+</sup> T cell intrinsic defects, but may in fact be a combination of intrinsic and extrinsic CD4<sup>+</sup> T cell defects.
Similarities of Itk−/− mice to other signaling-deficient mice

Tec family kinases function in the phosphorylation and activation of PLC-γ1 via the formation of a multi-molecular signalosome complex that includes Grb2, Gads, SLP-76, LAT, and PLC-γ1. Recently, two independent groups, Aguado et al [214] and Sommers et al [215], described mice that expressed only a mutated form of LAT, LATY136F. Previous work had shown that Y136 of LAT is required for the binding and activation of PLC-γ1 in response to TCR stimulation [216-218]. Interestingly, the LATY136F mutant mice have a very similar biochemical and physiological phenotype when compared to Tec kinase-deficient mice. Similarly, mutation of another TCR signaling molecule, Vav1, which has also been shown to be important for the full activation of PLC-γ1 [219], results in mice with a similar T cell deficiency [220-222]. Based on their role in PLC-γ1 activation, it is not surprising that each of these molecules has also been shown to play a role in regulating calcium mobilization in T cells.

Downstream of PLC-γ1 activation, IP3 generation, and subsequent calcium mobilization, transcription factors of the NFAT family are rapidly dephosphorylated and activated by the calcium-dependent phosphatase, calcineurin. This dephosphorylation allows NFAT molecules to translocate to the nucleus where they bind to specific promoter sequences and activate transcription. The three NFAT family members expressed in T cells, NFATc1 (NFATc, NFAT2), NFATc2 (NFATp, NFAT1), and NFATc3 (NFAT4, NFATx) appear to have overlapping as well as unique functions, as has been elucidated by studies of mice with single as well as multiple mutations in NFAT family members [223]. Predictably, several groups including our own have found Itk−/− and Rlk−/−Itk−/− CD4+ T cells have a defect in NFAT activation and/or translocation.
following TCR stimulation, most likely as a result of the defects in calcium mobilization [72, 73, 94]. The resemblance of the phenotypes observed in Itk<sup>-/-</sup>, Rlk<sup>-/-Itk</sup><sup>-/-</sup>, as well as the LAT<sup>Y136F</sup> mice, with those observed in several NFAT knock-outs is quite intriguing, and further confirms the notion that disruption of PLC-γ1 activity results in defective NFAT activation, which in turn affects a number of T cell effector functions.

The most striking similarities seen among these signaling protein-deficient mice involves alterations in CD4<sup>+</sup> T cell homeostasis and the apparent in vivo skewing to a Th2-dominated environment in non-immunized mice. Two groups independently generated the LAT<sup>Y136F</sup> mice and both described the peripheral CD4<sup>+</sup> T cells isolated from these mice as having an activated phenotype (CD62L<sup>lo</sup>, CD44<sup>hi</sup>, CD69<sup>hi</sup>), showing defects in TCR-mediated cell death, and selective production of type-2 cytokines [214, 215]. Furthermore, in vitro, CD4<sup>+</sup> T cells from LAT<sup>Y136F</sup> mice are defective in calcium mobilization, IL-2 production, and FasL expression, analogous to the milder defects seen in Itk-deficient CD4<sup>+</sup> T cells. Unlike Itk<sup>-/-</sup> and Rlk<sup>-/-Itk</sup><sup>-/-</sup> cells, the ERK pathway remained intact in the LAT<sup>Y136F</sup> T cells for reasons that are not clear. Interestingly, both groups alluded to the fact that, as a result of impairments in thymic selection, cells reactive towards self-ligands may be escaping from the thymus. While both studies described similar phenotypes of the LAT<sup>Y136F</sup> mice, Aguado et al maintained their mice on the Balb/c background, whereas Sommers et al maintained their mice on the C57Bl/6 background. Thus, genetic differences between these two mouse strains that affect Th1 vs. Th2 differentiation in other circumstances appear unrelated to the phenotype observed in the LAT<sup>Y136F</sup> mice.
Mice deficient in NFAT family members also share some of the common phenotypes with the Tec family-deficient and LAT*Y136F mice, but the relationships are more complex. NFATc1−/− mice are unable to induce Th2 responses, as demonstrated by decreased IL-4 production by T cells from these mice [195, 224]. Remarkably, while CD4+ T cells from NFATc1-deficient mice possess a reduced proliferative capacity in vitro, they exhibit no defect in IL-2, IFN-γ, nor TNF-α production, when skewed in Th1 conditions in vitro. A second NFAT family member, NFATc2, which is expressed at high levels in resting T cells and then downregulated following activation [225], has been found to directly regulate the expression of NFATc1 by transactivating the NFATc1 promoter following TCR activation [226]. Interestingly, lymphocytes from mice possessing mutations in NFATc2 exhibit an activated phenotype, show moderate increases in IL-4 production, and have defects in activation-induced cell death in addition to severe eosinophilia in the lungs [227]. Further studies by Hodge et al [228] and Kiani et al [229], demonstrated that NFATc2 is required for the termination of the late-phase of IL-4 transcription, and ultimately for the down-regulation of the Th2 response. NFATc3, comparable to NFATc2, is expressed in resting T cells and is downregulated following activation [225]. NFATc3-deficient peripheral T cells, although they lack defects in cytokine production, have an activated phenotype and exhibit increased apoptosis as a result of elevated FasL expression [230]. These latter data are consistent with the notion that NFATc3 may function to repress FasL expression and thus, may play an important role in T cell survival.

Mice containing combined mutations in NFAT family members have proven to be invaluable in the dissection of NFAT-regulated pathways. NFATc2/NFATc3
doubly-deficient mice develop a lymphoproliferative disorder, as a result of increased resistance to apoptosis. These mice have allergic blepharitis, interstitial pneumonitis, and 1000-10000-fold increase in serum IgG1 and IgE levels, secondary to a dramatic and selective increase in Th2 cytokines [225]. In contrast, T cells from mice containing combined mutations in both NFATc1 and NFATc2 exhibit an activated phenotype, but show substantial defects in the development of multiple effector functions, such as cytokine production, surface effector molecule expression, and cytolytic activity [231].

All of the above findings suggested that, while NFATc1 may play a positive role in proliferative functions, NFATc2 and NFATc3 essentially function to repress specific T cell functions. These data imply that, depending on the strength or quality of the TCR signal, some NFAT family members may be selectively activated relative to others. Furthermore, it is evident that lymphoid homeostasis and Th2 development require a critical balance among NFAT family member activities.

Alterations in the balance of NFAT family member activation is likely to affect T helper cell differentiation and effector function. Based on the observations arising from analyses of NFAT-deficient T cells, it seems likely that upon stimulation of T lymphocytes lacking Itk, Rlk and Itk, or LAT, there would be an initial defect in NFATc2 activation, followed by a more substantial defect in NFATc2-induced NFATc1 upregulation. In addition, as a result of their impairment in NFAT activation, Itk−/− and Rlk−/−Itk−/− T cells may also show decreased repression mediated by NFATc2 and NFATc3, providing an explanation for many of the observed defects in T cell effector functions seen in these cells, as well as those expressing LATY136F. The more severely impaired Rlk−/−Itk−/− CD4+ T cells may be more akin to the NFATc1/c2 double knockout
cells, where there is a more profound defect in NFAT-mediated effector functions. The relationship between Itk, Rlk, and LAT, signaling molecules that function to amplify calcium signals, and NFATs, calcium sensitive transcription factors, as well as the relationship between NFATs and specific effector genes, may not be a simple one. To obtain a clearer picture of the aforementioned relationships, it will be necessary to determine how individual NFAT molecules are spatio-temporally regulated in T cells during T cell stimulation, expansion, and contraction.

Lastly, another T cell signaling protein, JNK (c-jun N-terminal kinase), has also been implicated in pathways downstream of Itk activation and other proximal TCR signaling events. We have shown that Itk−/− CD4+ T cells are defective in the activation of the JNK pathway, a defect which is likely to contribute to the impaired FasL expression in these cells [232]. CD4+ T cells from JNK1-deficient mice synthesize augmented levels of IL-4, but normal levels of IFN-γ, when stimulated in vitro in non-skewing conditions [233]. NFATc1 has been identified as a substrate for JNK1, and may function to inhibit Th2 responses [233]. Consistent with this, JNK1−/− mice are susceptible to Leishmania infection, which is not due to their inability to make a Th1 response, but rather is due to their inability to suppress the development of a Th2 response [234]. Exactly how disregulation of JNK1 signaling in Itk−/− mice contributes to the T helper imbalance seen in these mice is unclear.

Despite all of the intriguing similarities and differences that exist between the aforementioned signaling protein-deficient mice, all of the current models of TCR signaling, in regards to T cell differentiation, fail to reconcile many of the in vivo findings in these knock-out mice. Perhaps a better understanding of the complex interactions and
cross-talk between all these signaling molecules through gene expression array analyses will aid in our future understanding of how these signaling molecules and pathways integrate to affect T cell activation and differentiation.

While T helper cell differentiation can clearly be altered by modification of genes within the T cells themselves, it is clear that environmental factors, especially cytokines, elicited from other cell types can also influence this process. *In vivo*, in addition to T cells, mast cells are believed to be another major source of IL-4. Interestingly, Tec family kinases, the adaptors SLP-76 and LAT, and the NFAT family of transcription factors, are all involved in signaling within mast cells. Thus, it is possible that, along with intrinsic T cell changes in the signaling mutants discussed, disruption of signaling in mast cells may affect the T cell phenotype observed in these mutant mice. Mast cell defects may indirectly result in imbalances in the cytokines that are available to T cells during T cell activation, thereby altering the differentiation process. This argument will be discussed below.
Table II: Similar phenotypes of mice deficient in T cell signaling proteins

Chart compares biochemical defects as well as the *in vivo* phenotypes of various T cell signaling molecule knockouts. ND = Not Done, or unpublished. NA = Not Applicable. Biochemical findings were either done in peripheral T cells or thymocytes with the exception of those for SLP-76, which were done in a SLP-76 deficient Jurkat T cell line.
### Similar phenotypes of mice deficient in T cell signaling proteins

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<th>Biochemical Defects</th>
<th>Distal Events</th>
<th>In vivo phenotype</th>
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<td>Proximal Events</td>
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<td>Calcium flux</td>
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<td>NFAT activation</td>
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<td>Alterations in cytokine production</td>
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<th>Defects in AICD</th>
<th>Increase in serum IgE/IgG1 levels</th>
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<td>IgG1: 400X IgE: 5,000X</td>
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**Proximal Events**
- Defects in PLCγ activation
- Defects in calcium flux
- Defects in ERK activation
- Defects in NFAT activation

**Distal Events**
- IL-2
- Multiple cytokines defective
- IL-2, IL-4, & IFNγ
- NFAT activation

**In vivo phenotype**
- CD4⁺ cells have activated phenotype
- CD4⁺ cells produce Th2-type cytokines
- Defects in AICD
- Increase in serum IgE/IgG1 levels
- Eosinophilia
A role for Itk in the regulation of Mast Cell function?

A role for mast cells in the Th2-type skewing observed in the mutant mice described above is a likely possibility, as mast cells play an integral role in Th2 effector responses. Cross-linking of the high affinity IgE receptor, FceRI, activates mast cells by initiating signal transduction events that result in two general responses; an early degranulation response in which molecules such as TNF-α, IL-4, and histamine are immediately released, as well as a response where mediators such as other interleukins and chemokines are newly synthesized. Two Src family kinases, Fyn kinase and Lyn kinase, initiate signaling downstream of the FceRI. However, evidence from several labs has indicated that the Tec kinase, Btk, functions in a Lyn-dependent pathway, that involves the action of SLP-76, LAT, Vav1, as well as PLC-γ1, which ultimately leads to calcium mobilization [235, 236]. In addition to Btk, Itk is also expressed in mast cells and becomes phosphorylated following cross-linking of FceRI [237, 238]. There is substantial evidence indicating a role for Btk in mast cell function [239], but little is currently known about the role of Itk in these cells. Both PLC-γ1, which is activated by Itk in T cells, and PLC-γ2, which is activated by Btk in B cells, associate with this complex, suggesting a possible role for both of these Tec kinases in mast cell signaling. In mast cells, the activation of both of these PLC-γ isoforms is believed to be important for calcium responses and for the activation of the NFAT family of transcription factors, signals that are critical for optimal mast cell function and cytokine production (See [236] for review). While the role for Itk in mast cells remains elusive, leaving open the possibility that the disregulation of mast cells in the absence of Itk might affect T cell homeostasis and/or differentiation, it has been found by Nadler et al that Lyn-deficient
mast cells in fact possess enhanced degranulation responses [240]. Thus, it is possible that Itk may play a negative regulatory role in mast cells. Future work will address this possibility.

**Tec kinases and T cell anergy**

As discussed earlier, self-reactive lymphocytes are typically deleted during thymic development, however, T lymphocytes can be tolerized to self antigens in the periphery via a number of different mechanisms. The maintenance of tolerance in the peripheral immune system is vital for homeostasis. Needless to say, mechanisms of immune tolerance are an intense area of investigation due to its clinical importance in the treatment of autoimmunity and transplantation. One mechanism of tolerance involves a subset of CD4$^+$ T lymphocytes that exhibit regulatory properties, such as the ability to produce immunosuppressive cytokines such as TGF-β and IL-10 [241]. Another mechanism, termed anergy, involves the uncoupling of the TCR and the activation of specific downstream signaling pathways. The tolerizing stimulus to the T cell typically commences upon partial activation as a result of an imbalance in TCR signals without costimulatory signals. Ultimately, this process induces the T cell to enter a long-lasting state of unresponsiveness, making these cells refractory to TCR and costimulatory signals [242-244]. More specifically, anergic T cells fail to produce IL-2 upon stimulation with the cognate antigen, however, the anergic state can be overcome upon exposure to IL-2 [245]. To date, there have been no studies addressing the function of Tec family kinases in either mechanism of T cell tolerance. The function of Itk in T cell tolerance may be an exciting area to pursue in future studies for reasons that will be discussed below.
One of the characteristic features of a tolerizing stimulus that will ultimately induce anergy is the ability of this stimulus to activate the calcium pathway. Several reports have demonstrated that by treating T cells with the calcium ionophore, ionomycin, can induce anergy. This induction can be blocked upon treatment with the chelating reagent EGTA as well as with the calcineurin inhibitor, cyclosporin A (CsA) [242]. Stimulation of T cells with an altered peptide ligand (APL) possessing weak agonistic properties, can elicit a weak calcium signal. This type of T cell stimulation has also been implicated in T cell anergy induction [190, 243, 244]. As discussed in great detail earlier, calcium mobilization results in the calcineurin-dependent dephosphorylation of cytoplasmic NFAT. Following its nuclear translocation, NFAT cooperatively interacts with AP-1 (Fos/Jun dimer) to induce several cytokine and effector genes. Calcium mobilization is completely dependent on TCR signals alone and is not coupled to costimulatory molecules. Thus, NFAT activation is virtually independent of costimulatory receptors and dependent on the TCR alone. Costimulation, however, is required for the efficient activation of AP-1 and NF-κB activity. Interestingly, T cells lacking NFAT1 are resistant to the induction of anergy. Macian et al revealed that NFAT1 induces T cell anergy when restricted from interacting with AP-1, its transcriptional partner [246]. This information leads me to speculate that Itk may play an important role in the induction of anergy. Itk plays an important role in the efficient mobilization of intracellular calcium. Given that sufficient calcium signals are required for the induction of anergy, one could surmise that Itk-deficient cells may in fact be more resistant to tolerization.
T regulatory cells (T_{Reg}), a subset of CD4^+ cells important for homeostasis, are characterized by the cell surface expression of CD25 as well as CTLA-4 [247]. Chen et al have demonstrated that upon cross-linking of CTLA-4 is able to induce the secretion of TGF-β, an immunoregulatory cytokine. The role of Itk in the development and function of T_{Reg} cells is presently unknown. Furthermore, whether Itk functions in signals downstream from costimulatory molecules, such as CTLA-4, as well as in the signaling pathway that leads to TGF-β production remains to be learned.

**Tec family members may directly affect gene expression**

Recent progress has provided additional insights into Tec kinase functions within immune cells. As mentioned earlier, Rlk can translocate to the nucleus upon TCR stimulation. A recent paper from Takeba et al demonstrated that Rlk can bind to regions of the IFN-γ promoter and increase promoter activity. Interestingly, this event is dependent on Rlk phosphorylation, presumably in response to TCR engagement [39, 40]. Other Tec family members, such as Btk and Bmx, have been previously shown to interact with transcription factors (reviewed in [44]), suggesting that these proteins may play more direct roles in affecting gene transcription. Btk, which is mainly localized to the cytoplasm in resting cells, translocates to the cell membrane following BCR or growth factor stimulation. In addition, a recent study has found that Btk can undergo nucleocytoplasmic shuttling in a nuclear export signal (NES)-dependent manner [248]. The role of Btk in the nucleus was clarified by the findings of Egloff et al., who showed that Btk could associate with and phosphorylate a transcriptional regulator, BAP/TFII-I, in B cells following BCR engagement [249]. Likewise, Btk was also found to associate
with another transcription factor, STAT5A, following the engagement of the BCR [250]. These finding support a previously unrecognized role for Tec family kinases as direct modulators of gene expression.

This newly proposed function for Tec kinases in gene regulation appears to be family-wide. Recent work from Perez-Villar et al demonstrated that Itk can associate with a nuclear importin protein, termed Rch1α/karyopherinα, thereby allowing it to translocate to the nucleus [41]. Furthermore, Laurie Glimcher and colleagues have observed a direct interaction of Itk with the Th1-specific transcription factor, T-bet. Preliminary data from her laboratory suggests that Itk may in fact regulate T-bet function by a phosphorylation event (personal communication). Needless to say, much work is required to understand how Tec family kinases can not only function in cytoplasmic events, such as the activation of PLC-γ and downstream, signaling pathways, but also in nuclear events, such as the activation or regulation of transcription factors.

Final word

T cell signaling research today seeks to clarify the intersecting and diverging branches of antigen receptor signaling pathways in order to understand how distinct immune outcomes are regulated at the molecular and cellular level. Ultimately, this will allow researchers to develop new methods that allow the elicitation of the desired immune response to control disease. Determination of the precise and possibly unique role of each Tec family kinase in T cells will require further investigations using a combination of genetics and biochemistry. While one caveat of these genetic studies is the possibility that when one member of the Tec family is absent, compensatory
mechanisms may emerge to counteract the imbalance, we favor the notion that under normal physiological circumstances, each Tec kinase family member has a specific and non-redundant role in hematopoietic cell signaling. Supporting this idea, individual Tec kinases show distinct requirements for activation, such as the dependence on PI3K, in addition to the common need for phosphorylation by a Src kinase. Furthermore, activation of Tec kinases in T cells leads to unique downstream consequences, as illustrated by the observation that Rlk, but not Itk, can translocate to the nucleus and act as a transcription factor. Future work with Tec family kinases should begin to reveal more precisely the roles of Itk, Rlk and Tec in T cell signaling.

Collectively, all of the data presented and discussed in this thesis underscore the important role of TCR signals in maintaining the appropriate balance of T cell survival, activation, and differentiation, and further emphasize the importance of Tec kinases in regulating these processes that are crucial for proper immune function.
CHAPTER VI.

MATERIALS AND METHODS
Mice

In chapters II and III, 5C.C7 TCR transgenic mice [107] on the B10.BR (H-2k) background were crossed to Itk−/− mice [60]. Itk+/− and Itk−/− mice, which had been backcrossed to the C57BL/10 background for at least 8 generations were used for SEB injection experiments. In chapter three, Itk+/− and Itk−/− mice, which had been backcrossed to the C57BL/10 background for at least 10 generations were used. Itk−/− mice on the Balb/c background used in chapter three were backcrossed at least 8 generations (a gift from D. Fowell [74]). All mice used were between 6 and 10 weeks of age and maintained in a specific-pathogen free facility. In chapter IV, Itk-deficient mice previously generated in our laboratory [60] were backcrossed to the C57BL/10 background >10 generations. These mice were crossed to 5C.C7 TCR transgenic RAG+/− mice (Jackson Laboratories, Bar Harbor, ME) to ultimately generate H-2k 5C.C7 Itk+/− RAG+/− and 5C.C7 Itk−/− RAG−/− mice. All mice used for these experiments were between 6 to 10 weeks old and were maintained in a specific-pathogen-free facility.

Purification of CD4+ T cells

For chapters II and III, spleens and lymph nodes were removed from 6- to 10-week old 5C.C7 Itk+/− and 5C.C7 Itk−/− littermates. Following RBC lysis, single-cell suspensions were incubated with anti-CD4-coated magnetic microbeads and passed through LS+ columns according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). In chapter IV, spleens and lymph nodes were removed from 5C.C7 Itk+/− RAG+/− and 5C.C7 Itk−/− RAG−/− littermates. Following RBC lysis, spleen and lymph node cells were pooled and single-cell suspensions were incubated with anti-CD4 magnetic microbeads. The
cells were then run through a Miltenyi Biotech AutoMACs separation system to positively select CD4$^+$ cells according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). Purified CD4$^+$ T cells were pooled and resuspended in RPMI 1640 (Gibco, Bethesda, MA) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 2mM L-glutamine, 100 U penicillin, 100μg/mL streptomycin, 10mM HEPES, and 50μM β-ME. This purification routinely yielded 90-97% CD4$^+$ Vα11$^+$ T cells. On the Rag-deficient background, all purified CD4$^+$ cells were consistently >96% naïve (CD44lo, CD62Lhi), and expressed the 5C.C7 TCR, which is specific for moth cytochrome C (MCC93-103).

