THE ROLE OF TEC KINASES IN CD4$^+$ T CELL ACTIVATION

A Dissertation Presented

By

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ABSTRACT

The Tec family tyrosine kinases Itk, Tec and Rlk are expressed in T cells. Previous studies have established that these kinases are critical for TCR signaling, leading to the activation of PLCγ1. To further understand the functions of Tec kinases in T cell activation, we took three different approaches. First, we performed a thorough analysis of CD28-mediated signaling events and functional responses with purified naïve T cells from *Itk*$^{-/-}$ mice and a highly controlled stimulation system. Data from this set of studies definitively demonstrate that CD28 costimulation functions efficiently in naïve CD4^{+} T cells in the absence of Itk. Second, in order to further study the functions of Tec kinases *in vivo*, we generated transgenic mouse lines expressing a kinase-dead (KD) mutant of *Tec* on the *Itk*$^{-/-}$*Rlk*$^{-/-}$ background, hoping to study mice that are functionally deficient for all three Tec kinases. The results hint the importance of the Tec kinases in T cell development and/or survival. Finally, in order to identify potential transcriptional targets of Itk, we used microarray technology to compare global gene expression profiles of naïve and stimulated *Itk*$^{-/-}$ versus *Itk*$^{+/-}$ CD4^{+} T cells. This analysis provided a short list of differentially expressed genes in *Itk*$^{-/-}$ versus *Itk*$^{+/-}$ CD4 T cells, providing a starting point for further studies of Itk in T cell activation. Collectively, these studies clarified the role of Itk in CD28 signaling, revealed some unexpected aspects of Tec family kinases in T cells, and indicated potential targets of Itk-dependent signaling pathways in T cells.

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ATTRIBUTIONS

Chapter III

The micro-injection of transgenic constructs was performed by the University of Massachusetts Medical School Transgenic Animal Modeling Core.

Chapter IV

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IN MEMORY OF MY GRAND PARENTS

To my wife, Shuang Wang.

CHAPTER I

INTRODUCTION

Introduction

Over millions of years of selection, vertebrates have acquired an immune system, known as the adaptive immune system, as an addition to the innate immune system present universally (1). In the innate immune system, cells nonspecifically cope with invading organisms (pathogens), mainly by phagocytosis, and eliminate them. The innate immune response is not enhanced when the body is infected again by the same pathogen. In contrast, the adaptive immune system consists of specialized cells, known as T and B lymphocytes, that recognize antigens from invading pathogens through specific receptors expressed on their surface. Following the initial encounter with a pathogen, the adaptive immune system responds more rapidly and more vigorously when the same pathogen invades the body again. Thus, specificity and memory are the hallmarks of the adaptive immune system.

The specificity of the adaptive immune system is achieved through unique receptors that recognize foreign antigens. These antigen receptors are termed T cell receptor (TCR) and B cell receptor (BCR) for T and B lymphocytes, respectively. The expression of TCR or BCR in each individual cell results from the rearrangement of gene segments that encode parts of the receptors during development. For instance, the TCR on αβ T cells, which comprise the major T cell population in the body, is a heterodimer that consists of an α chain and a β chain; the expression of TCR α chain is the result of rearranging of Vα, Jα, and Cα segments in the α chain gene locus, while the expression of TCR β chain is the result of rearranging of Vβ, Dβ, Jβ, and Cβ segments in the β chain

gene locus. This rearrangement event plus possible imprecise joining among genomic segments result in an enormously diverse repertoire of receptors. For instance, the human TCR repertoire has been estimated to be on the order of 10^7 (2). While the BCR recognizes and binds directly to antigens that include proteins and pathogenic particles, the TCR can only recognize antigenic peptides processed in and presented by antigen-presenting cells (APCs). APCs process antigens to peptides and present the antigenic peptides on molecules known as major histocompatibility (MHC) molecules. There are two types of MHC molecules. Class I MHC molecules are present on the surface of almost all nucleated cells, and class II MHC molecules are present on specialized immune cells known as professional APCs, such as dendritic cells (3). Thus, TCRs recognize not only the foreign antigenic peptide, but the self MHC molecules as well. In addition to TCR, T cells also express one of two coreceptor molecules, CD4 or CD8, that recognize class II or class I MHC molecules, respectively. These coreceptors facilitate the interaction between T cells and APCs. Thus, $\alpha\beta$ T cells can be distinguished as $CD4^+$ and $CD8^+$ lineages by coreceptor expression.

When B cells are activated by binding to antigens through BCRs, they undergo clonal expansion and secrete a soluble form of BCR, known as antibody, which in turn binds and neutralizes antigens. Neutralized antigens are more easily recognized and destroyed by the innate immune cells. During B cell responses, BCRs also undergo an "editing' process, known as affinity maturation, through somatic hypermutation of the coding sequence, resulting in the best possible affinity to the antigen. On the other hand,

when T cells are activated by antigens presented on APCs, they also undergo clonal expansion; in addition, they differentiate into functional subsets. Some T cells act directly on the antigen bearing cells, as when cytotoxic T cells kill virus-infected cells. Other subsets of T cells function as regulators that orchestrate the immune response through secreting soluble small proteins, known as cytokines, as well as through cell-cell interaction. However, it is generally accepted that activated T cells do not undergo receptor affinity maturation, which means that the TCR on a given mature T cell will not change regardless of the activation status of the T cell.