**In vitro activation of CD4$^+$ T cells**

For in vitro activations in chapters II and III, 1 X 10^6 CD4$^+$ T cells were cultured in 24-well plates with 2μM of MCC93-103 peptide (DLIAYLQATK)(Tufts Microchemistry Facility, Medford, MA) plus 1 X 10^6 Mitomycin C-treated (Calbiochem, La Jolla, CA) IEd B7.1-expressing CHO cells [251]. In chapter II, cultures were all performed under Th1-skewing conditions (anti-IL-4 (1μg/mL) and rIL-12 (1ng/mL) (R&D, Minneapolis, MN)). After 24 hours, the cells were transferred into and expanded in fresh medium containing 5 ng/mL of IL-2 (Pharmingen, San Diego, CA). Addition of IL-2 immediately upon primary stimulation had no effect on the secondary responses of Itk$^{+/−}$ or Itk$^{-/-}$ cells. Following stimulation, cells were maintained in medium supplemented with IL-2 until restimulation. Itk$^{+/−}$ and Itk$^{-/-}$ T cells expanded comparably with these stimulation conditions, and similar numbers of cells were recovered from both types of cultures prior to secondary stimulation.
In chapter IV, 1x10^6 purified 5C.C7 Itk^{+/} RAG^{+/-} and 5C.C7 Itk^{+-} RAG^{+/-} cells were activated with 1x10^6 Mitomycin-C treated CH27 cells (B cell lymphoma line, IE^{k+} and B7^+) in a variety of different conditions. For Th1-skewing conditions, cultures were supplemented with rIL-12 (1ng/ml) and anti-IL-4 antibody (1μg/ml), while Th2-skewing conditions included rIL-4 (10ng/ml) and anti-IFN-γ antibody (0.1μg/ml; R&D, Minneapolis, MN). Cells stimulated in skewing conditions were activated with 10nM of MCC_{93-103} peptide plus CH27 cells. Cells stimulated in a variety of non-skewing conditions (no exogenous cytokines or antibodies added) were stimulated with the indicated amounts of MCC_{93-103}, MCC_{1028}, or MCC_{A968} peptide plus CH27 cells as APCs. After 24 hours, culture media for both skewed and non-skewed conditions was supplemented with rIL-2 (5 ng/ml). Cells were expanded and maintained in their respective culture conditions until restimulation.

**Antibodies and Flow Cytometry**

Cells were stained with the indicated antibodies in HBSS supplemented with 3% FCS for 30 minutes on ice. Cells were then washed and analyzed on a Becton Dickinson FACSCalibur. Data was analyzed using CellQuest software. The antibodies and flow cytometry reagents used were anti-CD4-CyChrome, anti-Vα11-FITC, anti-Vβ8.1/8.2-FITC, anti-Vβ6-FITC, anti-Fas-biotin, anti-CD69-PE, anti-CD44-FITC, anti-CD62L-PE, anti-CD25-PE, anti-CD8-APC, anti-CD11c-FITC, anti-CD11b-PE, anti-CD8-Cychrome, anti-CD40-PE, anti-B7.1-PE, anti-B7.2-PE, anti-CD62L-PE, anti-IL-2-PE, anti-IL-4-PE, and anti-IFNγ-APC, streptavidin-PE (Pharmingen), and anti-FasL-PE (eBioscience, San Diego, CA).
In vitro Proliferation Assay

For primary proliferation assays, 5 X 10^4 CD4^+ Vα11^+ T cells were stimulated with MCC_93-103 peptide plus 5 X 10^4 Mitomycin C-treated CHO cells expressing IE^k and B7.1 in a volume of 200μL for 48 hours. As a control, cells were stimulated with PMA (Sigma; 2.5ng/mL) and Ionomycin (Calbiochem; 375ng/mL): 3[H]thymidine (NEN, Boston, MA) was added at 1μCi/well and incubated for an additional 20 hours, and plates were harvested on a Tomtec Harvester 96 and 3[H]thymidine incorporation was quantified on a Perkin Elmer Trilux microbeta counter. Secondary proliferation assays were performed on day 14 post-initial stimulation, the point at which the cells were no longer dividing. Previously-activated cells were removed from culture, washed, counted, and as in the primary proliferation assay, 5 X 10^4 CD4^+ Vα11^+ T cells were stimulated. For the blocking of FasL-mediated AICD in vitro, cells were stimulated in the same conditions as above with the addition of anti-FasL (MFL3) or an isotype control Ig (eBioscience) added at 5μg/mL to each well at 0 and 20 hours.

Functional Assay for FasL-induced apoptosis

The induction of FasL-mediated cell death was determined by Annexin V-FITC (Pharmingen) and propidium iodide (Sigma) staining 15 to 20 hours after 3 X 10^5 T cells were stimulated with 5 X10^4 CHO (IE^k- and B7.1-positive) cells and MCC_93-103 peptide. 5μg/mL of anti-FasL or an isotype control Ig was added at 0 and again at 10 hours post-stimulation where indicated. Soluble FasL (sFasL) was pre-bound for 30 minutes, followed by addition of a cross-linking enhancer Ig (Alexis Corp., San Diego, CA) at 2
hours post-stimulation. Cells were immediately analyzed by flow cytometry. Specific apoptosis was determined by calculating the ratio of live cells in the treated wells to live cells in the wells incubated in the absence of MCC peptide.

**Intracellular Cytokine Staining**

In chapter II, 3 X 10⁵ T cells were cultured with 5 X 10⁴ (IEk and B7.1-expressing) CHO cells and MCC₉₃-₁₀₃ peptide, or PMA (2.5ng/mL) and Ionomycin (375ng/mL) for 6 hours in a 96-well plate. In chapter IV, 3x10⁵ T cells were restimulated with 1x10⁵ CH27 cells and the indicated concentrations of MCC₉₃-₁₀₃ for 6 hours in a 96-well plate. Golgi Stop™ and/or Golgi Plug™ (Pharmingen) were added for the last three hours. All cells were stained for approximately 15 minutes, fixed for 20 minutes, then permeabilized and stained intracellularly with either anti-IL-2-PE, anti-IL-4-PE, and/or anti-IFN-γ-APC according to the Cytofix/Cytoperm™ kit protocol (Pharmingen). Cells were immediately analyzed by flow cytometry on a BD FACSCalibur. Ten thousand CD4⁺ events were typically collected.

In chapter III, for the *ex vivo* stimulation of splenic CD4⁺ cells, following the purification of CD4⁺ cells from both Itk⁺/⁻ and Itk⁻/⁻ mice, 1 X 10⁶ cells were stimulated with PMA (2.5ng/mL) and Ionomycin (375ng/mL) for 6 hours. To assess the differentiation status of T cells initially stimulated in non-skewing conditions, 3 X 10⁵ T cells were restimulated with 5 X 10⁴ (IE⁻ and B7.1-expressing) CHO cells and MCC₉₃-₁₀₃ peptide, or PMA and Ionomycin for 6 hours in a 96-well plate. Golgi Stop™ and/or Golgi Plug™ (Pharmingen) were added for the last two hours. For *ex vivo* stimulation, cells were
stained with anti-CD44-FITC and anti-CD4-Cy. For in vitro restimulation, cells were stained with anti-Vα11-FITC and anti-CD4-Cy. Similar procedures were followed as indicated above.

**Calcium Flux**

Day 8 post-initial stimulation with peptide and APCs, 5 x 10⁶ 5C.C7 Itk⁺/⁻ and 5C.C7 Itk⁻/⁻ CD4⁺ cells were incubated with 3μg/mL Fluo-3 and 5μg/mL of Fura-Red (Molecular Probes, Eugene, OR) in RPMI containing 3%FCS for 45 minutes. Cells were washed twice and incubated in the dark at room temperature for 30 minutes. 1 X 10⁶ cells were placed in 1mL of 37°C serum-free RPMI and analyzed on a Becton Dickinson Flow Cytometer. Baseline calcium was measured, cells were then stimulated with anti-CD3ε-biotin (145-2C11; 25μg) (Pharmingen) for 45 seconds, followed by streptavidin (Gibco; 40μg) cross-linking for 5 minutes. Ionomycin (1μg) was added at 6 minutes. Data were analyzed by calculating the mean fluorescence ratio of Fluo-3 (FL1) and Fura-Red (FL3) using FACSAssistant® software.

**Western Blot analysis**

In chapter II, on day 14 post-stimulation with 2μM of MCC93-103, 3 X 10⁶ 5C.C7 Itk⁺/⁻ and 5C.C7 Itk⁻/⁻ CD4⁺ T cells were incubated on ice in 120μL of RPMI-0 containing 25μg/mL of biotinylated α-CD3ε for 10 minutes. Cells were quickly spun and resuspended in 120μL of RPMI-0 containing 50μg/mL of streptavidin and incubated in a 37°C water bath for 0, 2, 5, or 10 minutes. As a positive control, cells were stimulated with PMA (2.5ng/mL) and Ionomycin (375ng/mL) for 15 minutes at 37°C. Ice-cold 1X
PBS containing 20mM NaF and 1mM Na₃VO₄ was added to stop the reactions. Cells were quickly spun and lysed for 15 minutes on ice in lysis buffer containing 25mM Hepes (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 1mM PMSF, 1mM Na₃VO₄, and 10μg/mL leupeptin. Total lysates were cleared and resolved by 10% SDS-PAGE, transferred to an Immobilon-P membrane (Millipore, Bedford, MA), blocked, and blotted for Phospho-p44/42 MAP Kinase or Phospho-SAPK/JNK (Cell Signaling, Beverly, MA). Following incubation with an HRP-conjugated secondary antibody, membranes were developed by ECL, stripped, and re-probed for total p44/42 MAP Kinase or SAPK/JNK protein (Cell Signaling).

In chapter IV, for the analysis of Tec family protein levels, 3.5x10⁶ cells were removed from culture at indicated time points and lysates prepared as described below. The AE7 Th1 clone (gift from Vijay Kuchroo) [252] and lysates from Rlk⁻/⁻Itk⁻/⁻ were used as positive and negative controls, respectively. For the analysis of signaling events following TCR cross-linking, 5C.C7 Itk⁺/⁺ RAG⁻/⁻ and 5C.C7 Itk⁻/⁻ RAG⁺/⁺ Th1 and Th2 cells were restimulated on Day 11 following primary stimulation as follows. Cells were incubated on ice in 120μL of RPMI-0 containing 25μg/mL of biotinylated α-CD3ε for 10 minutes, quickly spun, and resuspended in 120μl of RPMI-0 containing 50μg/mL of streptavidin and incubated in a 37°C water bath for 0, 1, or 2 minutes. Ice-cold 1X PBS containing 20mM NaF and 1mM Na₃VO₄ was added to stop the reactions. Cells were quickly spun and lysed on ice in buffer containing 25mM Hepes (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 1mM PMSF, 1mM Na₃VO₄, 10μg/mL aprotinin, and 10μg/mL leupeptin for 15 minutes. Cell lysates were cleared and run on a
10% SDS-PAGE, transferred to an Immobilon-P membrane (Millipore, Bedford, MA), blocked, and blotted with the indicated antibody in 1% BSA. For Tec family protein levels, membranes were blotted with anti-Itk (10B2 and 2F12 mAb mix)[253], or anti-Rlk (Santa Cruz). For the examination of signaling events in wild type and Itk<sup>−/−</sup> Th1 and Th2 cells, membranes were blotted for Phospho-783 PLC-γ1 (Biosource International), or Phospho-p44/42 MAP Kinase (Cell Signaling, Beverly, MA). Following incubation with an HRP-conjugated secondary antibody, membranes were developed by ECL, stripped, and re-probed for total PLC-γ1 protein (Upstate Biotech, Inc.), p44/42 MAP Kinase (Cell Signaling), or anti-Itk (10B2 and 2F12 mAb mix)[253].

**Real-time Quantitative PCR**

In chapter II, purification of naïve CD4<sup>+</sup> T cells was performed by staining MACS-purified CD4<sup>+</sup> cells with anti-CD4-Cy and anti-CD44-FITC, and sorting for CD4<sup>+</sup> CD44<sup>lo</sup> cells on a Becton Dickinson FACS Star. 2 X 10<sup>6</sup> sorted cells were incubated in media containing 5μg/mL of biotinylated anti-CD3ε for 20 minutes on ice, followed by resuspension in media containing 2.5μg/mL of streptavidin and 5ng/mL of IL-2 for 0, 6, 18, or 48 hours in a 24-well plate. For secondary stimulations, 2 X 10<sup>6</sup> previously activated T cells were restimulated as above for 0, 1, 2, 4 or 6 hours.

In chapter IV, for the analysis of cytokine gene expression during secondary stimulation, 3x10<sup>6</sup> T cells were restimulated with 5x10<sup>5</sup> CH27 cells and the indicated concentrations of MCC<sub>93-103</sub> in a 48-well plate for 6 hours. For the analysis of gene expression levels of IFN-γ, IL-4, T-bet, and GATA-3 after the primary stimulation, 4x10<sup>5</sup> purified naïve
CD4$^+$ T cells were stimulated with 3x10$^5$ Mitomycin C-treated CH27 cells and the indicated concentration of peptide for either 0, 12, or 24 hours in a 96-well plate. For the analysis of Tec family members during T cell differentiation, CD4$^+$ T cells were skewed as above, and cells were removed from culture at the indicated time points.

Following each time point, cells were removed from the plate and total RNA was isolated using the Qiagen RNeasy kit (Valencia, CA) according to the manufacturer’s protocol. Following DNase treatment (Promega, Madison, WI), RNA was reverse transcribed into cDNA using Superscript II and Random Hexamers (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Real-time quantitative PCR amplification was performed on a Bio-Rad iCycler$^\text{TM}$ using SYBR$^\text{®}$ Green PCR Core Reagents (PE Applied Biosystems, Foster City, CA). To quantify the amount of cDNA for an individual transcript, SYBR$^\text{®}$ Green fluorescence, was measured at the end of each cycle. The cycle threshold ($C_t$), the cycle at which exponential growth of the PCR product is first detected, was determined for known concentrations of plasmid DNA, and a standard curve was created. Template copy numbers were calculated for each sample by interpolating the $C_t$ values on the standard curve using the iCycler$^\text{TM}$ software. All samples and standards were run in triplicate for any given experiment. In chapter II, the values of FasL, Egr3, and Egr2 were normalized to $\beta$-actin by dividing the average copy number of the respective transcript by the average copy number of $\beta$-actin in the respective sample. From 1$\mu$g of RNA, we consistently found there to be approximately 5 X10$^7$ copies of $\beta$-actin in naive cells and 1.3 X 10$^8$ copies in restimulated cells.
For details of the PCR reactions presented in chapter II refer to [71]. For PCR reactions presented in chapter IV, template DNA was initially denatured at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 25s, 25s of primer annealing at 62°C, 58°C, 60°C, 59°C, 53°C, 60°C, 61.7°C, 54°C, 58°C, or 63°C for β-actin, GAPDH, GATA-3, T-bet, c-MAF, Itk, Rlk, Tec, IFN-γ, and IL-4 respectively, and lastly a 25s extensions step at 72°C. Primers sequences were as follows, β-actin sense 5'-CGAGGCCAGAGCAAGAGAG-3', antisense 5'-CGGTTGGCCTTAGGGTTTCAG-3', GAPDH sense 5'-ATGTCTCGTGAGTGATCTGA-3', antisense 5'-CCTGCTTCACCCACCTTGAT-3', IFN-γ sense 5'-CCTGCAGAGCCAGATTATCTC-3', antisense 5'-CCTTTTTTCGCTTGCTGTGC-3', IL-4 sense 5'-CGAAGAACCACCACACAGAGTGAGCT-3', antisense 5'-GACTCATTCATGGTGAGCTTATCG-3', GATA-3 sense 5'-GAAGGCAAGGAGTGTGTGAA-3', antisense 5'-TGTCCTGCTCCTCCTTGCTGTG-3', T-bet sense 5'-GGGCTGCGAAGACATGGCA-3', antisense 5'-GGCTCGGGATAAGAAACG-3', c-MAF sense 5'-AGCAGTTGGTGACCATTGTGG-3', antisense 5'-TTGGAGATCTCTCCTGCTTGAG-3', Itk sense 5'-CTCCGCTATCCAGTTTGTGCTC-3', antisense 5'-GTCCTTTGAGCCAGTAGCC-3', Rlk sense 5'-TCAATCAACCAGAGCGGG-3', antisense 5'-CCGCTCTTCCTAGTGCCAA-3', Tec sense 5'-GTGTGGATGTAGATGGGCG-3', antisense 5'-GGTAACAGATGTAGATGGGCG-3'. Specific products were verified by melt-curve analysis and gel electrophoresis. For the generation of standard curves, plasmids containing cDNA clones of Egr3 (gift from Jeffrey Milbrandt, Washington University School of Medicine, St. Louis, MO), FasL (gift from Ann Marshak-Rothstein, Boston University Medical Campus, Boston, MA), and β-actin (gift from Rachel Gerstein, University of Massachusetts Medical School, Worcester,
MA) were utilized. A 190bp fragment of Egr2 (135-324) was cloned into pGEM®-T Easy (Promega) and used for the generation of a standard curve, GATA-3 (gift from Ken Murphy, Washington University School of Medicine, St Louis, MO), T-bet (gift from Nezih Cereb of Histogenetics, Inc. and Center for Genetic Polymorphisms, Hawthorne, NY), c-MAF (gift from Laurie Glimcher, Harvard School of Public Health, Rlk (gift from Pam Schwartzberg, National Institutes of Health, Bethesda, MD), IFN-γ (gift from Anjana Rao, Harvard Medical School), and IL-4 (gift from Mark Bix, University of Washington, Seattle, WA) were used. Full-length cDNA clones of Itk and Tec were generated in our laboratory [254, 255].

SEB-induced deletion in vivo

Itk+/− and Itk−/− littermates were injected intravenously with 75µg of SEB (Toxin Technology, Sarasota, FL) on Day 0. Mice were tail bled on days −1, 3, 7, 11, and 15 into Alsever's solution. Following red blood cell lysis, cells were stained with anti-Vβ8.1/8.2-FITC or anti-Vβ6-FITC, and anti-CD4-PE. Ten thousand live CD4+ events were collected on a flow cytometer and the percentage of CD4+ cells expressing Vβ8 or Vβ6 was determined.

Isolation of Splenic Dendritic Cells

Spleens from either wild type or Itk−/− mice were removed, teased apart with forceps, and collagenase digested in RPMI-2% FCS with DNase at 37°C for 40 minutes. 10mM EDTA was added for the last 5 minutes. Cells were pipetted vigorously, and then filtered over a wire mesh and a nylon mesh into a fresh tube. Following two washes in
HBSS/EDTA/FCS, cells were resuspended in cold 14.5% Nycodenz (Sigma), and spun for 15 minutes at 1500g at 4°C. The low density fraction was obtained from the interface, which contains approximately 30% CD11c⁺ cells.

**Cytokine ELISA**

5x10⁴ T cells that were initially stimulated in Th1- and Th2-skewing conditions were restimulated with 5x10⁴ CH27 cells and the indicated concentrations of MCC₉₃-₁₀₃ for 24 hours in a 96-well plate. Supernatants were serially diluted and assayed for IFN-γ, IL-4, IL-5, and IL-10 using cytokine detection kits from Becton Dickinson.
CHAPTER VII.

REFERENCES CITED


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APPENDIX I.

PUBLICATIONS
Defective Fas Ligand Expression and Activation-Induced Cell Death in the Absence of IL-2-Inducible T Cell Kinase

Andrew T. Miller and Leslie J. Berg

The Tec family tyrosine kinase, IL-2-inducible T cell kinase (Itk), plays an important role in TCR signaling. Studies of T cells from Itk-deficient mice have demonstrated that Itk is critical for the activation of phospholipase C-γ1, leading to calcium mobilization in response to TCR stimulation. This biochemical defect results in reduced IL-2 production by Itk-deficient T cells. To further characterize the downstream effects of the Itk deficiency, we crossed Itk−/− mice to a TCR-transgenic line and examined T cell responses to stimulation by peptide plus APC. These studies show that Itk is required for maximal activation of early growth responses 2 and 3 and Fas ligand transcription after TCR stimulation. These transcriptional defects lead to reduced activation-induced cell death of stimulated Itk−/− T cells, both in vitro and in vivo. Together these studies define an important role for Itk in TCR signaling, leading to cytokine gene expression and activation-induced cell death. The Journal of Immunology, 2002, 168: 2163–2172.

The signaling pathways that lead to the activation of the FasL gene in T cells have been a major focus of investigation in recent years. However, this pathway is still not well understood. Several lines of evidence have suggested that specific TCR signaling pathways induce the expression of FasL. For instance, Lck and ZAP-70, members of the src and syk family of nonreceptor protein tyrosine kinases, respectively, have been shown to be critical for TCR-mediated FasL expression; in contrast, FasL expression is not dependent on Fyn, another proximal tyrosine kinase (6, 7). Furthermore, both calcineurin and members of the extracellular signal-related kinase (ERK) MAP kinase (MAPK) and c-Jun N-terminal kinase (JNK) families have been recognized as important members of the signaling pathway leading to the induction of FasL expression (8–12). In this study, we focused on the involvement of a Tec family kinase, IL-2-inducible T cell kinase (Itk), in the TCR-mediated up-regulation of FasL and the subsequent induction of AICD.

The importance of Tec family kinases in the immune system is exemplified by Bruton’s tyrosine kinase (Btk), a relative of Itk that is expressed in B cells and mast cells. Mutations in Btk have been linked to X-linked agammaglobulinemia in humans and X-linked immunodeficiency (xid) in mice (13–15). Biochemical studies have indicated a similar role for Itk and Btk in Ag receptor signal transduction in T cells and B cells, respectively (16). Specifically, mice deficient in Itk exhibit defects in T cell development and function. This is manifested as reduced numbers of peripheral CD4+ cells, indicating a defect in thymic positive selection, as well as reduced cytokine production by peripheral itk−/− T cells (17, 18). Biochemical studies have shown that Itk-deficient CD4+ T lymphocytes are defective in proximal TCR-initiated signaling events, such as the activation of phospholipase Cγ1 and calcium mobilization (17). Furthermore, Fowell et al. (19) demonstrated that Itk-deficient CD4+ T cells possess defects in the nuclear translocation of NF-ATc following TCR ligation, which consequently results in the inability to produce IL-4 and to elicit Th2-type responses in vivo. Because NF-AT proteins are essential transcription factors for many effector genes such as IL-2, FasL, and CD40 ligand (CD154), in addition to IL-4, these findings suggested that in the absence of Itk, other T cell effector functions that are dependent on signals downstream of PLCγ1/Ca2+/NF-AT may be affected.
To further characterize the role of Itk in CD4+ T cell effector function, we crossed itk-/- mice to 5C.C7 TCR-transgenic mice (20), expressing a TCR specific for a moth cytochrome c peptide (MCC3-10) bound to the MHC class II molecule, IE6 (21). These mice have provided a system for examining T cell signaling events and effector function in response to the natural receptor-ligand interaction. In this report, we show that in addition to calcium defects, itk-/-/CD4+ T cells are defective in the activation of the ERK/MAPK and JNK pathways, the expression of early growth response (Egr) 3 and Eg2r, and consequently FasL expression. Moreover, we demonstrate the physiological consequence of these defects in vivo, where T cells in itk-/- mice are unable to undergo efficient AICD in response to a superantigen, staphylococcal enterotoxin B (SEB).

**Materials and Methods**

**Mice**

5C.C7 TCR transgenic mice (22) on the B10.BR (H-2^d) background were crossed to itk-/- (17) and itk-/- mice, which had been backcrossed to the C57BL/6 background for at least eight generations, were used for SEB injection experiments. All mice were used between 6 and 12 wk of age and maintained in a specific-pathogen-free facility.

**Preparation and activation of CD4+ T cells**

Spleens and lymph nodes were removed from 6- to 12-wk-old 5C.C7 itk-/- and 5C.C7 itk/- littermates. After RBC lysis, single-cell suspensions were incubated with anti-CD4-coated magnetic microbeads and passed through LS^+ columns according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). Purified CD4+ T cells were pooled and resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (HyClone, Logan, UT), 2 mM L-glutamine, 100 U penicillin, 100 mg/ml streptomycin, 10 mM HEPES, and 50 mg/ml B-ME. This purification routinely yielded 90-97% CD4+ Vα11+ T cells (the 5C.C7 TCR is Vα11). For in vitro activations, 1 x 10^6 CD4+ T-cells were cultured in 24-well plates with 2 x 10^5 MCC3-10 peptide (DL1AY LKQATK; Tufts Microchemistry Facility, Medford, MA) plus 1 x 10^6 mitomycin C-treated CD4+ T-cells (Calbiochem, La Jolla, CA) IE6.7.1-expressing Chinese hamster ovary (CHO) cells (23). Cultures were all performed under Thl-skewing conditions (anti-IL-4 (1 mg/ml) and IL-12 (1 mg/ml), R&D, Minneapolis, MN). After 24 h, the cells were transferred into and expanded in fresh medium containing 5 ng/ml IL-2 (BD Pharmingen, San Diego, CA). Addition of IL-2 immediately on primary stimulation had no effect on the secondary responses of itk-/- or itk-/- cells. After stimulation, cells were maintained in medium supplemented with IL-2 until restimulation. In this report, T cells expanded comparably with these stimulation conditions, and similar numbers of cells were recovered from both types of cultures before secondary stimulation.

**Abs and flow cytometry**

Cells were stained with the indicated Abs in HBSS supplemented with 3% FCS for 30 min on ice. Cells were then washed and analyzed on a BD Biosciences (San Jose, CA) FACS Calibur. Data were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA). The Abs and flow cytometry reagents used were anti-CD4-PE-Chromeo (Cy), anti-Vα11-FITC, anti-Vβ8.1/2-FITC, anti-Vβ6-FITC, anti-Fas-biotin, anti-CD69-PE, anti-CD44-FITC, anti-CD25-PE, streptavidin-PE (BD Pharmingen), and anti-Fas-PE (eBioscience, San Diego, CA).

**In vitro proliferation assay**

For primary proliferation assays, 5 x 10^4 CD4+ Vα11+ T cells were stimulated with MCC3-10 peptide plus 5 x 10^5 mitomycin C-treated CHO cells expressing IE6 and B7.1 in a volume of 200 ml for 48 h. As a control, cells were stimulated with PMA (Sigma; 2.5 ng/ml) and ionomycin (Calbiochem; 2.5 ng/ml). [3H]Thymidine (NEN, Boston, MA) was added at 1 μCi/well and incubated for an additional 20 h. Plates were harvested on a Tomtec Harvester 96 (Orange, CT), and [3H]Thymidine incorporation was quantified on a Trilux microbeta counter (PerkinElmer, Wellesley, MA). Secondary proliferation assays were performed on day 14 after initial stimulation, the point at which the cells were no longer dividing. Previously activated cells were removed from culture, washed, and counted, and, as in the primary proliferation assay, 5 x 10^4 CD4+ Vα11+ T cells were stimulated. For the blocking of FasL-mediated AICD in vitro, cells were stimulated in the same conditions as above with the addition of anti-FasL (MFL3) or an isotype control Ig (eBioscience) added at 5 μg/ml to each well at 0 and 20 h.

**Functional assay for FasL-induced apoptosis**

The induction of FasL-mediated cell death was determined by annexin V-FITC (BD Pharmingen) and propidium iodide (Sigma) staining 15-20 h after 3 x 10^5 T cells were stimulated with 5 x 10^5 CHO (IE6 and B7.1 positive) cells and MCC3-10 peptide. Anti-Fasl or an isotype control Ig (5 μg/ml) was added at 0 and again at 10 h poststimulation where indicated. Soluble Fasl (sFasl) was prebound for 30 min, followed by addition of a cross-linking enhancer Ig (Alexis, San Diego, CA) at 2 h poststimulation. Cells were immediately analyzed by flow cytometry. Specific apoptosis was determined by calculating the ratio of live cells in the treated wells to live cells in the wells incubated in the absence of MCC peptide.

**Intracellular IL-2 staining**

Cells were stained 24 h after restimulation with anti-CD4-PE and anti-Vα11-FITC, and intracellular IL-2 was stained using the Cytofix/Perm/kit protocol (BD Pharmingen). Cells were immediately analyzed by flow cytometry. Ten thousand CD4+ Vα11+ events were collected.

**Calcium flux**

Day 8 post-primary stimulation with peptide and APCs, 5 x 10^5 5C.C7 itk-/- and 5C.C7 itk-/-/CD4+ cells were incubated with 3 μM fluo-3 and 5 μM fura-Red (Molecular Probes, Eugene, OR) in RPMI containing 3% FCS for 45 min. Cells were washed twice and incubated in the dark at room temperature for 30 min. Cells (1 x 10^6) were placed in 1 ml of 37°C serum-free RPMI and analyzed on a BD Biosciences flow cytometer. Baseline calcium was measured, and cells were then stimulated with anti-CD3e-biotin (145-2C11; 25 μg/ml) (BD Pharmingen) for 45 s, followed by 45 s stimulation with SEB (Life Technologies; 40 μg/ml) and propidium iodide (Sigma) (1 μg/ml) was added at 6 min. Data were analyzed by calculating the mean fluorescence ratio of fluo-3 and fura-Red using FACSAssist software (BD Biosciences).

**ERK and stress-activated protein kinase (SAPK)/JNK phosphorylation**

On day 14 after stimulation with 2 μM MCC3-10, 5 x 10^5 5C.C7 itk-/- and 5C.C7 itk-/-/CD4+ T cells were incubated on ice in 120 μl serum-free RPMI containing 25 μg/ml of biotinylated anti-CD3e for 10 min. Cells were quickly spun and resuspended in 120 μl serum-free RPMI containing 50 μg/ml streptavidin and incubated in a 37°C water bath for 0, 2, 5, or 10 min. As a positive control, cells were stimulated with PMA (2.5 ng/ml) and ionomycin (375 ng/ml) for 15 min at 37°C. Ice-cold 1 x PBS containing 20 mM NaF and 1 mM NaN_3 was added to stop the reactions. Cells were quickly spun and lysed for 15 min in ice by buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4 and 10 μg/ml leupeptin. Total lysates were cleared and resolved by 10% SDS-PAGE, transferred to an Immobilon-P membrane (Millipore, Bedford, MA), blocked, and blotted for phospho-p44/p42 MAPK or phospho-SAPK/JNK (Cell Signaling, Beverly, MA). After incubation with an HRP-conjugated secondary Abs, membranes were developed by ECL, stripped, and reprobed for total p44/p42 MAPK or SAPK/JNK protein (Cell Signaling).

**Real-time quantitative PCR**

Purification of naive CD4+ T cells was performed by staining MACS-purified CD4+ cells with anti-CD4-Cy and anti-CD44-FITC and sorting for CD4+CD44low cells on a BD Biosciences FACStar. Sorted cells (2 x 10^6) were incubated in medium containing 5 μg/ml biotinylated anti-CD3e for 20 min on ice, followed by resuspension in medium containing 2.5 μg/ml streptavidin and 5 μg IL-2 for 0, 6, 18, or 48 h in a 24-well plate. For secondary stimulations, 2 x 10^6 previously activated T cells were restimulated as above for 0, 1, 2, 4, or 6 h. Cells were removed from the plate and total RNA was isolated using the Qiagen RNeasy kit (Valencia, CA) according to the manufacturer's protocol. After DNase treatment (Promega, Madison, WI), 1 μg of total RNA was reverse transcribed into cDNA using Superscript II and Random Hexamers (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Real-time quantitative PCR amplification was performed on a Bio-Rad iCycler using SYBR Green PCR Core
Reagents (PE Applied Biosystems, Foster City, CA). To quantify the amount of cDNA for an individual transcript, SYBR Green fluorescence was measured at the end of each cycle. The cycle threshold (Ct) value at which exponential growth of the PCR product is first detected, was determined for known concentrations of plasmid DNA, and a standard curve was created. Template copy numbers were calculated for each sample by interpolating the Ct values on the standard curve using the iCycler software. All samples and standards were run in triplicate for any given experiment. The values of GAPDH, Egr3, and Egr2 were normalized to β-actin by dividing the average copy number of the respective transcript by the average copy number of β-actin in the respective sample. From 1 μg RNA, we consistently found there to be ~5 × 10⁶ copies of β-actin in naïve cells and 1.3 × 10⁶ copies in restimulated cells.

The PCR were as follows: templates were initially denatured at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 20 s, 25 s of primer annealing at 62°C, 65°C, 58°C, or 51°C for β-actin, FasL, Egr3, and Egr2, respectively, and finally a 72°C extension for 25 s. Primers were: FasL sense 5′-TGGACACCTCTGATCTTGC-3′; antisense 5′-GCTTCTGGGTTTGC-3′; Egr3 sense, 5′-GCCCTTGCTCTGA GTCTCTG-3′; antisense, 5′-CCCTTCTCGACTTCTCCTC-3′; Egr2 sense, 5′-CTTAGACAAAATCCCGTAA-3′; antisense, 5′-TCCTTT CTCCTGACTGCTG-3′; β-actin sense, 5′-CAGGGCCCGAGGCAA GAGAG-3′; antisense, 5′-CGTGGGCTCTAGGCAGT-3′. Specific products were verified by melt-curve analysis and gel electrophoresis. For the generation of standard curves, plasmids containing cDNA clones of Egr3 (gift from J. Milbrandt, Washigton University School of Medicine, St. Louis, MO), FasL (gift from A. Mariak-Rothstein, Boston University Medical Campus, Boston, MA), and β-actin (gift from R. Gerstein, University of Massachusetts Medical School, Worcester, MA) were used. A 190-bp fragment of Egr2 (135–324) was cloned into pGEM-T Easy (Promega) and used for the generation of a standard curve.

SEB-induced deletion in vivo

ilek−/− and ilek+/+ littermates were injected i.v. with 75 μg SEB (Toxin Technology, Sarasota, FL) on Day 0. Mice were tail bled on days −1, 1, 3, 7, 11, and 15 into Alsever’s solution. After RBC lysis, cells were stained with anti-Vβ8.1/2-FITC or anti-Vβ6-FITC and anti-CD4-PE. Ten thousand live CD4+ events were collected on a flow cytometer, and the percentage of CD4+ cells expressing Vβ8 or Vβ6 was determined.

Results

Naive ilek−/−CD4+ T cells have defects in IL-2 production and proliferation in response to MHC/peptide stimulation

Numerous studies over the past few years have indicated that the recruitment and activation of specific signaling pathways in T lymphocytes are determined by the nature of the TCR-peptide-MHC interaction (reviewed in Ref. 3). Nonetheless, prior in vitro studies that have focused on elucidating the role of ilek in T cell signaling and effector function have largely used Abs to T cell surface receptors, such as CD3 and/or CD28, to trigger TCR/costimulation signaling events. Moreover, although initial studies demonstrated that ilek-deficient CD4+ cells have functional defects in response to anti-CD3 stimulation (17, 18), ilek has also been implicated as a negative regulator of CD28 cosimulation (25). Therefore, we were interested in examining the role of ilek in TCR-mediated signaling events in response to the physiological receptor-ligand interaction. To accomplish this, we crossed ilek−/− mice to mice transgenic for the SC.C7 TCR, which is specific for a MCC peptide, MCC93-103, bound to MHC class II IEα (21, 22).

To first determine the functional responses of TCR-transgenic ilek−/− T cells, purified CD4+ T cells from SC.C7 ilek−/− or SC.C7 ilek−/− mice were stimulated in vitro with MCC93-103 peptide plus APC (IEβ and B7.1-expressing CHO cells). As shown in Fig. 1A, we found a modest (−2-fold) decrease in the Ag-induced proliferative responses of ilek−/−CD4+ T cells compared with control T cells at all peptide concentrations tested. In contrast, stimulation with a phorbol ester, PMA (P), plus a calcium ionophore, ionomycin (I), induced comparable levels of proliferation, confirming previous data indicating that these pharmacological agents bypass the ilek−/− defect by directly activating the protein kinase C (PKC)/Ras and calcium pathways, respectively (18). Previous studies had also indicated a defect in anti-CD3 Ab-induced IL-2 secretion by ilek−/− T cells. To re-examine this issue with primary TCR-transgenic T cells, we determined the extent of IL-2 production by SC.C7 ilek−/− or SC.C7 ilek−/− cells after stimulation with MCC93-103 peptide and APCs. For these studies, we used intracellular staining of permeabilized cells with an anti-IL-2 Ab. As shown in Fig. 2A, B, and C, ilek−/− cells produced ~50% less IL-2 than ilek+ + cells. Interestingly, these rather modest differences in the percent of responding cells, as measured by intracellular cytokine staining, correlate with much greater differences in IL-2 secretion as measured by ELISA (data not shown). Thus, these data are in close accordance with previously published experiments, using anti-
CD3 or anti-CD3 plus anti-CD28 Abs, showing decreased IL-2 production by naive *itk*<sup>−/−</sup> T cells (17, 18).

*itk*<sup>−/−</sup> CD4<sup>+</sup> T cells proliferate more vigorously than control T cells on secondary stimulation

Prior studies that have investigated the role of Itk in T cell signaling pathways have focused primarily on the responses of naive T cells that lack Itk. Therefore, we were interested in determining how *itk*<sup>−/−</sup> cells respond on secondary stimulation. To accomplish this, 5C7 *itk*<sup>−/−</sup> and 5C.C7 *itk*<sup>−/−</sup> CD4<sup>+</sup> T cells were stimulated in vitro with 2 μM MCC<sub>93-103</sub> peptide plus APCs and expanded in medium containing IL-2 until restimulation. In addition, to ensure a homogeneous population of activated T cells, these stimulations were performed in Th1-skewing conditions (IL-12 plus anti-IL-4 Ab). On day 14 after the initial stimulation, cells were restimulated with a range of concentrations of MCC<sub>93-103</sub> peptide plus APCs, and T cell proliferation was assessed. Interestingly, *itk*<sup>−/−</sup> T cells proliferated far more vigorously than control (*itk*<sup>+/+</sup>) T cells on secondary stimulation, indicating that the control T cells had either failed to proliferate or had undergone AICD (Fig. 2A). To ensure that these previously activated *itk*<sup>−/−</sup> T cells still possessed defects in IL-2 production in a secondary response, we performed intracellular IL-2 staining. These assays indicated that previously activated *itk*<sup>−/−</sup> T cells consistently produced reduced levels of IL-2 over a wide range of peptide concentrations (Fig. 2B). These data support the notion that *itk*<sup>−/−</sup> CD4<sup>+</sup> T cells retain a TCR signaling defect after secondary in vitro stimulation.

**FasL up-regulation is defective in *itk*<sup>−/−</sup> CD4<sup>+</sup> T cells**

FasL, transcription is regulated by a number of factors, including NF-κB, NFAT, AP-1 (*fos/jun*), and Egr families members, all of which are activated in response to TCR stimulation (26–31). A previous study has demonstrated that *itk*<sup>−/−</sup> CD4<sup>+</sup> T cells are impaired in their ability to efficiently translocate cytoplasmic NF-AT to the nucleus on TCR stimulation (19). Together with our observation that *itk*<sup>−/−</sup> T cells proliferate more vigorously on secondary stimulation compared with *itk*<sup>+/+</sup> T cells, this finding suggested that *itk*<sup>−/−</sup> T cells may be impaired in the expression of FasL. As an initial effort to assess whether proliferative differences between control and *itk*<sup>−/−</sup> T cells were due to differences in Fas/FasL-mediated AICD, we repeated the secondary in vitro proliferation assays in the presence of a neutralizing anti-FasL Ab (Fig. 2C). These experiments indicated that the presence of anti-FasL Ab, but not an isotype control Ab, blocked AICD and restored the proliferative capacity of control (*itk*<sup>+/+</sup>) T cells. In contrast, the anti-FasL Ab had no effect on the proliferative responses of *itk*<sup>−/−</sup> T cells. Interestingly, at high peptide concentrations (100 nM), both wild-type and *itk*<sup>−/−</sup> T cells undergo AICD in the presence of anti-FasL Ab. This is likely due to the up-regulation of Fasl in the *itk*<sup>−/−</sup> T cells in response to very strong TCR signaling (100 nM peptide vs 10<sup>−8</sup> or 10<sup>−11</sup> nM peptide), which may be more difficult to block with the concentrations of anti-Fasl. Ab used. Nonetheless, at lower concentrations of peptide, it appears that *itk*<sup>−/−</sup> T cells fail to up-regulate FasL after stimulation.

As an additional measure of FasL up-regulation, previously activated T cells were restimulated with peptide and APCs for 9 h, stained for surface Fas and FasL, and analyzed by flow cytometry. As shown in Fig. 3, we observed induced surface expression of FasL on *itk*<sup>−/−</sup> T cells at all peptide concentrations, with maximal levels at the highest peptide concentration tested (100 nM). In contrast, *itk*<sup>−/−</sup> T cells failed to detectably up-regulate FasL, except perhaps at the highest peptide concentration where a slight shift in FasL staining can be seen. Both *itk*<sup>+/+</sup> and *itk*<sup>−/−</sup> T cells show no difference in the expression of Fas upon stimulation, indicating that differences in AICD between control and *itk*<sup>−/−</sup> T cells are not due to differences in surface expression of Fas.

To confirm that the decreased proliferative responses of *itk*<sup>−/−</sup> T cells and the increased induction of FasL expression correlated with increased apoptosis, *itk*<sup>+/+</sup> and *itk*<sup>−/−</sup> T cells were stained with annexin V and propidium iodide after stimulation. As shown in Fig. 4, a substantial degree of apoptosis is induced in control T cells after peptide stimulation, whereas *itk*<sup>−/−</sup> T cells require stimulation with 10<sup>5</sup>- to 10<sup>6</sup>-fold higher concentrations of peptide to induce a comparable degree of apoptosis. The presence of a neutralizing anti-Fasl Ab was able to increase cell viability, by 2.4-fold in stimulations of control T cells. In contrast, cell viability was only increased by 1.3-fold when anti-Fasl Ab was included in cultures of *itk*<sup>−/−</sup> T cells. This observation further supports the conclusion that greater levels of functional FasL are expressed on *itk*<sup>−/−</sup> compared with *itk*<sup>+/+</sup> previously activated CD4<sup>+</sup> T cells. These data are also consistent with a previous finding that thymocytes from *itk*<sup>−/−</sup> mice are defective in activation-induced cell death in response to anti-CD3 Ab stimulation (32).

**FIGURE 2.** A and B, 5C.C7 *itk*<sup>−/−</sup> and 5C.C7 *itk*<sup>−/−</sup> CD4<sup>+</sup> T cells that were initially stimulated with 2 μM MCC<sub>93-103</sub> were restimulated on day 14, and proliferative responses were measured. B, Cells were restimulated on day 12 for 6 h and analyzed for IL-2 production by intracellular staining. C, Proliferative responses of 5C.C7 *itk*<sup>−/−</sup> and 5C.C7 *itk*<sup>−/−</sup> CD4<sup>+</sup> T cells that were restimulated in the presence of anti-Fasl Ab or an isotype control Ig (5 μg/ml). Abs were added at time 0 and again at 20 h. P + 1, PMA and ionomycin; Ctrl, control.
FIGURE 3. Previously activated SC.C7 itk+/– and SC.C7 itk−/−CD4+ T cells were restimulated on day 14 with APCs and the indicated concentrations of MCC3-103 peptide. After 9 h, cells were stained for CD4, Vα11, Fas, and FasL and analyzed by flow cytometry. Ten thousand CD4+ Vα11+ events were collected. Dotted line, Nonstimulated cells; bold line, staining of stimulated cells. These data are representative of three independent experiments.