T cell development and maturation occur in the thymus, where T lineage-committed cells go through CD4CD8 double negative (DN) and CD4CD8 double positive (DP) stages to the mature CD4 single positive ($CD4^+$ SP) or CD8 single positive ($CD8^+$ SP) stage. During thymic development, T cells are selected so that only those that express TCRs with appropriate affinity to self-MHC can survive and mature (4). The rearrangement and selection events ensure a highly diverse T cell repertoire that can recognize foreign peptides presented on self MHC molecules while not responding to self-peptides presented on self-MHC. When T cells are mature, they leave the thymus and enter the secondary lymphoid organs (*i.e.* spleen, lymph nodes, and Peyer's patches). Before a T cell encounters its specific antigen, it is called a "naïve" T cell. Naïve T cells continuously migrate from one lymphoid organ to another via blood and lymph (5, 6). In the secondary lymphoid organs, T cells scan APCs for antigens. If a T cell does not recognize its specific antigen, it migrates to the next secondary organ. This continuous

migration allows T cells to make rapid contact with antigens. When a T cell recognizes an immunogenic peptide presented on APCs through TCR-MHC/peptide interaction, it is activated. The signals from TCR and some accessory receptors, known as costimulatory molecules, are transduced through the cell to the nucleus, where *de novo* expression of a variety of genes is activated. This gene expression is vital for the function of activated T cells.

TCR signaling and T cell activation

T cell activation is initiated by the interaction between the TCR on the T cell and the MHC/peptide complex that is present on the APC (7). This signal first induces a reorganization of the plasma membrane and membrane associated signaling proteins, followed by the formation of a series of signaling complexes, which in turn facilitate the activation of effector signaling molecules such as phospholipase C-γ1 (PLCγ1). Subsequently, the signal transduced in this way induces the activation and nuclear translocation of many important transcription factors, such as NFAT, AP-1, and NFκB. These signaling events culminate in the activation of the expression of a variety of genes that are crucial for effector functions of activated T cells, such as interleukin-2 (IL-2), a critical T cell growth factor. This series of events is described sequentially as follows (Figure 1-1).

In addition to the binding of the TCR to the MHC/peptide complex, the T cell-APC interaction also requires the binding of coreceptors CD4 or CD8 to MHC class II or class I molecules, respectively, and the interactions between adhesion molecules, such as LFA-1 on T cells and ICAM-1 on APCs. Imaging studies have demonstrated that a special structure, termed the immunological synapse (IS), forms in the area where these interactions occur (8, 9). Biochemical studies have shown that upon ligation, TCR molecules associate to cell membrane domains, known as lipid rafts, which are critical for TCR signaling (10-13). These rafts contain enriched glycosphingolipid, and are relatively resistant to non-ionic detergents, such as Triton X-100, at low temperatures

(14). A number of intracellular proteins that transduce TCR signals associate with the rafts, including the Src family tyrosine kinase Lck and the transmembrane adaptor protein Linker for Activation of T cells (LAT) (15, 16).

While the αβ heterodimer of a TCR is responsible for the specific recognition of and interaction with the MHC/peptide complex, transduction of the signal from this interaction depends on the intracellular non-polymorphic components of the TCR/CD3 complex. In addition to the αβ chains, this complex is comprised of a TCRζ chain dimer, a CD3 γ chain, a CD3 δ chain, and two CD3 ε chains (17). The intracellular components of these chains contain a unique motif, the Immunoreceptor-based Tyrosine Activation Motif (ITAM) with a consensus YXXI/L$X_{(6-8)}$YXXI/L sequence (18, 19). There are three ITAM motifs on each TCRζ chain, and there is one such motif on each of the γ, δ, and ε chains. A number of studies have shown that the ITAM motifs are both necessary and sufficient for transducing the signals initiated by TCR ligation (20, 21).

The earliest experimentally detectable event after TCR ligation is the phosphorylation and activation of the Src family tyrosine kinases, such as Lck. As mentioned above, Lck is targeted to the plasma membrane via its N-terminal myristylation (22), and is further localized to the activated TCR complex by its association with the coreceptors CD4 or CD8 (23). The first substrates of activated Lck are thought to be the ITAM motifs on the intracellular components of the TCR complex. Another Src family tyrosine kinase, Fyn, functions similarly to Lck (24). Phosphorylated

tyrosine residues on the ζ chain ITAMs then recruit the Syk family tyrosine kinase ZAP-70 (zeta-chain-associated protein of 70kDa) to the complex, through the binding of tandem Src homology 2 (SH2) domains on ZAP-70 to the two phosphorylated tyrosine residues on the ITAM (25, 26). Once bound to the ζ chain in this fashion, ZAP-70 is activated by Src kinase-mediated tyrosine-phosphorylation of the kinase domain activation loop (27, 28). Subsequently, activated ZAP-70 undergoes autophosphorylation, which allows binding to other SH2 domain-containing proteins (29). Activated Src and Syk tyrosine kinases are then able to phosphorylate other kinases as well as a number of molecules, known as adaptor proteins, that lack enzymatic activities but function to mediate interactions among other signaling proteins (30). One such adaptor protein is LAT, which was originally identified as a 36-38 kDa protein that is rapidly phosphorylated after TCR ligation and that associates with a number of other signaling molecules including PLCγ1 and Grb2 (31-33).