One previous study has indicated that another Tec kinase family member, Btk, functions as an inhibitor of signaling through Fas in B cells (33). Therefore, to determine whether signaling through Fas is altered in itk−/− T cells, cells were treated with a Fas agonist, sFasL. As shown in Fig. 4B, stimulation with sFasL led to comparable levels of apoptosis in both itk+/– and itk−/− T cells. These data indicate that signaling through Fas is unperturbed in itk−/− T cells.

Although IL-2 is commonly recognized as a growth-promoting cytokine that triggers survival and proliferative signals upon binding its receptor, IL-2 can also potentiate AICD by inducing maximal FasL expression (34, 35). This is thought to occur through the action of IL-2R-mediated transcription factors such as SP-1 (36). Furthermore, IL-2R signals have also been shown to down-regulate Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (FLIP), an anti-apoptotic molecule (37). Thus, signals through the IL-2R can cooperate with TCR signals to provide a feedback mechanism that renders activated T cells more susceptible to apoptotic death. In light of these data, we were interested to determine whether the reduced ability of itk−/− T cells to undergo AICD was due, in part, to decreased levels of IL-2 production (Fig. 2B). To address this issue, exogenous IL-2 was added to cultures during restimulation. We found that addition of exogenous IL-2 did not enhance FasL-induced cell death or FasL surface expression on itk−/− T cells (data not shown), indicating that the defect in FasL expression is not secondary to the decreased levels of IL-2 production seen in itk−/− CD4+ T cells. Furthermore, because both itk+/– and itk−/− T cells are cultured in an excess of exogenous IL-2 during the primary stimulation, differences in IL-2R signaling are unlikely to account for differential expression of FasL or responsiveness to AICD during the subsequent in vitro stimulations.

Calcium, ERK, and JNK pathways are defective in previously activated itk−/− CD4+ T cells

Stimulation of the TCR leads to the activation of signaling pathways that ultimately result in the generation of active transcription factors leading to new gene expression (38). Previous biochemical studies have demonstrated that Itk plays a role in the phosphorylation and activation of PLCγ1 following stimulation of the TCR (17, 39). Activated PLCγ1 then converts the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, into inositol 1,4,5-triphosphate, an activator of calcium release channels in the endoplasmic reticulum, and 1,2-diacylglycerol, an activator of the
CD4 T cells require Itk for efficient FasL expression and AICD

Ras and PKC pathways. A sustained increase in intracellular calcium concentrations after TCR stimulation leads to the calciuneurin-dependent dephosphorylation of cytoplasmic NF-AT, resulting in NF-AT translocation to the nucleus (40-42). To confirm that previously activated Itk−/−CD4+ T cells retain the biochemical defects characterized in primary resting Itk−/−CD4+ T cells (17), intracellular calcium mobilization was measured upon restimulation of 5C.C7 Itk−/− T cells initially stimulated with 2 μM MCC957-103 plus APCs and cultured for 10 days in exogenous IL-2. As demonstrated in Fig. 5A, Itk−/− T cells show a significant defect in calcium mobilization compared with Itk+ T cells after stimulation through the TCR. These data indicate that previously activated Itk−/− T cells exhibit a comparable defect in signaling compared with freshly isolated ex vivo Itk−/− T cells.

Recently, several reports focusing on Itk biochemistry have demonstrated that Itk is recruited to the linker of activated T cells/SH2 domain-containing 76-kDa leukocyte protein (SLP-76) complex in response to TCR signaling, thereby providing a scaffold for Itk to activate PLCγ1, potentially by direct phosphorylation. These data place Itk intermediate between proximal TCR signaling events and downstream events such as the activation of the Ras pathway (Refs. 43-46; reviewed in Ref. 16). Activated Ras is known to activate the ERK/MAPK pathway, which subsequently leads to the transcription of fos proteins, and ultimately to the formation and activation of AP-1 complexes (reviewed in Ref. 47). In addition, the ERK proteins, ERK1 and ERK2, have recently been shown to play a role in AICD by inducing FasL transcription (9). Consistent with these findings, the FasL promoter was also found to possess target sites for AP-1 transcription complexes (30).

In light of these data, we were interested in determining whether the reduced ability of Itk−/−CD4+ T cells to up-regulate FasL in response to TCR signaling was due, in part, to defective activation of the ERK/MAPK pathway. As shown in Fig. 5B, after anti-CD3 stimulation, Itk−/− T cells failed to achieve maximal levels of phosphorylated ERK proteins over the course of a 10-min stimulation. In contrast, treatment with PMA and ionomycin, which bypass the proximal signaling events, induced comparable levels of phosphorylated ERK in both Itk+ and Itk− T cells, demonstrating that there is no intrinsic defect in the ability of the Ras pathway to activate ERK in the absence of Itk. These data are consistent with previous reports by Schaaff er et al. (32, 48), who demonstrated that Itk−/−/− T cells and thymocytes show a marked reduction in ERK phosphorylation after TCR stimulation.

Activated Ras also plays a role in the activation of the MAPK kinase kinase-1/JNK pathway, which is essential for transcriptional activation of the FasL promoter via the activation of c-Jun (8, 49). To further assess the role of Itk in the activation of the Ras pathway and its downstream effectors, we examined the level of JNK/SAPK phosphorylation in Itk−/− cells upon TCR stimulation. As demonstrated in Fig. 5B, the activation of the JNK/SAPK pathway is also impaired in Itk−/−CD4+ T cells. Similar to ERK phosphorylation, treatment with PMA and ionomycin induced comparable levels of SAPK/JNK phosphorylation in Itk−/− and Itk−/− cells. Collectively, these biochemical data strongly suggest that Itk−/−CD4+ T cells are unlikely to mount normal levels of active c-Fos and c-Jun and thus are likely to have reduced levels of AP-1 complexes after TCR stimulation.

Reduced Egr2, Egr3, and FasL transcription after TCR stimulation of Itk−/− T cells

Several transcription factors such NFAT, NF-κB, Egr2, as well as Egr3, have been implicated in the TCR-mediated activation of the FasL promoter. In fact, the FasL promoter contains consensus sequences for NF-AT, NF-κB, and Egr, as well as the AP-1 factors, c-Fos, and c-Jun (27, 29, 50, 51). The Egr2 and Egr3 genes are normally expressed at low basal levels in resting T cells, and are transcriptionally induced following TCR stimulation (52). Furthermore, the Egr2 and Egr3 promoters themselves are targets of NF-AT proteins and in turn function as strong trans activators of the FasL promoter (31). Consistent with these findings, the over-expression of either Egr2 or Egr3 in T cell hybridomas or HeLa cells induces FasL transcription (27, 50). Additional evidence also indicates that the induction of Egr2 and Egr3 transcription, and consequently FasL expression, is inhibited by the calcineurin inhibitor, cyclosporin A (27, 50). Despite these compelling data, there have been conflicting results regarding which factor, Egr2 or Egr3, is more critical for FasL transcription. Nonetheless, the bulk of the evidence indicate that NF-AT and Egr factors act synergistically in the activation of the FasL promoter.

Recent studies have demonstrated that Itk−/−CD4+ T cells are defective in the nuclear translocation of NF-AT upon TCR stimulation (19); furthermore, dominant-negative Itk can inhibit TCR-induced NF-AT-dependent transcription (43). Given these observations and the fact that the Egr2 and Egr3 promoters are regulated by NF-AT, we reasoned that Egr2 and Egr3 transcription might be defective in Itk−/− T cells, resulting in impaired FasL transcription. To test this idea, we used real-time quantitative PCR analysis to determine the mRNA levels of Egr2, Egr3, FasL, and β-actin in resting and stimulated T cells.
Since Fas-dependent death can occur at low levels in naive CD4+ T cells upon strong stimulation of the TCR within the first 18 h (53), we first sought to determine the expression levels of the Egr2, Egr3, and FasL transcripts in naive SC.C7 itk+/− or itk−/− CD4+ T cells that were stimulated with anti-CD3 Ab for 0, 6, 18, or 48 h. For these experiments, CD4+CD44hi T cells were sorted from mice of each genotype, to prevent ambiguities caused by the presence of activated/memory T cells in the unsorted populations. For the analysis of previously activated T cells, SC.C7 itk+/− and SC.C7 itk−/− CD4+ T cells were stimulated with 2 μM MCC18+3 peptide plus APCs in Th1-skewing conditions and then expanded in IL-2. These cells were then restimulated on day 14 with anti-CD3 Ab for 0, 1, 2, 4, and 6 h.

Fig. 6A shows an example of raw data obtained from this analysis. The amount of PCR product present, as measured by fluorescence intensity, is indicated for each PCR cycle. As can be seen, the β-actin curves for both samples (itk+/− and itk−/−) are virtually superimposable, indicating nearly identical amounts of cDNA in these samples. In contrast, the FasL curves do not superimpose, indicating a difference in the copy number of FasL transcripts between the stimulated itk+/− and itk−/− T cells. By interpolation of these data to a standard curve, absolute values for FasL copy numbers can be obtained for each sample. To normalize for the amount of cDNA present in each sample, a ratio of the average copy numbers of Egr2, Egr3, and FasL to β-actin copy numbers was calculated for each data point. The data from a representative experiment of each type are shown in Fig. 6B. This analysis demonstrated decreased levels of Egr2, Egr3, and FasL transcripts in primary ex vivo itk−/−CD4+ T cells after stimulation, although the magnitude of this reduction is modest. A more striking deficit was seen in the analysis of previously activated itk−/− T cells, where we observed, on average, a 5- and 6-fold decrease in Egr2 and Egr3 levels, respectively, and a 3-fold decrease in levels of FasL transcripts compared with the levels in the itk+/− T cells at the peak of the response. Interestingly, these data also clearly demonstrate the dramatic enhancement in TCR-mediated Egr3 and FasL transcription in previously activated compared with naive CD4+ T cells. For example, the peak of Egr3 expression in previously activated itk−/− T cells is nearly 40-fold higher than in naive T cells, as is the case for FasL as well. In contrast, peak Egr2 levels only increase by ~2-fold in previously activated compared with naive CD4+ T cells. These data are consistent with a more important role in FasL transcription for Egr3 than for Egr2. Together these data clearly indicate that signaling through Itk plays an important role in the TCR-induced up-regulation of Egr factors and that impaired expression of Egr2 and Egr3 in itk−/− T cells correlates with impaired FasL expression.

**CD4+ T cells in itk−/− mice are defective in AICD in vivo**

It has been well documented that mice deficient in the expression of Fas (lpr) or FasL (gld) possess profound defects in the peripheral deletion of activated lymphocytes and develop severe autoimmune disorders as a result of a failure to maintain peripheral T cell tolerance (reviewed in Ref. 2). Furthermore, CD4+ T cells...
from mice bearing the lpr or gld mutations are resistant to TCR-mediated apoptosis upon anti-CD3 or superantigen stimulation (54–57). When injected into mice, SEB, a bacterial superantigen, selectively activates Vβ8+ T cells. This leads first to the expansion of Vβ8+ T cells, followed by a steep decline in the percentage of these cells as a result of Fas-mediated cell death (58, 59). Thus, we were interested in determining whether Itk-deficient T cells would possess defects in AICD in vivo, as a result of the defective FasL expression we observed in vitro. To assess this, we injected itk+/− and itk−/− mice with SEB and examined peripheral blood T cells on days 3, 7, 11, and 15 postinjection. Fig. 7 shows the percentage of CD4+ cells bearing Vβ8, or Vβ6 as a control, over the course of the response to SEB. Interestingly, itk+/− and itk−/− mice initially responded similarly to SEB, as seen by the equivalent increases in Vβ8+CD4+ T cells on day 3 after injection. Following the expansion phase of the response, itk−/−/Vβ8+ T cells underwent deletion, as has previously been reported. In contrast, itk−/−/Vβ8+ T cells survived to a much greater degree than control cells, indicative of reduced AICD. Fig. 7B demonstrates that both itk+/− and itk−/− mice had comparable percentages of the control Vβ6+CD4+ T cells, which are not reactive to SEB. Overall, these data suggest that itk−/− CD4+ T cells are inefficient at undergoing AICD, most likely as a result of reduced FasL expression. Consistent with this conclusion, we routinely observe a 2-fold increase in the proportion of CD4+ T cells with an activated/memory phenotype in itk−/− compared with itk+/− control mice (data not shown). However, we cannot rule out the possibility that the levels of IL-2 in vivo may be decreased in SEB-injected itk−/− mice, thereby rendering itk−/−/CD4+ T cells less susceptible to FasL-mediated death.

Discussion

Aberrant regulation of the Fas/FasL system has detrimental effects on the health of an organism. Mutations in the Fas or FasL genes result in autoimmunity and lymphadenopathy in mice and autoimmune lymphoproliferative syndrome in humans (3–5). Conversely, increased expression of FasL in HIV-1-infected T cells has been found to be a factor in the T cell depletion that ultimately causes AIDS (60, 61). Currently, the signaling requirements for proper FasL expression are not completely understood. Therefore, investigation of the molecular mechanisms regulating this pathway is warranted. In our efforts to elucidate the role of a Tec family kinase, Itk, in CD4+ T cell effector function, we discovered that Itk plays a crucial role in the signaling pathway that induces FasL expression.

Previous studies by several groups have established that Itk is important for T cell effector functions, including cytokine production as well as the development of protective immunity to pathogen infections (17–19, 48). The data presented in this article address the role of Itk in T cell homeostasis, as opposed to effector function, and demonstrate that Itk is required for efficient apoptosis induced by TCR stimulation. These experiments further strengthen the notion that Itk is a crucial component of the TCR signaling cascade required for the transcription of genes important for proper immune function. The physiological relevance of these findings is reflected in the altered response of itk−/− mice to the superantigen, SEB.

These results also support the notion that Itk may be involved in setting the threshold for TCR signaling (32). In the absence of Itk, the efficiency of TCR signaling is reduced, as assessed by a variety of biochemical and functional readouts, including reduced PLCγ1 activation, calcium mobilization, MAPK activation, and cytokine production. This reduced signaling lowers the overall effector response of the cell. Given that the ability of CD4+ T cells to up-regulate FasL is dependent on the integration of TCR signals, itk−/− T cells may require more receptor stimulation to attain a threshold of signals great enough to induce FasL up-regulation. This is consistent with our observation that, at high peptide concentrations, itk−/− T cells do up-regulate low levels of FasL and can undergo AICD.

These experiments led to the surprising finding that, during the initial phase of the response, itk−/− T cells expand comparably with itk+/− T cells in response to SEB injection in vivo. Given that previous studies have documented both reduced IL-2 production and proliferation of itk−/− T cells in vitro, these findings were somewhat unexpected. One interesting explanation for this discrepancy is the possibility that SEB produces such a strong activation signal in naïve T cells that FasL expression may be induced early during the activation process. Thus, the net expansion of Vβ8+ T cells in the control mice may reflect the combined effects of proliferation being offset by some apoptosis. Consistent with this possibility, upon infection of lpr/lpr mice with lymphocytic choriomeningitis virus, there is a notable increase in the rate of expansion of antiviral CTLs during the initial phase of the response compared with what is typically observed in wild-type mice (62). Thus, T cells in itk−/− mice may proliferate more poorly but may also undergo less apoptosis, yielding the same net outcome of Vβ8+ T cell numbers at the peak of the response as are found in itk+/− mice.

Although our data directly demonstrate reduced transcription of FasL in stimulated itk−/− T cells in vitro, the interpretation of the in vivo experiments is clearly more complex. Activation-induced cell death can involve the action of other molecules in addition to Fas/FasL, such as TNF-α and Bcl-2 family members. We have not fully ruled out the possibilities that itk−/− T cells are defective in the expression of one or more of these additional molecules and that such differences might also contribute to the decreased AICD
of itk−/− cells in vitro and in vivo. Studies addressing this possibility are currently underway. In addition, it is also possible that itk−/− T cells express greater amounts of FLIP, an inhibitor of the Fas pathway. Because IL-2R signaling inhibits FLIP transcription (37), it is possible that reduced production of IL-2 by itk−/− T cells in vivo may result in increased FLIP, thereby rendering itk−/− T cells less susceptible to Fas-mediated apoptosis. Although differences in FLIP expression in vivo between control and itk−/− T cells will be interesting to examine in the future, this concern is unlikely to be relevant to our in vitro studies, because stimulated T cells were cultured in an excess of exogenous IL-2. Another concern is the observation by Bonfoco et al. (63) that nonlymphoid FasL is essential for the deletion of SEB-reactive T cells. These investigators also demonstrated that T cell activation was necessary for the induction of nonlymphoid FasL transcription. Therefore, we cannot fully rule out the possibility that itk−/− mice may be defective in the expression of cytokine(s) or effector molecule(s) necessary to induce the up-regulation of nonlymphoid FasL. Again, this possibility applies only to the in vivo studies and is not relevant to our in vitro studies using purified T cells.

Finally, we also considered the possibility that reduced AICD in vivo by itk−/−/CD4+ T cells may reflect reduced activation and/or preferential differentiation of itk−/− T cells into Th2 effectors that express FasL poorly (64, 65). These possibilities were tested by examining VB8+CD4+ T cells at the peak of the response (day 3) after SEB injection. When examined by flow cytometry for a panel of activation markers, both itk+/+ and itk−/− T cells showed comparable percentages of activated T cells. Furthermore, in vitro stimulation of these cells followed by intracellular staining for IL-2, IFN-γ, and IL-4 indicated no increased proportion of IL-4-producing cells among itk−/− T cells compared with controls (data not shown). Thus, we find it unlikely that the reduced AICD we observe in vivo in itk−/− mice is due to a skewed differentiation of itk−/− T cells into Th2 effectors.

Similar to the FasL gene, the Fas gene is transcriptionally regulated by factors such as c-Fos and c-Jun. A report by Li et al. demonstrated that, although PKC and JNK are involved in the activation of the Fas gene upon TCR stimulation, phosphatidylinositol 3-kinase, calcineurin, and ERK kinases play no role in the signaling pathway leading to Fas transcription (66, 67). These findings suggest that the TCR signaling pathways leading to Fas and FasL transcription are distinct. Our data support the notion that Itk does not play a role in the TCR-mediated up-regulation of Fas. Another recent study indicated that Btk, a Tec kinase family member, acts as an inhibitor of the Fas-signaling pathway in B cells (33). Based on our observations that itk−/− T cells are equally susceptible to apoptosis after treatment with sFasL, the possibility that Itk plays a comparable role in the Fas-signaling pathway in T cells seems unlikely.

On the basis of the data presented here, we speculate that itk−/− mice may have altered susceptibility to autoimmune disease. For instance, experimental autoimmune encephalomyelitis (EAE), which can be induced in mice upon adoptive transfer of myelin-specific Th1 cells, is a result of Th1 CD4+ T cellsinitiating tissue damage to the CNS (68, 69). Interestingly, mice possessing the bpr or gld mutations are resistant to the induction of EAE (70). These findings suggest that FasL-expressing T cells may mediate apoptosis within a target tissue, thereby contributing to the pathology of this disease. Given these observations, it is possible that itk−/− mice may also show decreased susceptibility to EAE. Alternatively, because the Fas/FasL pathway has been shown to be crucial for the removal of autoreactive T cells in the periphery (71), it is also possible that itk−/− mice may be more susceptible to other types of autoimmune diseases. For instance, FasL-expressing CD4+ T cells have been shown to be important in the deletion of autoreactive B cells (72), raising the intriguing possibility that itk−/− mice might have increased susceptibility to autoantibody-mediated autoimmune diseases.

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References


New insights into the regulation and functions of Tec family tyrosine kinases in the immune system
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The Tec family of protein tyrosine kinases play an important role in signaling through antigen-receptors such as the TCR, BCR and Fcε receptor. Recent studies have generated new insights into the domains in Tec kinases that take part in intramolecular and intermolecular binding. Furthermore, the consequences of these domain interactions for Tec activation and downregulation have been better defined. Genetic studies of kinase-knockout mice have emphasized the importance of Tec kinases in lymphocyte development, differentiation and apoptosis.

Abbreviations
BH  Btk homology
CyA  cyclosporine A
ERK  extracellular related kinase
FasL  Fas ligand
I Btk  inhibitor of Btk
LAT  linker of activated T cells
PH  pleckstrin homology
PI3-K  phosphoinositide 3-kinase
PIP2  phosphatidylinositol (4,5)-bisphosphate
PIP3  phosphatidylinositol (3,4,5)-trisphosphate
PKC  protein kinase C
PLC-Y  phospholipase C γ
PRR  proline-rich region
SH1  Src homology 1
SLP-76  SH2-domain-containing leukocyte protein of 78 kDa
Tec  tyrosine kinase expressed in hepatocellular carcinoma
TH  Tec homology
xid  X-linked immunoedeficiency
XLA  X-linked agammaglobulinemia

Introduction
Over the past twenty years, significant progress has been made in elucidating the signaling pathways that are triggered by engagement of immune cell antigen-receptors, such as the TCR, the BCR and the Fcε receptor. In lymphocytes, several protein tyrosine kinases are activated following receptor engagement and are crucial components of receptor-induced signal transduction cascades. Signaling pathways that are initiated by these tyrosine kinases can lead to a variety of outcomes, including cytokine production, clonal expansion, cell differentiation, and apoptosis. A major focus of recent studies in this area has been to clarify the intersecting and diverging branches of antigen-receptor signaling pathways, to provide a better understanding of how distinct immune outcomes are regulated.

Two of the initial cytoplasmic signaling molecules that were discovered and characterized in lymphocytes, Lck and Fyn, were revealed to be members of the largest family of non-receptor protein tyrosine kinases, the Src family. In 1990, almost twelve years following the discovery of the Src kinase, a novel type of non-receptor protein tyrosine kinase, termed Tec (tyrosine kinase expressed in hepatocellular carcinoma), was discovered in an effort to identify tyrosine kinases involved in hepatocarcinogenesis [1]. Shortly thereafter, several other related proteins were discovered and grouped into what is now recognized as the second largest family of non-receptor tyrosine kinases, the Tec family.