After being phosphorylated by ZAP-70 (34), LAT recruits a number of enzymes and adapter proteins, including Grb2 which brings the small G protein Ras activator SOS. Another important adaptor protein, SLP-76 (SH2-domain-containing leukocyte protein of 76kDa), joins the complex via its interaction with Gads (35). SLP-76 can bind multiple effector molecules, and thus, bring them to the signaling complex. These molecules include Vav and Itk (see below). Another critical membrane-proximal event is the activation of Phosphoinositol-3 kinase (PI3K). PI3K activation is thought to require Lck activity (36, 37). Activated PI3K phosphorylates the D-3 position of

phosphotidylinositol (4,5)-bisphosphate (PIP_2) to generate phosphotidylinositol (3,4,5)-trisphosphate (PIP_3). Importantly, this provides a binding site at the membrane for a number of signaling molecules that contain pleckstrin homology (PH) domains, such as PLCγ1 and Itk.

PLCγ1 is a central effector signaling molecule in T cells. As mentioned previously, PLCγ1 is recruited to the signaling complex by interacting with LAT via its SH2 domains and additionally by binding to PIP_3 via its PH domain. Activation of PLCγ1 requires the phosphorylation of multiple tyrosine residues on this molecule. Subsequently, activated PLCγ1 catalyzes the hydrolysis of phosphotidylinositol (4,5)-bisphosphate (PIP_2) to generate two products, inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to its receptor in the endoplasmic reticulum (ER), and induces the release of calcium stored in the lumen of the ER. This release of stored calcium then activates calcium channels in the cell membrane to open, resulting in an influx of calcium from the extracellular environment. The elevated cytoplasmic calcium concentration is crucial for functions of activated T cells. The other product of PLCγ1, DAG, activates the protein kinase C (PKC) family serine/theronine kinases, which in turn activate NFκB. Moreover, DAG can directly activate another Ras activator, RasGRP, leading to the activation of Ras/MAPK pathways (38, 39).

Figure 1-1 A schematic view of TCR signaling pathways

Engagement of the TCR leads to rapid activation of Lck and Zap-70, which phosphorylate numerous downstream targets, including the adapter molecules LAT and SLP-76. Together, these adapters form a platform for the accumulation of molecules into a signaling complex that includes PLCγ1, Grb2, Gads, Vav, Tec family kinases, and other associated molecules. Itk (and probably other Tec kinases) physically interact with SLP-76 and possibly LAT, bringing Itk into this complex where it can phosphorylate PLCγ1. Activation of PLCγ1 by Itk leads to the generation of IP_3 which is required for Ca^{2+} flux within the cell and DAG which activates members of the PKC family and RasGRP. This results in the downstream activation of mitogen-activated protein kinases, such as JNK, ERK1 and ERK2, and other effectors that direct gene transcription.

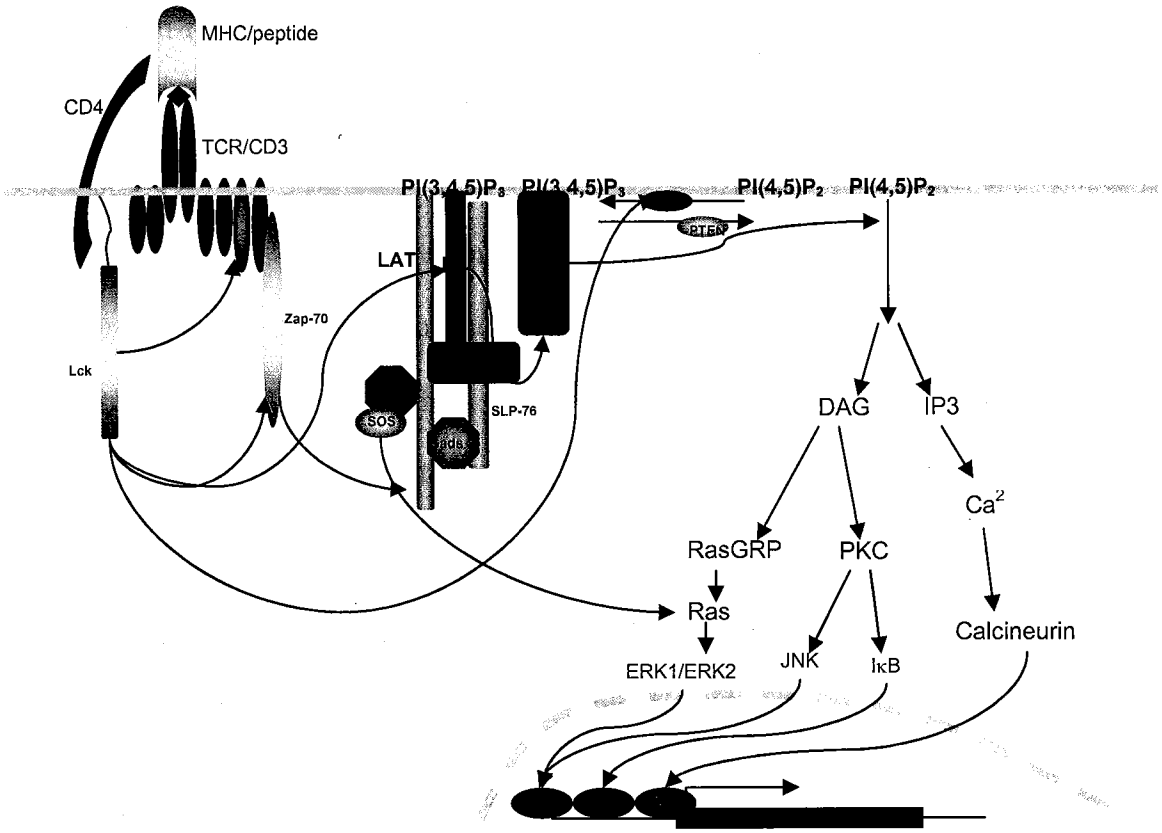


Figure 1-1 A schematic view of TCR signaling pathways

The signals that are generated on the cell membrane by the interaction of TCR and APC/peptide complex are then transduced to stimulate the activation of a variety of transcription factors. Among these are the nuclear factor of activated T cells (NFAT), NFκB and AP-1. NFAT plays a crucial role in the induction of gene transcription after T cell activation (40). The inactive form of NFAT is hyperphosphorylated and resides in the cytosol. After TCR stimulation, elevated calcium levels lead to the activation of a calcium-dependent phosphatase, calcineurin, which in turn dephosphorylates NFAT. Dephosphorylated NFAT is then able to translocate into the nucleus and induce transcription of NFAT-dependent genes, such as IL-2, in cooperation with other transcription factors. Sustained elevation in intracellular calcium is required for NFAT-dependent gene expression, as NFAT is rapidly phosphorylated in the nucleus by kinases, such as GSK3, and exported back to the cytosol (41, 42).

The transcription factor AP-1 is a heterodimer of members of the Fos and Jun families. AP-1 activity is regulated both at the level of transcription and post-translational modification. For instance, Fos expression requires signals transduced by the mitogen-activated protein (MAP) kinase, the extracellular signal-regulated protein kinase (ERK), which is downstream of the small G protein Ras (43). On the other hand, to be transcriptionally active, Jun family proteins need to be phosphorylated. This requires another MAP kinase, c-Jun N-terminal kinase (JNK), whose activation needs Rho family small G proteins (44, 45). Different combinations of specific Fos and Jun family members may have distinct effects in transcriptional regulation.

The transcription factor NFκB consists of homo- or hetero-dimers of the Rel family proteins, which have five major members: RelA (p65), RelB, c-Rel, NFκB1 (p50 and its precursor p105), and NFκB2 (p52 and its precursor p100). In resting cells, NFκB is tightly controlled by IκB proteins. Upon T cell activation, IκB molecules are phosphorylated by IκB kinases (IKK), ubiqitinated, and degraded, allowing NFκB to translocate to the nucleus. The mechanisms of the activation of IKK after TCR ligation are as yet not fully elucidated. However, PKCθ and the CARMA1-Bcl10-MALT1 complex are apparently required (46). Furthermore, recent data have implicated caspase-8 (47) and PDK1 (48) in NFκB activation through Bcl10-MALT1.

Once in the nucleus, activated transcription factors cooperate in activating *de novo* gene transcription. Significantly, the promoter of the *IL-2* gene contains binding sites for several families of transcription factors including NFAT, AP-1, and NFκB. IL-2 is a T cell growth factor that is vital for T cell survival and proliferation, and is thus crucial for an effective adaptive immune response. Lack of IL-2 will lead T cells to a state of unresponsiveness. Therefore, production of IL-2 is considered a hallmark or a gold standard of T cell activation.

Costimulation in T cell activation

In addition to the signal initiated by the interaction of TCR with MHC/peptide, optimal activation of T cells requires a second signal, known as costimulation. If T cells are stimulated through Their TCR without costimulation, they may enter a state of unresponsiveness, known as anergy (49, 50). Costimulation provides additional signals that enhance cell survival, promote cell proliferation, and enhance effector functions such as cytokine production, among others. There are two major families of receptors that have been shown to provide critical costimulatory signals to T cells: One is the tumor necrosis factor receptor (TNFR) family that includes OX40, CD27, CD30, 4-1BB, and HVEM (51); the other is the CD28 family that includes CD28 and inducible costimulator (ICOS), which belongs to the immunoglobulin superfamily (52, 53). Additionally, recent studies also suggest possible crosstalk between members of the TNFR and CD28 families (54, 55).