In 1993, the importance of Tec family kinases was highlighted by the discovery that point mutations or disruptions in the gene encoding one family member, Btk, lead to immunodeficiency diseases in both humans and mice (X-linked agammaglobulinemia [XLA] and X-linked immunodeficiency [xid], respectively) [2-4]. To date, the Tec family consists of eight members, only five of which are expressed in mammals: Bmx/Etk, Btk/Atk, Itk/Ik/Emt, Tec and Rlk/Txk.

The Tec and Src family members have similar overall structures. Whereas each Src family kinase contains a Src homology 1 (SH1) (kinase domain), SH2 and SH3 domain, members of the Tec family possess additional domains with distinctive functions. With the exception of Rlk, all Tec kinases have an amino-terminal pleckstrin homology (PH) domain, which associates with phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and plays a role in targeting the proteins to the membrane. Most Tec family members also possess a Tec homology (TH) domain, which contains a Zn2+-binding Btk homology (BH) motif, followed by one or two proline-rich region(s) (PRR). Furthermore, Tec family kinases lack the carboxy-terminal negative regulatory tyrosine found in Src kinases, suggesting a distinct mode of regulation for these proteins (see modular structure in Figure 1a).

In general, Tec family kinases are expressed in cells of the hematopoietic lineage, but are not restricted to a particular cell type. For instance, Tec is expressed in all hematopoietic cells, whereas Itk and Rlk are restricted to T lymphocytes, NK cells and mast cells. Btk is expressed in all hematopoietic cells, with the exception of T lymphocytes and plasma cells. A more detailed description of Tec family kinase expression patterns can be found in a recent review by Smith et al. [5*]. Interestingly, some Tec kinases are expressed in cells outside of the hematopoietic lineage; however, for the purposes of this review, we will only focus on those expressed in the immune system.
Prior reviews of Tec family kinases provide excellent insights into the mechanisms of Tec kinase activation and Tec kinase function within the multi-molecular signalosome, the effects of Tec kinases on biological processes such as lymphocyte development and differentiation, and also provide detailed comparisons of Tec family members to each other [5**,6**,7].

The first part of this review will focus on recent insights into the biochemical mechanism of Tec family kinase regulation and the role of specific protein domains in the functions of these molecules. In the second part of this review, we will discuss new data that demonstrate previously unappreciated or undiscovered functions of Tec kinases and steady-state intermolecular and intramolecular interactions of Tec family kinases. (a) Similar to Src kinases, Tec kinases possess a carboxy-terminal kinase domain, followed by an SH2 and an SH3 domain. The two families of kinases differ in their amino-terminal regions. Whereas Btk and Tec both possess two PRRs, and Itk and Rlk possess one, Bmx lacks a PRR in the TH region. A BH domain, the function of which is still not clear, and a PH domain are amino-terminal to the PRR of Btk, Tec, Itk and Bmx. Rlk is the only known Tec family member that does not possess amino-terminal BH and PH domains. Instead, Rlk possesses an amino-terminal cysteine-rich region which, following palmitoylation, targets Rlk to the membrane. At the carboxyl terminus, Tec family kinases lack the regulatory tyrosine found in Src family members. (b) Steady-state intermolecular and intramolecular interactions. In the inactive state, Btk can form symmetric and asymmetric homodimers via intermolecular SH3 domain and PRR interactions. Conversely, the SH3 domain and PRR of Itk can interact in an intramolecular association. Furthermore, a novel association of the SH2 domain and SH3 domains of Itk in an intermolecular interaction has also been described; however, whether the intramolecular SH3-PRR can occur simultaneously with the homodimerization is not known. References for studies of these domain interactions are described in the text.
kinases in immune cell signaling pathways. The majority of new information derives largely from analyses of Btk and Itk. The emphasis on these two members of the Tec kinase family is largely because of the obvious lymphocyte signaling defects that are observed in their absence. In contrast, the functions of Tec, Rlk and Bmx within the immune system are still not fully understood, as mice deficient in Tec or Rlk, for instance, have no apparent immune deficiencies. Interestingly, analyses of mice possessing combinatorial mutations have revealed both overlapping and unique functions between Tec and Btk, as well as between Rlk and Itk, as will be discussed in detail below.

**Review of Tec kinase activation downstream of antigen-receptor engagement**

The signals required for Tec kinase activation, or positive regulation, have been discussed in great detail in previous reviews [5**, 6**, 7**]; therefore, the section below will only briefly revisit the currently accepted mechanism of this process. For the sake of simplicity, this description will focus on events downstream of TCR engagement; however, comparable events also occur downstream of the BCR (see Figure 2).

Immediately following receptor stimulation, Src and Syk family kinases are activated (ZAP-70 is an example of the latter), leading to the phosphorylation of two adaptor molecules—linker of activated T cells (LAT), which is constitutively associated with lipid rafts, and SH2-domain-containing leukocyte protein of 76 kDa (SLP-76). Concurrently, stimulation leads to a phosphoinositide 3-kinase (PI3-K)-dependent increase in PIP3 levels within the cell membrane. The enrichment of PIP3 at the site of the activated receptor recruits Tec kinases, such as Itk or Tec, to the membrane in a PH-domain-dependent manner. Following this recruitment, a multimolecular signalosome complex consisting of a Tec family member, phospholipase C-γ1 (PLC-γ1), SLP-76, Cads and Grb-2 forms around LAT within lipid rafts. The Tec kinase is then transphosphorylated by a Src kinase. This is followed by Tec kinase autophosphorylation (H Wilcox, LJ Berg, unpublished data), resulting in complete activation of the kinase and, ultimately, to phosphorylation and complete activation of PLC-γ1, a crucial step in T cell activation [8**].

Activation of PLC-γ1 leads to hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP2) into inositol (3,4,5)-trisphosphate (IP3) and diacylglycerol (DAG), which lead to calcium mobilization and to activation of the protein kinase C (PKC)/Ras/Raf pathways, respectively. For previous reviews of the Tec kinase domains that are required for signalosome formation and enzyme function, see [5**, 6**, 7**].

**Intermolecular and intramolecular interactions regulate Tec kinases in the steady state**

The biological activity of tyrosine kinases is influenced by their interactions with activators, adaptors and substrates. These recognition events are dependent on the integrity and conformational state of specific domains within the protein. A number of studies over the last several years have indicated that protein tyrosine kinases are regulated, in part, via stabilizing intramolecular and/or intermolecular interaction(s) among their domains. For instance, in the resting state, Src kinases are folded such that their SH2 and SH3 domains are involved in interactions with their catalytic domain [9–12]. One important component of the Src kinase intramolecular interaction is the carboxy-terminal regulatory tyrosine in the catalytic domain which, when phosphorylated, binds to the Src SH2 domain. Unlike Src kinases, Tec kinases lack this carboxy-terminal regulatory tyrosine; nonetheless, several recent studies have indicated that domains of Tec kinases can undergo both intramolecular and intermolecular interactions [13, 14**, 15**, 16**, 17, 18**]. Although the details of the interactions differ from those found in Src kinases, the general principle that these interactions regulate kinase activity seems certain to hold for Tec kinases as well.

The importance of individual Tec kinase domains in enzyme regulation and signaling was first established by genetic data demonstrating that specific mutations in any of the five domains of Btk can result in XLA [19]. Initial biochemical and structural studies of Tec kinase regulation revealed that the SH3 domain of Itk associates intramolecularly with the PRR of the TH domain, and that this interaction could interfere with ligand binding via these domains [13]. Interestingly, sequence comparisons between Tec family members show that there are important differences in the TH domain, suggesting that individual Tec family members may engage in distinct domain associations, and thus may be subject to distinct modes of self-regulation (see Figure 1b).

For instance, unlike Itk, Btk and Tec each contain two PRRs within their TH domains. Independent studies have shown that both of these proteins can form homodimers as a result of stable intramolecular interactions between the PRR of one molecule and the SH3 domain of a second molecule [14**, 15**, 17]. In addition, Tec can also form intramolecular PRR-SH3 associations that occur with a lower affinity, suggesting potential roles for these different interactions in regulating enzymatic activity and protein localization [14**, 15**, 17]. A very recent report indicates that asymmetric homodimers of Btk can also form (see Figure 1b), adding further complexity to the modes of catalytic regulation and substrate specificity that may occur [16**]. Finally, a novel intermolecular association has been described for Itk, where the SH3 domain specifically interacts with the SH2 domain of a second molecule. This latter interaction may be an important step on the pathway to full enzyme activation, possibly by displacing the intramolecular interaction of the PRR with the SH3 binding pocket that occurs in the 'inactive state' [18**]. Although these data provide important clues to the protein domain interactions regulating Tec kinases, further studies will be essential to clarify the protein conformations that play important roles in intact cells.
Following the discovery that the xid defect in mice results from an amino acid substitution in the PH domain of Btk [4], it was further demonstrated that the PH domain functions to directly engage PIP3 in the plasma membrane. This finding provided a model to account for the localization of Btk and the other PH-domain-containing Tec
The role of Tec kinases in TCR and BCR signaling. Following the engagement of the TCR–CD3 complex, Src family kinases, such as Lck, are activated. Lck phosphorylates the ITAMs (immunoreceptor tyrosine activation motifs) within the CD3ε chains, which in turn recruits the Syk family kinase, ZAP-70, leading to ZAP-70 phosphorylation and activation by Lck. Activated ZAP-70 proceeds to phosphorylate the adaptor molecules SLP-76 and LAT. Concurrently, Tec-K is activated, which catalyzes the conversion of membrane-associated PIP_2 to PIP_3, the substrate of PH-domain-containing proteins such as Itk and PLC-γ1. CyA has been shown to be constitutively associated with inactive Itk via Itk’s SH2 domain. However, the mechanism by which CyA becomes dissociated is not known. Following Itk’s recruitment to the membrane via its PH domain, a number of complex interactions ensue. Whereas SLP-76 binds to the SH2 and SH3 domains of Itk, the interaction of SLP-76 with Gads leads to an interaction with LAT. Grb-2 can bind to the free PRR of Itk, which can also bind to LAT. Furthermore, PLC-γ1 recruitment to the membrane via its PH domain allows the amino-terminal SH2 domain of PLC-γ1 to bind to a phosphotyrosine on LAT. These interactions result in what is termed a signalosome complex. Lastly, following the phosphorylation of Itk by a Src kinase, Itk can in turn autophosphorylate, then proceed to phosphorylate and activate PLC-γ1. References for the novel aspects of this activation scheme are provided in the text. (B) Itk is thought to exist as either an asymmetric or symmetric homodimer via intramolecular SH3–PRR interactions in the inactive state (bottom left of figure). Furthermore, in the inactive state, Itk has been shown to bind the PH domain of Btk. Analogous to the TCR, upon stimulation of the BCR, Src family kinases, such as Lyn, are activated, which function to phosphorylate the ITAM motifs of the Igα and Igβ chains. As in T cells, the ITAM-K product, PIP_2, becomes available for PH-domain-containing proteins, such as Btk, to bind. Following BCR engagement, Itk becomes dissociated from Btk, via an unknown mechanism. Btk binds to PIP_3 and Grb_2 subunits through its PH domain. Concomitantly Igα and Igβ ITAM phosphorylation leads to the recruitment of the Syk kinase to the Ig chains via the Syk SH2 domains. BLNK/SLP-65 is recruited to a phosphorylated tyrosine on Igα that is distinct from the ITAM motif. Syk proceeds to phosphorylate BLNK/SLP-65, thereby providing binding sites for Btk and PLC-γ2. Upon the binding of Btk to BLNK/SLP-65 through its SH2 domain, Btk is phosphorylated by a Src kinase. Subsequently, Btk autophosphorylates and carries out its functions by phosphorylating PLC-γ2 at four tyrosine residues, which results in the complete activation of PLC-γ2. References for the novel aspects of this activation scheme are provided in the text.

These data fit quite well with earlier studies demonstrating that the PH domain of Itk is dispensable for the Src-kinase-induced phosphorylation and activation of Itk [23,27]. Both the PH and TH domains are also crucial for the recognition of specific substrates that can, in turn, bind and activate kinase activity. Data from Lowery et al. have demonstrated that the translocation of Btk to the membrane is not solely dependent on ITAK, but is also dependent on, and enhanced by, heterotrimeric G protein subunits. The direct binding of the Grb_2 subunits to the PH–TH module, as well as to the catalytic domain of Btk, was shown to directly activate kinase activity [28,29]. This cooperative mechanism may facilitate the interaction of Btk with SLP-65/BLNK, an important adaptor molecule that associates with Igα and is required for BCR signaling. The interaction of the Btk SH2 domain with BLNK has been shown to be required for phosphorylation and maximal activation of Btk kinase activity and, further, for the subsequent activation of PLC-γ2 [30–32,33,34].

Interestingly, Rlk, an additional Tec family kinase whose precise function remains unclear, lacks the amino-terminal PH domain common to the other Tec kinases. Instead, Rlk possesses a palmitoylated cysteine-string motif, suggesting a unique mechanism of regulation. Whereas both Itk and Rlk are phosphorylated and recruited to lipid rafts in response to TCR-induced Src kinase activity, the activation and membrane recruitment of Rlk is independent of ITAK activity [34,35]. Remarkably, the absence of Rlk from CD45 T cells does not appear to significantly diminish cell function; however, signaling defects observed in the absence of Itk are intensified in cells that lack both Itk and Rlk [34,36,37,38,39].

**Downstream effects of Tec kinase activation**

Many biochemical and genetic studies of Btk and Itk have established that these kinases are important for the phosphorylation and activation of PLC-γ2 in B and T cells, respectively, which in turn affect calcium mobilization, extracellular related kinase (ERK) and c-jun amino-terminal kinase (JNK) activation, and ultimately effector responses (for excellent reviews of previous work characterizing these findings, see [40,41]). One recent study has provided interesting evidence that CD28 signaling enhances the ability of Itk to activate PLC-γ1 in T cells [40]. In studies of BCR signaling, recent biochemical data have also identified the four Btk-dependent tyrosine phosphorylation sites in PLC-γ2. Furthermore, each of these sites is required for full BCR-coupled calcium mobilization [41,42].

New reports this past year have also connected the Tec-kinase-mediated activation of PLC-γ2 to the ultimate activation of the NF-κB and NFAT transcription factors [31,32]. Yamamoto et al. found that in transfected 293T cells, a non-lymphoid cell line, NF-κB and NFAT activation result from the cooperative interactions of Tec and Syk family members with the adaptor molecules SLP-76 and BLNK that play an important role in PLC-γ2 phosphorylation [43].

A second pathway involving Tec kinases has been identified downstream of antigen-receptor signaling, namely the cytoskeletal reorganization that occurs following TCR or...
BCR stimulation. Data from Woods et al. demonstrated that after anti-CD3 antibody stimulation of Jurkat T cells, Itk can activate β1 integrins and β-actin polymerization following its membrane translocation [44]. Previous data have also supported a role for Itk in cytoskeletal reorganization - Itk-deficient cells possess reduced cap formation following TCR stimulation (reviewed in [45]). Consistent with these data, previous studies on Btk have demonstrated that the PH domain of Btk binds filamentous actin and can regulate cytoskeletal reorganization [46,47], further supporting a role for Tec kinases in this pathway.

These findings are in contrast to those of Donnadieu et al., who investigated the roles of Lck and Itk in the different pathways downstream of the TCR [48]. These investigators found that whereas both kinases play an important role in initiating antigen-induced calcium responses, Lck, but not Itk, is required for a stable T-cell-APC (antigen-presenting cell) interaction and polarization of the T cell. Interestingly, Lck was found to be crucial for many intracellular signaling events, such as adhesion, cytoskeletal reorganization and calcium mobilization; however, Itk was discovered to augment calcium responses but was expendable for the other events studied [48].

Unlike the T cell studies mentioned above, which were performed on primary T cells from ltk-deficient mice or on the Jurkat T cell tumor line, the experiments of Donnadieu et al. were performed on a murine T cell hybridoma cell line [48]. Thus differences in the cells used may account for the discrepancies observed. Alternatively, as both the Jurkat and murine hybridoma experiments were performed using transfection of kinase-inactive Itk constructs to act as dominant-negative inhibitors of endogenous Itk in these cells, differences in the levels of inhibition achieved in these two systems may also contribute to the different outcomes observed.

In additional studies on non-lymphoid cells, Btk was found to associate with the actin-based cytoskeleton in activated (human) platelets [49]. Furthermore, Btk is substrate of calpain (calcium-dependent thiol protease — an important regulator of signaling events in platelets) in human platelets [50]. Although the physiological relevance of this latter finding is not clear, these data provide important insights into the function of Tec family members in other immune cells, such as platelets.

Although the discovery that Rlk translocates to the nucleus upon TCR stimulation [51] supports a potential role for Rlk in gene regulation, its targets and function within the nucleus are still unresolved (see Update). Tec family members such as Btk and Bmx have been previously shown to interact with transcription factors (reviewed in [6*]), suggesting that
these proteins may play more direct roles in affecting gene transcription. Btk, which is mainly localized to the cytoplasm in resting cells, localizes to the cell membrane following BCR or growth factor stimulation. In addition, a recent study has found that Btk can undergo nucleocytoplasmic shuttling in a nuclear export signal (NES)-dependent manner [52]. The role of Btk in the nucleus was clarified by the findings of Egloff et al., who showed that Btk could associate with and phosphorylate a transcriptional regulator, BAP/TFTI-I, in B cells following BCR engagement [53]. This finding supports a novel role for Tec family kinases as direct modulators of gene expression. Likewise, Btk was also found to associate with another transcription factor, STAT5A, following the engagement of the BCR [54].

This newly proposed function for Tec kinases in gene regulation is not restricted to Btk. Itk was also found to translocate to the nucleus in Jurkat cells via its association with a nuclear importin Rcl/X-karyopherin. This interaction is dependent on the Itk SH3 domain as well as a PRR motif in Rcl/X [55]. Future experiments will be required to elucidate the precise role(s) of Tec family kinases in the nucleus and to determine if they do indeed modulate gene expression.

**Negative regulatory mechanisms of Tec kinases**

Data from a number of laboratories have established that two events are required for the proper activation and function of Tec kinases: localization to the membrane and tyrosine phosphorylation. However, until recently, little was known about the mechanisms involved in downregulating Tec kinase activity. Several recent reports have identified proteins that inhibit the activity of Tec family kinases. For instance, IBtk (inhibitor of Btk) was found to bind to the PH domain of Btk and interfere with Btk-dependent calcium mobilization and NF-κB activation in B cells [56].

A more complex feedback loop regulating Tec kinase activity has been suggested by the studies of Kang et al. These investigators have presented evidence indicating that PKCβ, a classical PKC isofrom, phosphorylates a serine residue in the Btk TH domain thereby decreasing Btk membrane localization [57] (Figure 3). As PKC activation is one of the downstream consequences of PLC-γ activation, and thus is dependent on Btk activity, this mechanism provides an autoinhibitory loop that would function to downregulate Btk activity and thus terminate the signaling pathway. Interestingly, this region of the TH domain is conserved among all Tec kinases, suggesting that this mode of regulation may be in effect for the TCR signaling pathway as well.

More recently, biochemical and structural studies of Itk have demonstrated that Itk kinase activity is inhibited by a direct interaction between the Itk SH2 domain and the peptidyl-prolyl isomerase, cyclophilin A (CyA) [58]. Consistent with this, treatment of Jurkat T cells with Cyclosporin A, an immunosuppressive drug that binds CyA and inhibits calcineurin activity, disrupts this interaction and results in increased Itk phosphorylation and activation of downstream targets (e.g., PLC-γ1). Using NMR (nuclear magnetic resonance) structural analysis, this study also showed that the Itk SH2 domain can undergo a proline-dependent conformational switch via a single prolyl imide bond. Furthermore, this cis/trans isomerization within Itk, which is catalyzed by CyA, directly alters Itk binding specificity for phosphotyrosine-containing partners [59].

**Functional roles of Tec kinases in lymphocytes**

Significant attention over the past year has been focused on biochemical and functional defects that occur in the absence of Tec family members. These defects affect many complex biological processes, such as T and B cell development, activation, differentiation and effector function. For instance, the XLA syndrome is manifested by severe defects in early B cell development, resulting in a nearly complete absence of peripheral B cells and immunoglobulins [54]. A milder deficiency is observed in Btk−/− or xid mice, where the block in B cell development occurs at the later immature→mature B cell transition, resulting in the accumulation of substantial numbers of functionally impaired peripheral B cells in these mutant mice [60]. A recent report from Ellmeier and colleagues provided one potential explanation for the differences observed between mice and humans lacking Btk. These investigators generated Tec−/−Btk−/− mice, and showed that these doubly deficient mice have a much more profound block in B cell development than the Btk−/− mice [61]. These findings suggest that, in mice, Tec may partially compensate for the lack of Btk.

Additional studies of the role of Btk in B cell development have provided more-detailed information about the precise role of Btk in the regulation of the pro-B→pre-B cell transition that occurs in the bone marrow and in the later stages of B cell maturation in the periphery [62]. For recent, recent data indicate that Btk plays an important role in the regulation of the A locus for VDJ recombination in pre-B cells and in the T2→T3 transition with the pool of immature B cells. An additional study focusing on peripheral B cells showed that, following the export of immature B cells to the periphery, Btk plays a significant role in the immature→mature B cell transition occurring in the spleen.