The contribution of each receptor to T cell activation and effector function depends on its cell surface expression pattern and on the status of the T cells. For instance, with the exception of CD27 and HVEM, none of the TNFR family members is expressed on naïve T cells; the expression of these receptors is highly up-regulated in effector T cell (reviewed in (51)). This expression pattern suggests that perhaps the major role of the TNFR family costimulatory receptors is to enhance T cell effector functions. Similarly, the CD28 family member ICOS is not constitutively expressed on naïve T cells; its expression is induced after TCR activation (56). In contrast, CD28 is

constitutively expressed on naïve T cells, and provides the primary costimulatory signal for the activation of naïve T cells (see below). In addition to CD28 and ICOS, the CD28 family also has members that function to inhibit T cell activation, such as CTLA-4, PD-1 and BTLA. These negative costimulatory receptors, also referred to as "co-inhibitory" receptors are crucial to maintain homeostasis of the immune system after a response (57).

CD28 was the first receptor identified to provide a positive costimulatory signal to T cell activation (58), and CD28 costimulation is the best studied costimulatory pathway. Because CD28 is expressed constitutively on all $CD4^+$ T cells (59), it is able to provide the crucial primary costimulatory signal to naïve T cells. CD28 is a 44kDa glycosylated transmembrane protein belonging to the immunoglobulin superfamily and is expressed on T cells as a disulfide-linked homodimer. At the complementary determining region 3-like region (CDR3-like region) of its extracellular domain, there is a conserved MYPPPY motif. It is this MYPPPY motif that mediates the interaction of CD28 with its ligands B7.1 and B7.2, expressed on APCs such as dendritic cells and B cells (60). Antibodies that bind the MYPPPY region, such as the 37.51 clone, are used to stimulate CD28, mimicking the binding to its natural ligand (61). The intracellular tail of CD28 lacks any direct enzymatic activity. Thus, the signal transduced by CD28 is through the recruitment of other signaling molecules to its intracellular tail. Within the 41 amino acid residues of the CD28 intracellular tail, there are four tyrosine residues, one of which is in a conserved YMNM motif. Phosphorylation of the tyrosine in the YMNM motif provides a binding site for SH2 domain-containing signaling proteins, such as Grb2 and

the regulatory p85 subunit of PI3K (62). Moreover, there are two proline-rich motifs in the CD28 intracellular tail that provide binding sites for SH3 domain containing proteins (63).

Studies in which the CD28 gene is mutated or the interaction of CD28 with its ligands is disrupted have demonstrated that CD28 is crucial in many aspects of the generation of an efficient immune response. First, CD28 costimulation prevents T cell anergy. This has been demonstrated by *in vitro* experiments where CD28-B7 interactions are blocked by either antibodies or soluble CTLA4-Ig fusion proteins. Under these conditions, T cells enter an anergic state, which can be rescued only by delivering CD28 costimulatory signals or by providing extra IL-2 (64-67). These data demonstrate that CD28 signaling plays a critical role in preventing T cell anergy and controlling T cell responsiveness. Second, in the presence of suboptimal TCR/CD3 signaling, CD28 ligation promotes IL-2 production by enhancing IL-2 transcription, mRNA stability and translation. In addition to IL-2, CD28 costimulation also enhances production of other cytokines, such as IL-4, IL-5, IFNγ, and GM-CSF (68, 69). CD28 costimulation also up-regulates the expression IL-2 receptor α and β chains (70, 71). Increased IL-2 and IL-2 receptor interactions then enhance proliferation of activated T cells. Furthermore, CD28 costimulation can promote cell cycle progression by down-regulating the negative regulators GSK3 and $p27^{Kip}$ (72). It has also been established that the signal through CD28 promotes survival of activated T cells, through up-regulation of the anti-apoptotic Bcl-2 family member, Bcl-xL (73). In addition, after CD28 ligation, activated Akt can

prevent Fas-mediated apoptosis by inhibiting the formation of the death-inducing signaling complex (DISC) (74), providing another mechanism by which CD28 costimulation promotes T cell survival. Finally, CD28 costimulation promotes energy metabolism in activated T cells by increasing their glucose uptake and glycolytic rate in response to activation. These responses require the activation of PI3K-Akt after CD28 stimulation (75, 76). Increased metabolism would provide the energetic and biosynthetic needs of sustaining a response by activated T cells.