Whereas the generation of Rlk−/− mice has revealed a seemingly insignificant role for Rlk in the development and function of immune cells, the generation of Rlk−/−Itk−/− double-knockout mice has enhanced our understanding of the function(s) of Tec kinases in T cells [37]. Upon comparison
of TCR-induced signaling events in Tec family knockouts, it is evident that there are graded defects where the most severe defects are observed in the Rlk-/-Itk-/- double knockouts, modest defects in Itk-/- and minimal defects in Rlk-/- alone. Furthermore, combined studies of Itk-/- and Rlk-/-Itk-/- mice have indicated an important role for Tec kinase mediated signaling pathways in positive and negative selection the thymus ([36]; JA Lucas, L Atherley, LJ Berg, unpublished data). In addition, both Itk-/- and Rlk-/-Itk-/- thymi show altered CD4:CD8 ratios, due to fewer mature CD4+ thymocytes and increased numbers of CD8+ thymocytes.

These defects in thymic selection correlate with reduced TCR signaling, resulting in reduced calcium mobilization and reduced ERK-MAPK (mitogen-activated protein kinase) activation in Itk-/- and Rlk-/-Itk-/- thymocytes [36]. Interestingly, in spite of the reduced ERK-MAPK signaling and the altered CD4:CD8 ratio, detailed analysis of several TCR transgenic lines on an Itk-/- background did not reveal any alterations in CD4:CD8 lineage commitment in these mice (JA Lucas, L Atherley, LJ Berg, unpublished data). Instead, the data suggest that, in the absence of Itk and/or Rlk, peripheral T cell homeostasis may be altered, leading to peripheral expansion of CD8+ T cells and their recirculation back into the thymus (JA Lucas, L Atherley, LJ Berg, unpublished data). These findings suggest a potential role for Tec kinases in regulating peripheral T cell homeostasis.

Studies of Itk-/- and Rlk-/-Itk-/- mice have also revealed additional roles for Tec kinases in regulating T cell effector functions. For instance, after TCR stimulation in vitro, Rlk-/-Itk-/- CD4+ cells are defective in their ability to secrete both Th1- and Th2-type cytokines, a finding that correlates with reduced activation of NFAT and AP-1 transcription factors [38*]. This report also showed that Itk-/- mice are unable to mount an effective Th2 response after infection with *Schistosoma mansoni*; remarkably, though, the compound deficiency in both Itk and Rlk restored the ability of the mutant mice to respond to this pathogen and produce Th2 effector cytokines [38*].

Additional studies have demonstrated that Itk-/- CD4+ T cells show a defect in activation-induced cell death (AICD) both in vivo and in vitro [66*]. This defect results from impaired apoptosis via the Fas–FasL (Fas ligand) pathway because of a substantial reduction in FasL expression by activated Itk-/- CD4+ T cells. The mechanism underlying this FasL deficiency appears to be the failure of Itk-/- T cells to efficiently upregulate Eg2, Eg3 and, consequently, FasL transcription after TCR stimulation [66*]. Together, these data demonstrate that the absence of Tec family kinases affects not only T cell activation and differentiation pathways, but also apoptosis effector pathways.

**Conclusions**

The past year has provided important new insights into the biology of Tec kinases. A number of studies, focusing on the regulation of Tec kinase enzymatic activity, have demonstrated intramolecular and intermolecular domain interactions that are likely to play a crucial role in regulating this process. Importantly, these studies have indicated that the detailed mechanisms regulating Tec kinase activity are clearly distinct from those described for the better-characterized Src family of tyrosine kinases. In addition, these data have shown that individual Tec family members have unique modes of regulation. Additional studies have also refined our understanding of the interactions between Tec kinases and other signal transduction molecules, and have provided compelling evidence that Tec kinases directly phosphorylate and activate PLC-γ in lymphocytes. Finally, genetic studies using mice and/or cell lines lacking individual or multiple Tec kinases have begun to elucidate the functional roles of these kinases in lymphocyte biology. Overall, these studies have highlighted the complex interactions between cellular activation, effector functions, homeostasis and apoptotic pathways.

**Update**

Recent progress has provided additional insights into Tec kinase functions within immune cells. As mentioned earlier, Rlk can translocate to the nucleus upon TCR stimulation; however, its targets within the nucleus had not previously been defined. A recent paper from Takeba et al. demonstrated that Rlk can bind to regions of the IFN-γ promoter and increase promoter activity [67]. Interestingly, this event is dependent on Rlk phosphorylation, presumably in response to TCR engagement.

Lastly, we discussed earlier that many Tec family kinases, including Btk, are dependent on a (PI3-K)-mediated increase in membrane PIP3 levels for recruitment to the membrane, activation and function. A recent study performed by Fruman et al. using DNA microarray technology identified the target genes that are shared by Btk- and PI3-K-dependent signaling pathways following BCR engagement [68]. This study showed that whereas PI3-K and Btk share many genes, as expected, PI3-K activity can affect other genes independent of Btk function.

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**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest


This is a great, comprehensive review of the genetics and biology of all Tec family members throughout mammalian and non-mammalian systems. This review also offers insight into the evolution of Tec kinases.


This review focuses primarily on the role of Itk, Btk, and the proteins they interact with in the signalling cascade. Furthermore, this review also sheds light on the downstream effects of PLC-g activation and the role of Tec kinases in other signalling pathways.


See annotation in [18].


This paper and [14] demonstrate how, for Btk, the intramolecular PRR and SH3 domain interactions function to stabilize the inactive kinase.


This paper is the first to demonstrate that a Tec family member can form both symmetrical and asymmetrical homo-dimers.


This paper describes a novel intramolecular interaction between the SH2 and SH3 domains of Itk, which may be required for full enzyme activation. This finding suggests that this intramolecular interaction may function to displace the Intramolecular interaction of the PRR with the SH3 binding pocket that predominates in the 'inactive state'.


This paper is the first to describe a role for the G protein zy subunits in the activation of Btk.


This paper demonstrates that the BCR signaling subunit, the transmembrane protein Igk, can function as an adapter molecule, reminiscent of LAT, by providing an anchor site for SL-p1/BLNK.


This paper highlights the importance of the Tc kinases Itk and Rlk in the differentiation of CD4+ T cells into the effector Th1 and Th2 subsets. Moreover, this paper provides mechanistic insights into the role of these kinases in the regulation of multiple downstream transcription factors crucial for T helper cell differentiation.


See annotation to [42].


This paper and [41] both define the Btk-dependent phosphorylation sites in PLCγ2 that are required for BCR-coupled calcium release.


See annotation to [55].


See annotation to [55].


See annotation to [55].


This paper and [52]-[54] all offer insights into the currently uncharacterized role for Tec kinases within the nucleus. Together, these studies suggest that Tec kinases may either function as direct modulators of gene expression or may affect other nuclear processes via nucleo-cyttoplasmic shuttling.


See annotation to [58].


See annotation to [58].


This paper and [56],[57] provide important insights into the mechanisms by which Tec family kinases are either inhibited or downregulated following receptor activation.


See annotation to [56].


See annotation to [56].


See annotation to [56].


This paper and [52]-[64] all contribute to a more detailed understanding of the role of Btk in B cell development.


This paper demonstrates a role for Itk in activation-induced cell death in T cells both in vivo and in vitro.


The role of Tec family kinases in T cell development and function

Summary: Three members of the Tec family kinases, Itk, Rlk and Tec, have been implicated in signaling downstream of the T cell receptor (TCR). The activity of these kinases in T cells has been shown to be important for the full activation of phospholipase C-γ1 (PLC-γ1). Disruption of Tec family signaling in Itk−/− and Rlk−/−Itk−/− mice has multiple effects on T cell development, cytokine production and T-helper cell differentiation. Furthermore, mice possessing mutations in signaling molecules upstream of PLC-γ1, such as Src homology 2 (SH2) domain-containing phosphoprotein of 76 kDa (SLP-76), linker for activation of T cells (LAT) and Vav1, or in members of the nuclear factor for activated T cells (NFAT) family of transcription factors, which are downstream of PLC-γ1, have been found to have similar phenotypes to Tec family-deficient mice, emphasizing the importance of this pathway in regulating T cell activation, differentiation and homeostasis.

Introduction

Six members of the Tec family of nonreceptor 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)-triophosphatate (PIP3). 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
within the kinase domain, appears to be necessary. Prior to Rlk activation (reviewed in 1-4).

Lucas et al. Tec kinases in T cell development and function

This form of the protein can be translocated to the nucleus of T cells following activation (reviewed in 1-4).

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 (5, 6) and is dependent on the activation of phosphoinositide 3-kinase (PI3K), which converts phosphatidylinositol (4,5)-biphosphate (PIP2) into PIP3, thereby generating the ligand for the Itk and Tec PH domains. 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 (7). Following membrane localization, the activation of Tec family kinases in T cells requires phosphorylation by an Src kinase, possibly Lck, of a conserved tyrosine within the activation loop of the kinase domain (8). 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) (9). 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 (5, 9).

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) (10, 11), which does bind LAT via its interaction with Gads (12-15) in a ZAP-70-dependent manner (16). Once the Tec family members have been activated and are bound to LAT via SLP-76, they are capable of phosphorylating phospholipase C-γ (PLC-γ) (11). This phosphorylation leads to the activation of PLC-γ1, which then hydrolyzes PIP2 into the second messengers inositol (3,4,5)-triphosphate (IP3) and diacylglycerol (DAG). The production of IP3 causes calcium mobilization in the T cells, while DAG activates protein kinase C (PKC) and Ras-GRF, thereby leading to the activation of Ras/Raf/mitogen-activated protein kinase (MAPK) pathways. Subsequent to Ca2+ 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 Rrk1/2, p38 and c-jun N-terminal kinase (JNK). These pathways culminate in the activation of additional transcription factors, such as NF-κB and Rlk-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.

Single knockouts of the genes encoding Itk, Rlk and Tec have all been generated in mice and characterized (17-20). 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 Rlk−/−/Itk−/− 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 (19, 21). Because of the close association of the Tec kinase deficiencies with impaired PLC-γ1 activation, this review focuses on the in vivo consequences of reduced PLC-γ1 activation on T cell development and function. We also compare the phenotypes of the Tec family knockouts to other mouse mutants with deficiencies in PLC-γ1-dependent signaling pathways.

The role of Tec family kinases in T cells development

The development of mature T cells from bone marrow precursors takes place in the thymus and is critically dependent on both the environment and signals through the pre- and mature TCRs. Thus making proteins that are important for TCR signaling play critical roles in T cells development. For instance, disruption of the gene encoding the protein tyrosine kinase Lck (22) or ZAP-70 (23, 24) results in severe defects in T cell development. For instance, disruption of the gene encoding the protein tyrosine kinase Lck (22) or ZAP-70 (23, 24) results in severe defects in T cell
Are Tec kinases important for pre-TCR signaling?  

The most immature T cells precursors in the thymus lack the expression of the T cells coreceptors 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-DN4, based on the expression of CD44 and CD25. At the DN3 (CD44+CD25+) stage of development, cells are undergoing rearrangement of their T cell receptor βchain genes. At this stage of development, the cells progressing down the TCR αβ lineage are dependent on signals transduced through the pre-TCR, a receptor comprising a functionally rearranged TCR βchain paired with an invariant pre-TCR ζchain. 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. 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 (29).

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 (30) and Rlk (31) mRNA can be detected by day 14 of fetal development, a time at which the thymus comprises solely DN1 and DN2 precursor cells. More recently, work in our laboratory has demonstrated the expression of transcripts for Itk, Rlk and Tec at all four adult DN stages by real-time quantitative PCR (Lucas, Felices and Berg, unpublished data). Despite these data, analysis of adult and fetal thymic development as assessed by CD4 and CD8 expression reveals that Itk+/−, Rlk−/− and Rlk+/−Itk−/− mice all have relatively normal numbers of both DN and DP thymocytes, suggesting that progression to the DP stage of development is unaffected by the absence of these Tec family proteins (17, 21). Closer inspection of the DN subsets in Itk−/− mice based on CD44 and CD25 expression is also 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 (Lucas, Atherly and Berg, unpublished data).

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 bone marrow chimeras with a 50:50 mix of wild-type and Itk−/− bone marrow. In our preliminary analysis of thymi from these mixed wild-type/Itk−/− chimeras, we have observed equivalent numbers of wild-type and Itk−/− cells at the DN1-DN3 stages of development. However, we find that there are significantly fewer DN4 cells of Itk−/− origin. This finding indicates that when Itk−/− cells develop in the presence of Itk-positive cells, they are at a disadvantage due to intrinsic defects in pre-TCR signaling. In addition, BrdU labeling suggests that the Itk−/− cells take longer to progress from the DN to the DP stage of development or that fewer of the Itk−/− cells survive this transition (Lucas and Berg, unpublished data). Further analysis of these chimeras as well as those generated with Rlk−/−Itk−/− bone marrow will be necessary to confirm these initial observations and to provide more definitive data addressing the role of Itk and/or Rlk in pre-TCR signaling.

Tec family kinases are important for positive and negative selection  

Once developing thymocytes progress through the pre-TCR checkpoint, they commence rearrangement of the TCR α-chain genes and initiate expression of both the CD4 and CD8 coreceptor genes. At this point in development, the cells undergo a process known as repertoire selection. One component of this process, referred to as positive selection, leads to the selective survival and differentiation of DP thymocytes whose TCRs are able to bind self-peptide/major histocompatibility complex (MHC) complexes present on thymic stromal cells. In contrast, cells that do not receive this selection signal due to the inability of their TCRs to bind self-peptide/self-MHC complexes do not undergo further differentiation, and eventually these cells die. Although it is necessary that thymocytes recognize self-peptide/MHC complexes to develop into functional mature T cells, it is also critical that the selected cells do not become activated by interaction with these same complexes once they are part of the peripheral circulating T cell pool. Therefore, to ensure self-tolerance, thymocytes whose
TCRs have strong interactions with self-peptide/MHC complexes undergo negative selection. Although this process can occur at any point after successful rearrangement of the TCR α chain, negative selection most often occurs late in the DP stage of development, with strong signals through the TCR leading to the induction of apoptosis.

The role of Tec family signaling during repertoire selection has mainly been studied using TCR transgenic model systems. However, the first suggestion that Tec family members may be important for thymocyte selection came from analysis of Itk−/− mice generated by Liao and Littman (17). Analysis of the T cell subsets in these mice based on CD4 and CD8 expression indicated a reduced CD4:CD8 ratio in the thymus and the periphery, together with a twofold reduction in absolute numbers of peripheral CD4+ T cells. Although these data initially suggested that there was a preferential defect in CD4 T cell development, analysis of itk−/− mice crossed to either an MHC class I (HY) (32) or an MHC class II (AND− H2b) (33) specific TCR transgenic line revealed that the absence of Itk affected both CD4 and CD8 T cell development. In both cases, very few cells that expressed the transgenic TCR developed in mice that lacked Itk, in contrast to the significant number of transgenic TCR+ cells that develop on a wild-type background.

Schaeffer et al. (21) extended these studies by comparing Rlk−/−, itk−/− and Rlk−/−Itk−/− mice. They found that Rlk−/− mice were almost indistinguishable from wild-type mice, whereas the Rlk−/−Itk−/− mice were similar to Itk−/− mice, in that they showed an altered CD4:CD8 ratio in the thymus and somewhat reduced numbers of peripheral CD4+ cells. However, the total thymocyte number was slightly increased in Rlk−/−Itk−/− mice compared to itk−/− mice. Despite this increase, when the Rlk−/−Itk−/− mice were crossed to the same TCR transgenics used in the Itk−/− analyses, positive selection of the doubly deficient transgenic T cells was more severely impaired than was selection of the single Itk−/− thymocytes.

In our laboratory we have examined Itk−/− mice that are transgenic for a variety of other TCRs (28). Our rationale for examining additional TCRs was that the selection of both and (H2b) and HY TCR transgenic cells, used in the previous studies, is inefficient compared to that seen in other TCR transgenic lines. This inefficient selection has been used to suggest that these TCRs have a low avidity for their selecting ligands in the thymus. In the light of data showing that the absence of Itk affects T cell activation and cytokine secretion to a greater extent when the TCR signals are suboptimal (Wilcox and Berg, unpublished data), we were interested in determining whether the development of TCR transgenic thymocytes deficient in Itk would vary based on TCR avidity. Therefore, we compared the positive selection of 2B4 (34), SC.C7 (35) and AND (33) (H2b) TCR transgenic cells that lacked Itk to their wild-type counterparts. These TCRs are all specific for a pigeon/moth cytochrome c peptide bound to IEd, and the proposed avidity hierarchy of these TCRs for positively selecting ligands in the thymus is 2B4 < SC.C7 < AND (36). In the 2B4 Itk−/− and SC.C7 Itk−/− mice, the development of TCR transgenic cells was significantly reduced compared to that seen in Itk-positive mice, in agreement with the results seen in previous studies examining AND (H2b) Itk−/− mice. However, despite this reduction, a considerable number of TCR transgenic cells in SC.C7 Itk−/− mice successfully underwent positive selection. Finally, in AND (H2b) Itk−/− mice, the number of transgenic CD4SP thymocytes and peripheral CD4+ T cells was comparable to the AND (H2b) Itk+/+ mice, although the efficiency of positive selection, as assessed by the DP:CD4SP ratio in the thymus, was still slightly reduced in the absence of Itk. Thus, positive selection of all class II MHC-specific TCRs is impaired in Itk−/− mice, although the severity of the defect is minimized as the strength of the TCR signal increases.

Although we initially focused on the development of class II-restricted T cells, we have also crossed the Itk−/− mice to mice transgenic for the OT-1 TCR transgenic mice (37). The OT-1 TCR is class I restricted and is thought to have a high avidity for its selecting ligand(s) in the thymus. Unlike the HY Itk−/− mice (17, 21), OT-1 Itk−/− mice develop a significant number of CD8 SP and peripheral cells that express high levels of the transgenic TCR. Similar to the AND (H2b) Itk−/− mice, the difference between OT-1 mice that express Itk and those that do not is less dramatic than that seen in previous studies; however, the efficiency of the selection of these cells is still reduced (Lucas and Berg, unpublished data). Thus, the conclusions stated above appear to hold true for MHC class I-specific TCRs as well, in that TCRs with higher avidity for selecting ligands in the thymus are less impaired in their positive selection in the absence of Itk. Together, these findings indicate that, in the absence of full PLC-γ1 activity, fewer thymocytes of any specificity are able to maintain the continuous low level of signaling that is required to complete positive selection. It is important to note, however, that to date all of the analyses of TCR transgenic mice deficient in Tec family kinases have been performed with mice that are RAG competent; thus, these data may be influenced by the expression of endogenous TCR α chains on the transgenic thymocytes. In this regard, the initial characterization of SC.C7 RAG−/−Itk−/− mice...
in our laboratory suggests that the impaired maturation of transgenic thymocytes in the absence of Itk, at least in this line of mice, may be more dramatic than was observed previously in RAG+/- mice (Miller and Berg, unpublished data). This finding implies that some of the SC.C7+ T cells that were seen in the initial studies may have survived selection by coexpressing an endogenous TCR alpha chain and were not selected on the basis of the SC.C7 TCR specificity.

Another approach used to assess the role of Tec family-dependent signaling pathways during T cell development has been to generate transgenic mice that overexpress Rlk in thymocytes and T cells (38). Based on CD4 vs. CD8 profiles of the thymus and lymph nodes in these mice, T cell development appeared unaltered. However, when the Rlk transgene was crossed to the AND (H2b) and HY TCR transgenic mice, Sommers and colleagues (38) observed that the number of TCR transgenic cells that developed was reduced compared to non-Rlk transgenic controls. Furthermore, the Rlk transgene was able to restore nearly normal levels of positive selection in HY Itk-/- and AND Itk-/- TCR transgenic lines. These data confirm that Itk and Rlk have at least partially redundant roles in signaling at the DP stage of development in agreement with the analysis of Rlk-/-Itk-/- mice. Finally, the reduced level of T cell maturation observed in Itk-deficient Rlk transgenic mice is interpreted as a shift toward negative selection (discussed later), rather than as an inhibition of positive selection.

A number of the TCR transgenic Itk- and Rlk/Itk-deficient mice that were used to study the role of Tec kinases in positive selection have also been used to study the role of these proteins in negative selection. Initial studies by Liao and Littman (17) and Schaeffer et al. (21) examined this issue using HY TCR transgenic male mice (39), in which the cognate antigen for the male-specific HY TCR is constitutively present. Once again, the most dramatic deficit was observed in mice that lacked both Itk and Rlk (21), while a more modest effect was seen in single Itk-/- mice. Specifically, the HY Rlk-/-Itk-/- mice had a significant increase in the percentage and number of DP and CD8 SP thymocytes and CD8+ peripheral T cells that expressed the HY TCR. Notably, these HY+ Rlk-/-Itk-/- T cells express significantly higher levels of the CD8 coreceptor than is seen on the HY+ cells that escape deletion in wild-type HY transgenic male mice.

In our laboratory we have 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, SC.C7 or AND; all Vp3+) along with an endogenous superantigen (SAg) that specifically deletes thymocytes whose TCRs use Vp3. 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 Vp region, the efficiency of SAg-mediated deletion in the different lines varied, even among Itk-sufficient mice (Fig. 1A). 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 SC.C7 and AND TCR transgenic lines seemed to occur later in development, as these mice still retained a significant proportion of DP thymocytes (Fig. 1A) and had smaller decreases in total thymic cellularity. When Itk was absent from either the SC.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 (Lucas and Berg, unpublished data). 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 (40), which does not seem to happen in AND Itk-/- mice (28). 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.

The second approach we used to examine negative selection in Itk-/- mice used 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 (41). These mice have low basal levels of expression of the fusion protein in the thymus, which can be increased when the mice are given Zn2+ to induce the metallothionein promoter. When the HEL-cyt mice are crossed to the SC.C7 TCR transgenic line, the majority of SC.C7+ T cells are deleted; however, due to
Lucas et al. - Tec kinases in T cell development and function

A.

![Bar graph showing the number of DP thymocytes in different genotypes and treatments.](image)

B.