The signaling mechanisms of CD28 costimulation have not yet been fully elucidated. No unique signaling molecule or pathway has been identified that is solely activated by CD28 stimulation alone. Stimulation of both TCR and CD28 is needed to effect T cell activation, which makes it difficult to dissect these two pathways. Perhaps this has led to the notion that CD28 signaling provides "quantitative" assistance to T cell activation. However, evidence has indicated a difference between CD28 and TCR signaling, in that CD28 signaling is insensitive to inhibition by cyclosporine A and thus is independent of calcium and calcineurin (60, 77, 78). Although many signaling molecules, such as Vav, SLP-76 and Grb2 have been implicated in transducing the CD28 signal, mounting evidence has suggested that PI3K and its down-stream pathways are crucial for CD28 costimulation. After CD28 ligation, the tyrosine residues on the CD28 intracellular tail, particularly the one in the YMNM motif, are phosphorylated. This phosphorylation requires the Src kinases Lck and Fyn (79). Phosphorylation of the tyrosine in the YMNM motif creates a binding site for the SH2 domain of the regulatory

subunit of PI3K, p85 (80-82); the binding affinity with p85 is 10-100 fold higher than that with Grb2 (83). More importantly, it has been demonstrated that ligation of CD28 by B7.1 or B7.2 can induce the accumulation of the products of active PI3K, D-3 phosphoinositide lipids including PIP_3 (84, 85).

The best studied down-stream signaling molecule of PI3K is the PH domain containing serine/threonine kinase Akt, whose activation, as measured by its phosphorylation at S473 and/or T308, is often used as an indication of PI3K activity. Ligation of CD28 can lead to Akt activation (86-88), and many of the above mentioned functions of CD28 costimulation can be attributed to Akt activation. The PI3K product PIP_3 induces localization of Akt to the membrane. Akt is then phosphorylated and activated by PDK1 (89). Activated Akt, in turn, has multiple down-stream targets, including transcription factors such as NFκB, FoxO and CREB, and other kinases such as GSK3 and mTOR (90). Through activating NFκB, activated Akt enhances transcription of genes encoding cytokines, such as IL-2, and other proteins like Bcl-xL. By inhibiting GSK3, signaling through activated Akt can help to maintain activated NFAT in the nucleus, since GSK3 has been shown to phosphorylate NFAT and facilitate its export from the nucleus (91). Through activating mTOR and down-stream p70S6K, activated Akt enhances protein translation. Thus, the PI3K-Akt signaling pathway can be viewed as a signature of CD28 costimulation (Figure 1-2).

Figure 1-2 PI3K pathways in CD28 costimulation

Stimulation of CD28 leads to the phosphorylation of tyrosine residues on its intracellular tail, especially the one in the YMNM motif. Phosphorylation on this site recruits and activates PI3K, which in turn generates PIP_3. Both PDK1 and Akt are recruited to the membrane via the binding of the PH domain and PIP_3. Subsequently, Akt is activated by PDK1, and then regulates multiple down-stream pathways, including transcription factors such as NFκB and FoxO, and other kinases such as mTOR and GSK3. In this way the PI3K pathway transduces the signal from CD28 to promote gene expression, translation, cell survival, cell cycle progression, and energy metabolism.

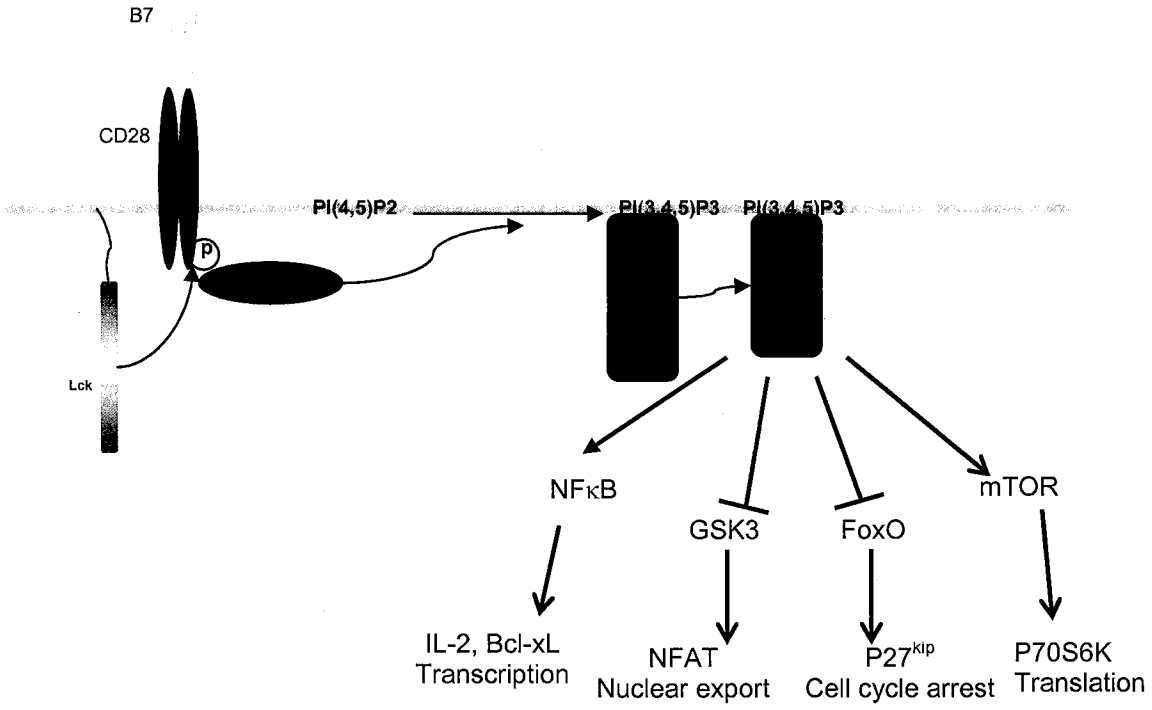


Figure 1-2 PI3K pathways in CD28 costimulation

As mentioned above, CD28 costimulation promotes transcription of a variety of genes, including cytokines like IL-2. Evidence indicates that NFκB is perhaps the most relevant transcriptional target of CD28 costimulation (92). A link between CD28 costimulation and NFκB was found initially with the identification of a CD28 responsive element (CD28RE) in the *IL-2* gene promoter. This element was found to specifically bind to nuclear complexes from anti-CD28 stimulated T cells. The sequence of this CD28RE is similar to the NFκB binding site (93-96). Subsequently, CD28RE has been identified in many other genes, including Bcl-xL, CD40 ligand (CD40L), IL-3, and GM-CSF (95, 97, 98). It is not quite clear how the CD28 signal induces NFκB activation in T cells. However, several studies have shown that IKK activity is increased by CD28 stimulation (98-100). Akt is also involved in IKK activation, although Akt may not directly phosphorylate IKK (90, 101). Furthermore, Akt has also been involved in directly regulating NFκB activity by phosphorylating RelA/p65 of NFκB (102, 103). In addition, a recent study demonstrated that a PI3K down-stream protein, PDK1, plays a critical role in activating IKK through regulating the activation of PKCθ and the recruitment of CARMA1-BCL10-MALT1 complex (48). Therefore, it seems that the PI3K and its down stream pathways play a major role in transducing CD28-induced NFκB activation.

Itk and Tec family tyrosine kinases

In 1990, a novel non-receptor tyrosine kinase was identified from a mouse liver cDNA library and was found to be expressed in hepatocellular carcinoma cell lines; this kinase was termed Tec (104). Subsequently, several other proteins were identified that share extensive sequence similarities with Tec, making the Tec family the second largest family of non-receptor kinases. There are five members of the Tec family, all of which are primarily expressed in hematopoietic cells. In addition to Tec, there are Btk (105, 106), Itk (also known as Tsk and Emt) (107-109), Rlk (also known as Txk) (110, 111), and Bmx (also known as Etk) (112). All Tec family members share the same overall domain structure (Figure 1-3). From the C-terminus, there is a kinase domain, followed by an SH2 domain and then an SH3 domain. This structure resembles that of the Src family of tyrosine kinases. However, Tec kinases lack the C-terminal regulatory tyrosine present in Src kinases, suggesting that these kinases require a distinct mechanism of regulation. N-terminal to the SH3 domain, there is a Tec homology (TH) domain, which comprises a Btk homology (BH) motif and one or two proline rich region(s) (PRR). Finally, at the N-terminus of the protein is a PH domain, which can bind to PIP_3 and facilitate membrane recruitment of Tec family kinases. There are some variations of this general domain structure. Specifically, Rlk does not possess an N-terminal PH domain. Instead, it has an N-terminal cysteine-string that can be palmitoylated and can result in Rlk recruitment to the plasma membrane. In addition, Bmx does not have a PRR in the TH domain.

Figure 1-3 Domain structures of Tec family kinases

Tec family kinases share an overall similar structure. This structure includes a C-terminal kinase domain followed by an SH2 and an SH3 domain. N-terminal to the SH3 domain, there is a Tec homology (TH) domain that consists of a Btk homology (BH) motif and one (Itk, Rlk) or two (Btk, Tec) proline rich region(s) (PRR). At the N-terminus of the protein, there is a pleckstrin homology (PH) domain. Exceptions to this structure include Rlk and Bmx. At its N-terminus, Rlk does not have a PH domain, but instead a cysteine-string. Bmx does not have a PRR motif in the TH domain.