![Scatter plots showing CD4 vs. CD8 profiles of thymocytes.](image)

Fig. 1. Negative selection in Itk-deficient mice. (A) The average number of DP thymocytes in 2B4, SC.C7 and AND (H2b) 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. (B) CD4 vs. CD8 profiles of thymocytes from SC.C7 Itk(+/+) and Itk(-/-) mice with and without HEL-cyt transgene. Thymocytes were stained with antibodies to CD4 and CD8 and analyzed by flow cytometry.

Despite the reduced degree of positive and negative 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 (21) stimulated thymocytes from wild-type and Rlk−/−ltk−/− mice with anti-CD3 antibodies and found that phosphorylation of PLC-γ1 was more severely impaired in the Rlk−/−ltk−/− thymocytes. This change in PLC-γ1 phosphorylation affects downstream pathways, namely Ca2+ mobilization and the Ras/MAPK pathway. For instance, in both Itk−/− and Rlk−/−ltk−/− thymocytes, defects in sustained Ca2+ elevation, but not in the initial Ca2+ spike, were observed upon TCR stimulation (21). 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 Rlk−/−ltk−/− thymocytes. These studies showed that there was no change in p38 activation, but reduced ERK activation was found in these cells (21).

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 (21). Current models propose 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 Rlk−/−ltk−/− 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 Rlk−/−ltk−/− 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 (H2b), 2B4 and HY 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 HY male mice that lack Itk or both Itk and Rlk, the number of CD8+ HY+ cells in the periphery is increased (21). In contrast, the 5C.C7/HEL-cyt Itk−/− mice had reduced numbers of peripheral CD4+ 5C.C7+ cells. Thus, in one system, the absence of Tec family kinases results in a shift from negative selection to positive selection, but in the other system 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 (21), which have been shown to be crucial for positive selection, while there is no effect on the activity of the p38 MAPK (21), 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 (43). Furthermore, maintenance of at least some TCR signals is dependent on sustained Ca2+ elevation, which is impaired in cells deficient in Tec family kinases due to reduced PLC-γ1 activity. Thus, Tec family kinases and PLC-γ1 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 Rlk−/−ltk−/− mice is reduced by 50%, while there are almost normal numbers of CD8+ cells. Therefore, the changes in negative selection in 5C.C7/HEL-cyt Itk−/− mice might be masked due to a more substantial defect in CD4+ T cell maturation or survival.

CD4/CD8 lineage commitment is unaffected by the loss of Tec family kinases

Concurrent with the processes of positive and negative selection is a third developmental process, referred to as CD4/CD8 lineage commitment. This process ensures that DP thymocytes that express MHC class II-specific TCRs develop into CD4+ SP cells, and those that express MHC class I-specific TCRs develop in CD8+ SP cells. The signals that determine the lineage choice of a given thymocyte are not completely resolved, but there is evidence that the levels of Lck activity (44, 45) as well as ERK-1/2 activity (46, 47) play a role, with high activity of both enzymes leading to CD4 SP development and low activity leading to CD8 SP development. These data suggested a possible role for Itk in this process, as Itk is directly activated by...
Thus, we were interested in determining whether addition, there are significantly more CDS periphery of Itk, Lck (S) and in turn plays a role in activating ERK-1/2 (21). In addition, there are significantly more CD8+ SP cells in the thymus and lower CD4/CD8 ratios in both the thymus and periphery of Itk−/− and Rlk−/−Itk−/− mice (17−19, 21, 28). Thus, we were interested in determining whether CD4/CD8 lineage commitment would be altered in Itk−/− mice or, more specifically, whether MHC class II-specific T cells would undergo aberrant selection into CD8+ cells in the absence of Itk. We used the Itk-deficient MHC class II-specific TCR transgenic lines that we generated to assess positive selection. In all five of the MHC class II-specific TCR transgenic systems we examined, we observed no increase in the number of TCR transgenic CD8+ SP thymocytes or CD8+ peripheral T cells, suggesting that the signals required for appropriate lineage commitment do not require intact Itk signaling (28).

Tec family kinases are important for peripheral T cell homeostasis and function

Many recent studies have implicated tonic low-level TCR signaling in the maintenance of peripheral T cell pools (48–53). Together with cytokine signals, these TCR signals are important for T cell survival, and they may also provide an enhanced level of reactivity to subsequent mitogenic TCR signals (48). Because of the role of Tec kinases in PLC-γ1 activation downstream of the TCR, it seemed likely that peripheral T cell homeostasis as well as bna file T cell activation would be impaired in Tec kinase-deficient mice.

Altered homeostasis in Tec family-deficient mice

The analysis of T cell subsets in Itk−/− mice has consistently revealed an increase in the proportions of both CD4+ and CD8+ T cells that have an activated or memory phenotype (CD4hi) when compared to cells from control littermates (Fig. 2). Although the exact proportion of these populations varies, especially with age, there is generally a two- to three-fold increase in the CD4hi subset among T cells from Itk−/− mice. For CD4+ CD44hi cells, this increase is observed in both nontransgenic mice as well as in Itk-deficient TCR transgenic RAG+ mice, but the increase is not seen in Itk-deficient TCR transgenic RAG− mice. Therefore, it seems likely that these cells arise following antigen-specific activation and persist to a greater extent in Itk−/− mice than in wild-type mice. One potential explanation for the persistence of these cells is that reduced numbers of CD4+ T cells in Itk−/− mice cause an increase in available ‘space’ or environmental factors that are necessary for CD4+ T cell survival and homeostasis. Another possible, but not mutually exclusive, explanation is suggested by the observations of Miller and Berg (54), which demonstrated a defect in activation-induced cell death (AICD) in Itk−/− CD4+ T cells. This reduction in AICD is due, at least in part, to a decrease in Fasl expression following activation of Itk−/− CD4+ T cells, a consequence of impaired NFAT activation and impaired upregulation of the Egr2/3 transcription factors. Thus, cells that would normally be eliminated in wild-type mice do not undergo apoptosis in the absence of Itk.

Within the peripheral CD8+ T cell compartment of Itk−/− mice, the percentage of CD44hi cells ranges from about 60−90% of the total pool. The prevalence of CD8+ CD44hi T cells in the periphery, along with the discovery that the increased numbers of CD8+ SP cells in the thymus were also CD44hi (28), at first suggested that the CD8+ CD44hi cells in Itk−/− mice may arise due to abnormal CD8+ T cell development. However, CD8+ CD44hi cells do not develop in fetal thymic organ culture of Itk−/− thymi and are absent from the thymus and periphery of Itk-deficient class I-restricted OT-1 TCR transgenic mice (Lucas, Atherly and Berg, unpublished data). Therefore, we favor the explanation that peripheral CD8+ T cell homeostasis is altered in Itk−/− mice. The precise events or mechanisms that cause this disruption in homeostasis and allow recirculation of
the CD8⁺ CD44hi cells back to the thymus in Itk-deficient mice are currently under investigation.

**CD8 effector function**

CD8⁺ T cells are at the forefront of the immune response to viral and some bacterial infections. During the course of infection, these cells differentiate into activated killer cytotoxic T lymphocytes (CTLs), whose main functions are the lysis of infected cells and the secretion of antiviral effector cytokines. There are two distinct mechanisms by which CD8 CTLs kill: perforin/granzyme exocytosis and Fas/FasL-mediated cytolysis (55). Both of these functions are dependent on activation of the CD8⁺ T cells through the TCR, leading to the generation of a sustained elevation in cytoplasmic Ca²⁺ subsequent to the activation of PLC-γ1 (56). Thus, FasL expression in CD8⁺ T cells is dependent on sustained calcium elevation and on the de novo synthesis of FasL protein (56). Both of these processes could potentially involve Tec kinase family members, as Itk and Rlk have been shown to be important for the efficient activation of PLC-γ1 and for the generation of a sustained calcium flux in CD4⁺ T cells (18, 19).

Despite the obvious implication of Tec kinase family members in CD8⁺ T cell function, studies addressing the possible role(s) of Tec kinases in CD8⁺ T cell development, signaling or function are lacking. An early study examined the role of Itk in CD8⁺ T cell function (57). In this study, Itk⁻/⁻ mice were infected with lymphocytic choriomeningitis virus (LCMV), vaccinia virus (VV) or vesicular stomatitis virus (VSV). As each of these viruses elicits different effector mechanisms involved in viral clearance, this study could assess CD8⁺ T cells, CD4⁺ T cells and B cell function in the absence of Itk. These data showed that the Itk⁻/⁻ mice generated impaired CTL responses to LCMV, VV and VSV, with CTL responses in the range of two- to sixfold reduced. However, despite these impaired CTL responses, the kinetics of viral clearance appeared comparable between Itk⁻/⁻ mice and wild-type mice. No other aspects of the antiviral responses were investigated.

Experiments currently being conducted in our laboratory are attempting to further define a role for Tec family kinases in CD8⁺ T cell function. Preliminary biochemical data suggest that Itk⁻/⁻ and Rlk⁻/⁻/Itk⁻/⁻ CD8⁺ T cells are impaired in TCR signaling downstream of PLC-γ1 activation, showing defects comparable to those observed in CD4⁺ T cells from these mice. For instance, the Tec kinase-deficient CD8⁺ T cells show a reduced level of sustained Ca²⁺ elevation in response to TCR signaling, and these cells have impaired phosphorylation of Erk1/2. These biochemical defects are functionally relevant, as direct in vitro stimulation of CD8⁺ T cells from Itk⁻/⁻ and Rlk⁻/⁻/Itk⁻/⁻ mice leads to reduced production of the effector cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α compared to wild-type mice (Atherly and Berg, unpublished data).

Our initial assessment of the antigen-specific antiviral response of Itk⁻/⁻ mice to LCMV infection provides an interesting comparison to the results reported by Bachmann et al. (57). In our hands, the CTL response to LCMV in Itk-deficient mice is comparable to that of wild-type mice. We also find that, in the absence of Itk, CD8⁺ T cells are impaired in their ability to expand during the early phase of the response to LCMV infection (Fig. 3A). This defect could be due to deficient interleukin (IL)-2 production by CD4⁺ or CD8⁺ T cells in the Itk⁻/⁻ mice, impaired turnover of the Itk⁺⁺ CD8⁺ T cells or death of the CD44hi CD8⁺ T cells in the initial few days following the infection. Differential kinetics of CD8⁺ T cell expansion may also account for the discrepancies seen in the CTL assays, as Bachmann et al. (57) used spleens from day 8 post-LCMV infection, a time-point at which the number and proportion of LCMV responsive cells in the Itk⁻/⁻ mice are greatly diminished in comparison to wild-type mice. In contrast, our CTL analysis was performed using splenocytes from day 9 and day 11 postinfection, time-points at which the proportions of LCMV responsive cells are more similar between wild-type and Itk⁻/⁻ mice (Fig 3B). Interestingly, we also observe a more substantial impairment in the antiviral response in mice deficient for both Itk and Rlk compared to those lacking Itk alone (Atherly and Berg, unpublished data).

**CD4 effector function and T helper cell differentiation is impaired in Tec kinase-deficient mice**

CD4⁺ lymphocytes play a critical role in the defense against many invading organisms. Following the engagement of the TCR/CD28 molecules on a naïve T cells with specific MHC/peptide/B7 complexes on antigen-presenting cells (APCs), a complex series of signaling cascades are activated in the T cell. These signals culminate in the transcription of genes that are involved in the differentiation of naïve CD4⁺ T cells into either T helper 1 (Th1) or Th2 effector cells, lineages that are characterized by their production of distinct sets of effector cytokines. Th1 cells preferentially produce IFN-γ and TNF-α, cytokines that support cell-mediated immunity; Th2 effector cells are characterized by the production of IL-4, IL-5 and IL-10, cytokines that function in humoral immunity. The ability of a naïve CD4⁺ T cell to differentiate into a Th1 or
Fig. 3. CD8 T cell responses to LCMV infection in Itk-deficient mice. (A) Expansion of CD4- and CD8 T cell subsets in wild-type and Itk-/- mice during a time-course of LCMV infection. Wild-type and Itk-/- mice were infected intraperitoneally with LCMV and spleens were harvested at various days postinfection. The expansion of the cell subsets was followed by CD4- and CD8 expression as analyzed by flow cytometry. Two mice were used per time-point. This experiment is one of three. (B) CTL assay using splenocytes from day 9 and day 11 LCMV-infected wild-type and Itk-/- mice. Whole splenocytes were incubated for 5 h at 37°C with RM target cells that were first loaded with the immunodominant LCMV-specific peptide gp33 and labeled with 51Cr. 51Cr release was measured and percent specific lysis calculated.

Th2 effector cell can be influenced by the cytokine milieu present during the initial stimulation. However, while many cell types such as dendritic cells, natural killer cells, and mast cells can serve as a source of effector cytokines that influence Thelper cell differentiation, the initial source of these cytokines in vivo is not clear. It is also possible that, depending on the characteristics of their interactions with APCs, T cells themselves produce cytokines that can affect their own fate.

The role of Tec family kinases in T cell effector function was initially discovered by Liao and Littman (17), who demonstrated that in the absence of Itk, CD4+ cells were defective in IL-2 production and consequently proliferation. Liu et al. (18) extended these findings by demonstrating biochemically that Itk is involved in proximal TCR-mediated signaling events, as Itk-deficient cells were specifically impaired in the phosphorylation of PLC-γ1, and as a result, in IP3 production and the mobilization of intracellular calcium (18). Schaeffer et al. (21) further extended these studies by demonstrating that CD4+ T cells from Rlk-/-Itk-/- mice had even more severe signaling defects than cells possessing a mutation in either Rlk or Itk alone.

Both Fowell et al. (58) and Schaeffer et al. (59) have shown that the biochemical defects observed in Tec kinase-deficient T cells translate in vivo to impaired immune responses to infection. These defects are observed in responses to pathogens requiring both Th1 and Th2 effector functions for clearance (Table 1). For instance, both Itk-/- and Rlk-/-Itk-/- mice succumb rapidly to infection by Toxoplasma gondii, a protozoan that normally induces a protective Th1 immune response in wild-type mice. These data indicate that Tec-deficient mice are incapable of mounting a protective Th1-type CD4+ T cell response to this pathogen.

In contrast, a second study found that Itk-/- mice on both the C57Bl/6 and Balb/c background were able to clear an infection of Leishmania major, a protozoan that also requires a Th1 response for protective immunity (58). This latter finding is particularly interesting as L. major normally elicits a protective Th1 response in C57Bl/6 mice and a nonprotective Th2 response in Balb/c mice. While at face value these two studies seem totally incompatible, it is possible that the discrepancy may result from the different requirements for protective immunity to these two pathogens. For instance, T. gondii infects many cell types, proliferates and spreads rapidly in the host and requires a fast and robust IFN-γ response to prevent lethality. In contrast, L. major infects primarily macrophages and dendritic cells, spreads slowly in the host and can be cleared by a slower and less robust Th1 response (60). Thus, it is possible that reduced kinetics, reduced efficiency and
Table 1. T helper responses in Itk<sup>-/-</sup> and Rlk<sup>-/-Itk<sup>-/-</sup></sup> mice

<table>
<thead>
<tr>
<th>Pathogen (Ref.)</th>
<th>Protective wild-type response</th>
<th>Response of Itk&lt;sup&gt;-/-&lt;/sup&gt; and/or Rlk&lt;sup&gt;-/-Itk&lt;sup&gt;-/-&lt;/sup&gt;&lt;/sup&gt;</th>
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<tr>
<td>L. major (58)</td>
<td>Th1 (B6)</td>
<td>Itk&lt;sup&gt;-/-&lt;/sup&gt; (B6/129) resistant</td>
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<td>Itk&lt;sup&gt;-/-&lt;/sup&gt; (Balb/c) resistant</td>
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<td>Rlk&lt;sup&gt;-/-Itk&lt;sup&gt;-/-&lt;/sup&gt;&lt;/sup&gt; (B6) susceptible (MST 69 days)</td>
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<tr>
<td>T. gondii (59)</td>
<td>Th1</td>
<td>Itk&lt;sup&gt;-/-&lt;/sup&gt; (B6) susceptible</td>
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<td></td>
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<td>Rlk&lt;sup&gt;-/-Itk&lt;sup&gt;-/-&lt;/sup&gt;&lt;/sup&gt; (B6) susceptible</td>
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<tr>
<td>N. brasiliensis (58)</td>
<td>Th2 (Balb/c)</td>
<td>Itk&lt;sup&gt;-/-&lt;/sup&gt; (Balb/c) susceptible</td>
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<tr>
<td>S. mansoni (59)</td>
<td>Th2</td>
<td>Rlk&lt;sup&gt;-/-Itk&lt;sup&gt;-/-&lt;/sup&gt;&lt;/sup&gt; (B6) susceptible</td>
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MST: mean survival time.

a reduction in the overall magnitude of the response in Itk<sup>-/-</sup> mice compared to wild-type mice might explain the different outcomes in response to these two different pathogens.

The responses of Itk<sup>-/-</sup> mice that require a Th2 effector response for protective immunity are more consistent. In response to the nematode Nippostrongylus brasiliensis, Balb/c mice make a protective Th2 response; however, Itk<sup>-/-</sup> Balb/c mice fail to clear the nematode and show a significant reduction in the number of cells making IL-4 compared to wild-type mice (58). Following this discovery, Schaeffer et al. (59) reported that Itk<sup>-/-</sup> mice on the mixed 129 x C57Bl/6 background were also unable to mount a sufficient Th2-type response to the helminth Schistosoma mansoni. Consistent with the overall reduction in the response to this pathogen, cells isolated from the lymph nodes of infected Itk<sup>-/-</sup> mice produced less of the Th2 cytokines, IL-4, IL-5 and IL-10, and more of the Th1 cytokine IFN-γ compared to cells from infected wild-type mice. Thus, Itk<sup>-/-</sup> mice seem to have a significant defect in making Th2 responses, presumably as a consequence of the CD4<sup>+</sup> T cells having an impaired ability to activate NFATc1 and ultimately to produce IL-4.

Paradoxically, the Schaeffer study (59) also showed that Rlk<sup>-/-Itk<sup>-/-</sup></sup> mice were able to elicit a normal Th2 response to S. mansoni, similar to that elicited in wild-type mice infected with this parasite (59). We have recently determined that Rlk is substantially downregulated following the stimulation of naïve wild-type cells in Th2-skewing conditions, while Itk is upregulated (Miller and Berg, unpublished data). Furthermore, Takeba et al. (61) recently demonstrated that in Jurkat cells, Rlk functions as a transcriptional regulator of the IFN-γ gene by binding to the IFN-γ promoter. Therefore, one potential explanation for these findings is that in the Itk<sup>-/-</sup> situation, Rlk is either upregulated or functions to compensate for the absence of Itk in CD4<sup>+</sup> T cells, resulting in cells that would selectively make IFN-γ upon differentiation. In contrast, the absence of Rlk in Rlk<sup>-/-Itk<sup>-/-</sup></sup> mice would cause a selective defect in IFN-γ production, potentially leading cells to default to a Th2 differentiation program. Consistent with this hypothesis is the observation that Rlk<sup>-/-Itk<sup>-/-</sup></sup> mice are unable to generate a Th1-mediated response to T. gondii, yet they produce a protective Th2-mediated response to S. mansoni (59).

To further dissect the role of Tec family kinases in T helper cell differentiation, a number of in vitro studies have examined the ability of Itk<sup>-/-</sup> CD4<sup>+</sup> T cells to differentiate under defined culture conditions. For the most part, the observations made by Fowell et al. (58) and Schaeffer et al. (59) and also in our own laboratory are consistent. However, some discrepancies with respect to the magnitude of the defects in IL-4 and IFN-γ production by Itk<sup>-/-</sup> CD4<sup>+</sup> T cells from mice of different genetic backgrounds have yet to be explained. Both Fowell et al. (58) and Schaeffer et al. (59) observed defects in IL-4 production by Th2 cells that were skewed in vitro. While Fowell et al. (58) observed a 100-fold decrease in the IL-4 production by Itk<sup>-/-</sup> Balb/c CD4<sup>+</sup> T cells, Schaeffer et al. (59) found approximately a 20-fold difference in IL-4 production by Itk<sup>-/-</sup> 129 x C57Bl/6 CD4<sup>+</sup> T cells compared to their wild-type counterparts. Even more strikingly, following the restimulation of cells primed in Th1 conditions, Fowell et al. (58) observed no defect in IFN-γ production by cells from Itk<sup>-/-</sup> Balb/c mice, whereas Schaeffer et al. (59) observed substantial defects (~12-fold) in cells from Itk<sup>-/-</sup> 129 x C57Bl/6 mixed mice. These differences suggested that the absence of Itk might have distinct effects in T cells from different strains of mice. One mechanistic explanation for this possibility is that Balb/c and mixed 129 x C57Bl/6 mice may have different basal expression levels of transcription factors, kinases or other signaling molecules that function in T helper cell differentiation pathways, and these distinctions may affect how CD4<sup>+</sup> cells respond to certain antigens. Evidence supporting this possibility was presented by Bix et al. (62), who identified two genetic loci in Balb/c mice that could influence the intrinsic ability of a CD4<sup>+</sup> T cell to produce IL-4. These identified loci were determined to influence IL-4 production prior to signals mediated by the IL-4 receptor.
Fig. 4. Increased effector/memory CD4+ T cells mice. Following the purification of splenic CD4+ T cells (Fig. 2), 1 × 10^6 cells were stimulated with PMA (2.5 ng/mL) and ionomycin (375 ng/mL) for 6 h in a 96-well plate. After 4 h, Golgi-blocking reagents were added to prevent cytokine secretion. The cells were then surface stained with antibodies to CD4 and CD44, fixed, permeabilized, and stained intracellularly with antibodies to IL-4 and IFN-γ. Cells were then immediately analyzed by flow cytometry.

To add further complexity to this story, we observe slight differences in Th helper cell differentiation using Itk-/- mice generated in our laboratory compared to those published by others. Our Itk-/- mice are completely backcrossed to the C57BL/6 background, and similar to the findings reported by Schaeffer et al. (59), we have found that CD4+ T cells from these mice possess defects in both IFN-γ and IL-4 production when skewed in Th1 and Th2 conditions, respectively. However, we have consistently observed an interesting phenomenon not reported by either Powell et al. (58) or Schaeffer et al. (59). As described above, Itk-/- have a substantial increase in the steady-state number of peripheral CD4+ T cells with an activated/memory phenotype. In an effort to determine the differentiation status of these activated cells in vivo, we stimulated CD4+ T cells from these mice ex vivo with phorbol myristate acetate (PMA) and ionomycin, and examined effector cytokine production by intracellular staining. This experiment indicated that many more CD4+ T cells in Itk-/- mice produce cytokines directly ex vivo (Fig. 4), and the cells responsible for the increased cytokine production were CD44hi. Of these CD44hi cells most are IFN-γ producers; however, a significant percentage of CD44hi cells from Itk-/- mice produce IL-4 in this assay. The striking aspect of this finding is that the number of IL-4-producing cells is substantially higher in the Itk-/- mice than in the control mice. Likewise, when stimulating purified CD4+ T cells from SC.C7 TCR transgenic Itk-/- mice intrinsically with peptide plus APCs in nonskewing conditions, we consistently observe the differentiation of Itk-/- cells into both Th1- and Th2-type cells (Fig. 5). Interestingly, the Itk-/- cultures always show increased percentages of cells that produce IL-4 compared to the control cultures where, under these nonskewing conditions, the T cells uniformly differentiate into IFN-γ producers. This increased tendency of cultured SC.C7 Itk-/- T cells to differentiate into IL-4 producers correlated with an increased percentage of cells that are CD44hi and Vα11hi in these mice. Together, these findings strongly suggest that T cells expressing endogenous TCR α-chains are becoming activated in the SC.C7 Itk-/- mice, differentiating in vivo into IL-4-producing cells and then skewing the in vitro cultures towards Th2 differentiation due to the elevated IL-4 production.

The origin and history of the IL-4-producing CD4+ T cells in the Itk-/- mice remain unclear. We favor the possibility that a repertoire of T cells is selected in the thymus of Itk-/- mice that are either autoreactive and become activated by self-antigen or, alternatively, are reactive to an environmental antigen that induces differentiation into the Th2 lineage. The explanation for why this occurs in Itk-deficient mice but not in wild-type mice has not been determined, but it may relate to defects in thymic selection or to an altered peripheral environment due to changes in the non-T cell populations that express Itk and/or Rlk. Nonetheless, several lines of evidence from Schaeffer et al. (59) and our own laboratory support the hypothesis that there is some type of Th2-biased immune response, autoimmune or otherwise, occurring in vivo. First, Schaeffer et al. (59) reported that Itk-/- mice have a fivefold increase in serum immunoglobulin E (IgE) levels, an IL-4-dependent antibody subclass. Second, we have observed a significant level of eosinophilia in lymph nodes of Itk-/- mice that is probably the result of Th2 effector cells in vivo (63). Third, the phenotype observed in Tec family kinase-deficient mice is strikingly similar to that seen in mice possessing mutations in signaling molecules such as LAT, JNK1 and certain NIFATs, all of which have all been implicated in the same pathway(s) as Itk, as is discussed later in greater detail.
Evidence implicating the PLC-γ1, calcium, NFAT pathway in regulating T cell development and differentiation

Tec family kinases are thought to function in the phosphorylation and activation of PLC-γ1 via the formation of a multimolecular signaling complex that includes Grb2, Gads, SLP-76, LAT and PLC-γ1 (for review of mechanism, see 1). Recently, two independent groups, Aguado et al. (64) and Sommers et al. (65), described mice that expressed only a mutated form of LAT, LAT\textsuperscript{Y136F}. Previous work had shown that Y136 of LAT is required for the binding and activation of PLC-γ1 in response to TCR stimulation (16, 66, 67). Interestingly, the LAT\textsuperscript{Y136F} mutant mice have a very similar biochemical and physiological phenotype when compared to the Tec kinase-deficient mice (Fig. 6). Similarly, mutation of another TCR signaling molecule, Vav1, which has also been shown to be important for the full activation of PLC-γ1 (68), results in mice with a similar T cell deficiency (69–71). Based on their role in PLC-γ1 activation, it is not surprising that each of these molecules has also been shown to play a role in regulating calcium mobilization in T cells.

Downstream of PLC-γ1 activation, IP\textsubscript{3} generation and subsequent calcium mobilization, transcription factors of the NFAT family are rapidly dephosphorylated and activated by the calcium-dependent phosphatase, calcineurin. This dephosphorylation allows NFAT molecules to translocate to the nucleus where they bind to specific promoter sequences and activate transcription. The three NFAT family members expressed in T cells, NFATc1 (NFATc, NFAT2), NFATc2 (NFATp, NFAT1) and NFATc3 (NFAT4, NFATx), appear to have overlapping as well as unique functions, as has been elucidated by studies of mice with single as well as multiple mutations in NFAT family members (72). Predictably, several groups including our own have found that Itk\textsuperscript{−/−} and Rlk\textsuperscript{−/−}Itk\textsuperscript{−/−} CD4\textsuperscript{+} T cells have a defect in NFAT activation and/or translocation following TCR stimulation, most probably as a result of the defects in calcium mobilization (58, 59, 63). The resemblance of the phenotypes observed in Itk\textsuperscript{−/−}, Rlk\textsuperscript{−/−}Itk\textsuperscript{−/−} and also the LAT\textsuperscript{Y136F} mice to those observed in several NFAT knockouts is intriguing, and this resemblance further confirms the notion that disruption of PLC-γ1 activity results in defective NFAT activation, which in turn affects a number of T cell effector functions.

With respect to T cell development, the clearest parallels are seen between mouse mutants carrying defects in signaling proteins that are upstream of PLC-γ1 activity. The absence of the adapter molecules SLP-76 and LAT has more severe effects on T cell development than those seen in Itk\textsuperscript{−/−} and Rlk\textsuperscript{−/−}Itk\textsuperscript{−/−} mice (21, 28). Specifically, Tec kinase-deficient mice have mild, if any, defects in development at the DN to DP transition, whereas thymocytes from both SLP-76\textsuperscript{−/−} (73, 74) and LAT\textsuperscript{−/−} mice (75) are blocked at this stage of development. These results are consistent with the fact that deficiencies in SLP-76 (76) or LAT (77) lead to more substantial reductions in PLC-γ1 activity than is seen in Tec family knockouts (19) and lead to a virtual absence of calcium mobilization in response to TCR signaling. The phenotype of the LAT\textsuperscript{Y136F} mice with respect to T cell development is similar to LAT\textsuperscript{−/−} mice, but less severe;
### Similar phenotypes of mice deficient in T cell signaling proteins

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<th>Biochemical Defects</th>
<th>In vivo phenotype</th>
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<td>Pre-TCR signaling</td>
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**Fig. 6. Similar phenotypes of mice deficient in T cell signaling proteins.** Chart compares developmental and peripheral cell phenotype of, as well as biochemical defects in cells from, various signaling molecule knockouts. ND, not done or unpublished; NA, not applicable. Biochemical findings were either in peripheral T cells or thymocytes with the exception of those for SLP-76, which were done in a SLP-76 deficient Jurkat T cell line (76).
instead of a complete block at the DN3 to DN4 stage of thymocyte development, young LAT\textsuperscript{Y136F} mice have a small population of DP cells, suggesting that a low level of pre-TCR signaling is retained in these mice (65). This early T cell development defect in LAT\textsuperscript{Y136F} mice confirms previous data indicating a role for PLC-γ1 in pre-TCR signals (29) and further implicates a role for Tec kinases at this stage of development.

The developmental and biochemical defects observed in Vav1\textsuperscript{−/−} mice show the greatest similarity to the defects seen in Itk\textsuperscript{−/−} and Rlk\textsuperscript{−/−}Itk\textsuperscript{−/−} mice (69−71). Vav1\textsuperscript{−/−} mice have deficiencies in both positive and negative thymic selection with no affects on lineage commitment (71), however, unlike the Tec kinases, Vav1 plays an indirect role in this process and may affect PLC-γ1 activity by at least two mechanisms (68). One mechanism is the requirement for Vav1 to activate PI3K, which in turn facilitates the activation of Itk and Tec via recruitment of these kinases to the activated receptor by the binding of their PH domains. This mechanism would then lead to the direct activation of PLC-γ1 by the Tec kinases. In support of this model, activation of both Itk and Tec is severely impaired in Vav1\textsuperscript{−/−} DP thymocytes. The second mechanism by which Vav1 is thought to regulate PLC-γ1 activation is PI3K independent, and is based on the requirement for Vav1 to promote PLC-γ1 association with SLP-76 and Gads. It is interesting to note that the developmental defects in Vav1\textsuperscript{−/−} mice are more severe than those seen in Itk\textsuperscript{−/−} mice, but they are similar to those seen in Rlk\textsuperscript{−/−}Itk\textsuperscript{−/−} mice. This similarity correlates well with the fact that both Itk and Rlk contribute to the full activation of PLC-γ1 in thymocytes and that the pathway involving Itk is PI3K dependent, unlike the pathway involving Rlk.

The most striking similarities seen among these signaling protein-deficient mice involve alterations in CD4\textsuperscript{+} T cell homeostasis and the apparent in vivo skewing to a Th2-dominated environment in nonimmunized mice. Both groups that generated LAT\textsuperscript{Y136F} mice described the peripheral CD4\textsuperscript{+} T cells isolated from these mice as having an activated phenotype (CD62L\textsuperscript{lo}, CD44\textsuperscript{hi}, CD69\textsuperscript{hi}), showing defects in TCR-mediated cell death and selectively producing type-2 cytokines (64, 65). Furthermore, in vivo, CD4\textsuperscript{+} T cells from LAT\textsuperscript{Y136F} mice are defective in calcium mobilization, IL-2 production and FasL expression, analogous to the milder defects seen in Itk-deficient CD4\textsuperscript{+} T cells. However, unlike Itk\textsuperscript{−/−} and Rlk\textsuperscript{−/−}Itk\textsuperscript{−/−} cells, the BER pathway remained intact in the LAT\textsuperscript{Y136F} T cells, although the explanation for this finding is not clear. Interestingly, both groups alluded to the fact that, as a result of impairments in thymic selection, cells reactive towards self-ligands may be escaping from the thymus. While both studies described similar phenotypes of the LAT\textsuperscript{Y136F} mice, Aguado et al. (64) maintained their mice on the Balb/c background, whereas Sommers et al. (65) maintained their mice on the C57Bl/6 background. Thus, genetic differences between these two mouse strains that affect Th1 vs. Th2 differentiation in other circumstances appear unrelated to the phenotype observed in the LAT\textsuperscript{Y136F} mice.

Mice deficient in NFAT family members also share some of the common phenotypes with the Tec family-deficient and LAT\textsuperscript{Y136F} mice, but the relationships are more complex. NFATc1\textsuperscript{−/−} mice are unable to induce Th2 responses, as demonstrated by decreased IL-4 production by T cells from these mice (79, 80). Remarkably, while CD4\textsuperscript{+} T cells from NFATc1-deficient mice possess a reduced proliferative capacity in vitro, they exhibit no defect in IL-2, IFN-γ or TNF-α production when skewed in Th1 conditions in vitro. A second NFAT family member, NFATc2, that is expressed at high levels in resting T cells and then downregulated following activation (81), has been found to directly regulate the expression of NFATc1 by transactivating the NFATc1 promoter following TCR activation (82). Lymphocytes from mice possessing mutations in NFATc2 exhibit an activated phenotype, show moderate increases in IL-4 production and have defects in activation-induced cell death in addition to severe eosinophilia in the lungs (83). Further studies by Hodge et al. (84) and Kiani et al. (85) demonstrated that NFATc2 is required for the termination of the late phase of IL-4 transcription and ultimately for the downregulation of the Th2 response. NFATc3, comparable to NFATc2, is expressed in resting T cells and is downregulated following activation (81). NFATc3-deficient peripheral T cells, although they lack defects in cytokine production, have an activated phenotype and exhibit increased apoptosis as a result of elevated FasL expression (86). These latter data are consistent with the notion that NFATc3 may function to repress FasL expression and thus may play an important role in T cell survival.

Mice containing combined mutations in NFAT family members have proven to be invaluable in the dissection of NFAT-regulated pathways. NFATc3/NFATc3 doubly-deficient mice develop a lymphoproliferative disorder as a result of increased resistance to apoptosis. These mice have allergic blepharitis, interstitial pneumonitis and 1000−10000-fold increase in serum IgG1 and IgE levels, secondary to a dramatic and selective increase in Th2 cytokines (81). In contrast, T cells from mice containing combined mutations in both NFATc1
and NFATc2 exhibit an activated phenotype but show substantial defects in the development of multiple effector functions, such as cytokine production, surface effector molecule expression and cytolytic activity (87). All of the above findings suggested that, while NFATc1 may play a positive role in proliferative functions, NFATc2 and NFATc3 essentially function to repress specific T cell functions. These data imply that, depending on the strength or quality of the TCR signal, some NFAT family members may be selectively activated relative to others. Furthermore, it is evident that lymphoid homeostasis and Th2 development require a critical balance among NFAT family member activities.

Based on the observations arising from analyses of NFAT-deficient T cells, it seems likely that upon stimulation of T lymphocytes lacking Itk, Rlk and Itk, or LAT, there would be an initial defect in NFATc2 activation, followed by a more substantial defect in NFATc2-induced NFATc1 upregulation. In addition, as a result of their impairment in NFAT activation, Itk−/− and Rlk−/−ltk−/− T cells may also show decreased repression mediated by NFATc2 and NFATc3, providing an explanation for many of the observed defects in T cells effector functions seen in these cells as well as those expressing LAT<sup>Y136F</sup>. The more severely impaired Rlk−/−ltk−/−CD4<sup>+</sup> T cells may be more akin to the NFATc1/c2 double knockout cells, where there is a more profound defect in NFAT-mediated effector functions. The relationships between Itk, Rlk and LAT, signaling molecules that function to amplify calcium signals, and NFATs, calcium sensitive transcription factors, and between NFATs and specific effector genes may not be simple. To obtain a clearer picture of these relationships, it will be necessary to determine how individual NFAT molecules are spatiotemporally regulated in T cells during T cell stimulation, expansion and contraction.

Another T cell signaling protein, JNK, has also been implicated in pathways downstream of Itk activation and other proximal TCR signaling events. We have shown that Itk<sup>−/−</sup> CD4<sup>+</sup> T cells are defective in the activation of the JNK pathway, a defect that is likely to contribute to the impaired FasL expression in these cells (54). CD4<sup>+</sup> T cells from JNK1-deficient mice synthesize augmented levels of IL-4 production but normal levels of IFN-γ production, when stimulated in vitro in nonskewing conditions (88). NFATc1 has been identified as a substrate for JNK1, and it may function to inhibit Th2 responses (88). Consistent with this, JNK1<sup>−/−</sup> mice are susceptible to Leishmania infection, which is not due to their inability to make a Th1 response but rather to their inability to suppress the development of a Th2 response (89). Exactly how dysregulation of JNK1 signaling in Itk<sup>−/−</sup> mice contributes to the T helper imbalance seen in these mice is unclear. Perhaps a better understanding of the complex interactions and crosstalk between all these signaling molecules through gene expression array analyses will aid in our future understanding of how these signaling molecules and pathways integrate to affect T cell development, activation and differentiation.

### Role of Tec kinase-dependent signals in non-T cells

Whereas T helper cell differentiation can clearly be altered by modification of genes within the T cells themselves, it is clear that environmental factors, especially cytokines, elicited from other cell types can also influence this process. Three of the most important cell types that have been shown to influence the T helper response are natural killer (NK) cells, NK-T cells and mast cells. Interestingly, Tec family kinases, the adapters SLP-76 and LAT and the NFAT family of transcription factors are all involved in signaling within these cells. Thus, it is possible that, along with intrinsic T cell changes in the signaling mutants discussed, disruption of signaling in other cell types may affect the T cell phenotype observed in these mutant mice.

Activation of the NK cell cytolytic machinery is mediated by signaling through the low-affinity FcyRIIIA complex on the cell surface. This receptor belongs to the same superfamily of receptors as the TCR, and it uses related signaling molecules and pathways to mediate its function (reviewed in 90). Among the signaling molecules that can associate with FcyRIIIA are Lck, ZAP-70 and SLP-76, all of which are molecules that influence Itk activity in response to signaling downstream of the TCR. Despite the familial similarities of receptors expressed on NK cells and T cells, the similar functions of NK cells and CD8 T cells and the expression of Itk in human NK cells, data from our own laboratory indicate that murine NK cells do not express Itk. Consistent with this, we have found that the functions of NK cells in Itk<sup>−/−</sup> mice are unimpaired in response to LCMV infection (Fig. 7). However, real-time quantitative PCR analysis on cDNA generated from IL-2-activated NK cells shows that NK cells do express transcripts for both Rlk and Tec (Atherly, Miller and Berg, unpublished data). Data from Schaeffer et al. (59) suggest that Rlk, like Itk, is not necessary for NK cell function in mice, as normal levels of serum IFN-γ are detected early after infection with T. gondii in both Rlk<sup>−/−</sup> and Rlk<sup>−/−</sup>ltk<sup>−/−</sup> mice. Therefore, as NK cell functions in Itk-deficient and Itk/Rlk-deficient mice seem to be normal, it is unlikely that alterations in the T helper cell phenotype observed in the periphery of these mice is due to aberrant NK cell function.

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A role for mast cells in the Th2-type skewing observed in the mutant mice described earlier is a possibility, as mast cells play an integral role in Th2 effector responses. Both Itk and Btk are expressed in mast cells and become phosphorylated following crosslinking of the high-affinity IgE receptor, FcεRI, present on mast cells (91, 92). There is substantial evidence indicating a role for Btk in mast cell function (93), but little is currently known about the role of Itk in these cells. Interestingly, many of the same molecules that are involved in the Tec kinase-dependent signal downstream of the TCR are found to associate following activation of FcεRI, including Vav1, SLP-76 and LAT. Additionally, both PLC-γ1, which is activated by Itk in T cells, and PLC-γ2, which is activated by Btk in B cells, associate with this complex, suggesting a possible role for both of these Tec kinases in mast cell signaling. In mast cells, the activation of both of these PLC-γ isoforms is thought to be important for calcium responses and for the activation of the NFAT family of transcription factors, signals that are critical for optimal mast cell function and cytokine production (reviewed in 94). Thus, a possible role for Itk in mast cells remains, leaving open the possibility that mast cell deregulation might affect T cell homeostasis and/or differentiation.

NK T cells are important regulators of T helper responses, and Itk has recently been shown to be important for the development and homeostasis of different NK T cell subsets (95). In this study, four stages/fractions of NK T cell development were defined based on the expression of various cell surface markers. Moreover, they found that fractions 1 and 2 predominately secrete IL-4, while fractions 3 and 4 express IFN-γ upon activation. Comparison of the NK T cell subsets present in young Itk-deficient mice and wild-type mice demonstrated that there is a partial block at the transition from fraction 2 to fraction 3 in the Itk−/− mice, with a twofold increase in the percentage of cells at fraction 2 and a reduction in fraction 3 cells. Additionally, as Itk-deficient mice age, they progressively lose a significant proportion of their NK T cells, suggesting a role for Itk in the maintenance of these cells. Based on these findings it seems likely that during the first few weeks of life, Itk−/− mice would have an increase in the immature IL-4-producing population of NK T cells as well as a progressive loss of this immunoregulatory population as a whole as the mice age. Thus, it seems possible that these effects on the NK T cell population could contribute to the Th2 skewed phenotype we observe in Tec family-deficient mice. Because of the limited studies carried out to address the role of various signaling molecules in NK T cell development, it remains to be seen whether changes in the population of these cells could be responsible for the phenotypes observed in other signaling molecule knockouts. Nonetheless, it will be interesting to determine whether or not the in vivo Th2 skewing observed in Tec family-deficient mice is T cell intrinsic, or is influenced by changes in the NK T cells, mast cells or both.

Conclusions

Determination of the precise and possibly unique role of each Tec family kinase in T cells requires further investigation using a combination of genetics and biochemistry. A complication of the genetic studies is the possibility that when one member of the Tec family is absent, compensatory mechanisms may emerge to counteract the imbalance. Consistent with this possibility, RNA isolated from lymphoid organs of Rlk−/− mice was found to have somewhat increased levels of Itk mRNA (19). In contrast, we have found that neither Rlk nor Tec mRNA expression is upregulated in Itk−/− CD4+ T cells (Miller and Berg, unpublished data). While this finding does not rule out the possibility that Rlk or Tec might compensate...
for Itk in its absence, we favor the notion that under normal physiological circumstances, each Tec kinase family member has a specific and nonredundant role in hematopoietic cell signaling. Supporting this idea, individual Tec kinases show distinct requirements for activation, such as the dependence on PI3K, in addition to the common need for phosphorylation by an Src kinase. Furthermore, activation of Tec kinases in T cells leads to overlapping as well as unique downstream consequences, as illustrated by the observation that Rlk but not Itk can translocate to the nucleus and act as a transcription factor. Future work with Tec family kinases should begin to reveal the precise roles of Itk, Rlk and Tec in T cell signaling.

The contradictory and somewhat mystifying data discussed above warrant further investigation of the role of Tec family kinases in CD4+ T cell differentiation. More specifically, a more complete understanding of the pathways from Tec kinase activation to transcription factor activation is needed. Despite many of the unknowns, all of the current data collectively emphasize the importance of Tec kinases in regulating T cell homeostasis as well as global immune responses. These data also highlight the role of Tec kinases in the regulation of Thelper cell differentiation. Finally, the data described here underscore the important role of TCR signal strength in maintaining the appropriate balance of T cell survival, activation and differentiation.

References