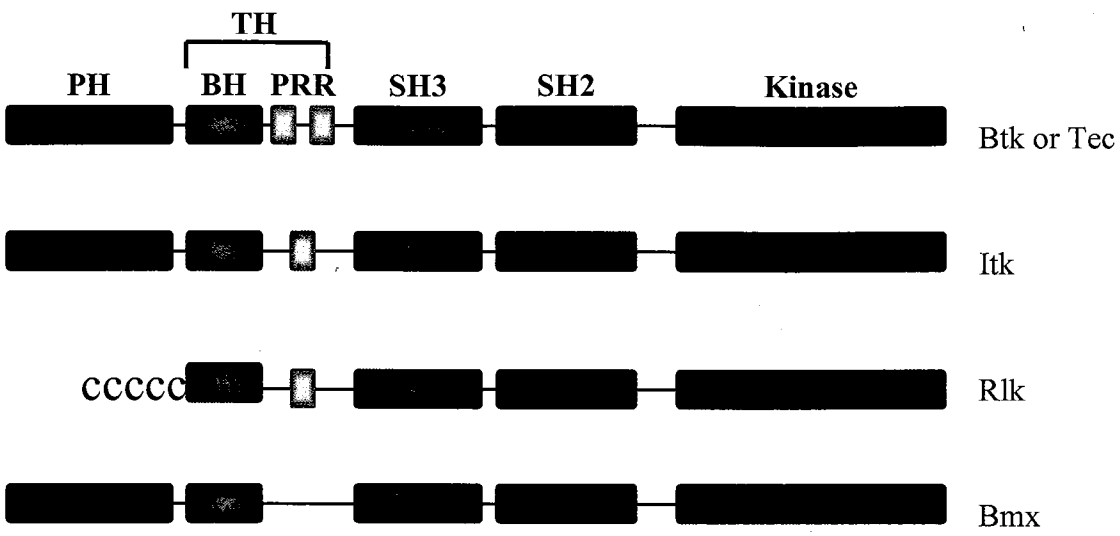


Figure 1-3 Domain structures of Tec family kinases

The importance of the Tec family kinases was first revealed when it was discovered that mutations in Btk were responsible for the human genetic disorder X-linked agammaglobulinemia (XLA) and the murine mutant X-linked immunodeficiency (*xid*), both of which are characterized by reduced serum immunoglobulins and defective B cell development (105, 106, 113, 114). At least three Tec family kinases are expressed in T cells; they are Tec, Itk and Rlk. So far, no mutation of these kinases has been related to any human T cell defects. However, studies of mice with targeted mutations in *Itk* or *Itk* and *Rlk* demonstrate that these Tec kinases are important in T cell development, activation and effector functions (115-122). Among these three Tec kinases, Itk seems to be predominant in T cells.

The activation of Itk is dependent on two major events: its localization to the plasma membrane and its phosphorylation at multiple sites. Upon TCR stimulation, the Src and Syk family kinases, as well as PI3K, are rapidly activated. Activation of Src and Syk kinases leads to the phosphorylation of the adaptor proteins LAT and SLP-76. Simultaneously, activated PI3K generates PIP_3. Increased PIP_3 recruits Itk to the membrane via the PH domain of Itk. This recruitment allows Itk to join a signaling complex consisting of SLP-76, Gads, Grb2, PLCγ1 and LAT (123-125). In this complex, Itk is able to be tyrosine-phosphorylated by the Src kinase Lck in its kinase domain (126). This is followed by Itk auto-phosphorylation on a tyrosine in its SH3 domain (127). These phosphorylation events result in full activation of the kinase. The localization of Itk to the membrane can be negatively regulated by the phosphatases PTEN and PHIP,

which dephosphorylate PIP_3 at the D-3 and D-5 positions, respectively, and convert PIP_3 back to PIP_2. This dephosphorylation thus prevents further recruitment of Itk to the membrane. It is believed that Tec is activated in the same manner as Itk. Because of the lack of PH domain, Rlk localization does not depend on PIP_3. Instead, palmitoylation of Rlk's N-terminal cysteine-string facilitates its recruitment to the membrane (128, 129). In addition, Itk is thought to be regulated by conformational changes directed by intra- and inter-molecular interactions involving its SH2 domain, SH3 domain and PRR motifs. For instance the PRR motif and the SH3 domain can mediate intra-molecular interaction within Itk, and this interaction is thought to be inhibitory (130). More recently, biochemical studies have shown that the peptidylprolyl isomerase cyclophilin A contributes to a conformation change in Itk via isomerization of a proline residue in its SH2 domain (131, 132).

Once the Tec kinases are activated, they are capable of phosphorylating and activating PLCγ1, which is also present in the LAT-Gads-SLP-76-Itk complex. Activated PLCγ1 then hydrolyzes PIP_2 to generate the second messengers IP_3 and DAG. As described above, IP_3 stimulates calcium mobilization and eventually leads to the activation of the NFAT transcription factors, while DAG activates PKC and RasGRP resulting in the activation of the NFκB, AP-1 and other pathways (Figure 1-1). Therefore, by activating PLCγ1, Tec kinases transduce TCR signals critical in regulating T cell development, activation, effector function and homeostasis. More recent studies have also implicated Itk in regulating actin polarization through interaction with Vav

after T cell activation, and this function seems not to require the kinase activity of Itk (133).

Consistent with a major role of Tec family kinases in regulating PLCγ1, T cells from *Itk*$^{-/-}$ or *Itk*$^{-/-}$*Rlk*$^{-/-}$ mice display diminished TCR-induced IP_3 production, calcium mobilization and MAP kinase activation (115, 117, 119). Functionally, these mice have impaired positive selection during thymocyte development. When *Itk*$^{-/-}$ or *Itk*$^{-/-}$*Rlk*$^{-/-}$ $CD4^+$ T cells are stimulated through the TCR *in vitro*, they produce decreased amounts of IL-2 and proliferate to a lower extent compared to wild-type T cells (115-117). Furthermore, these cells also show defective functional differentiation (119, 121, 134). Finally, T cells from *Itk*$^{-/-}$ mice also display a defect in activation induced cell death (AICD) (135). The *Tec* deficient T cells do not show appreciable defects (136). However, when wild type T cells were stimulated in the presence of antisense oligonucleotide against Tec, proliferation and IL-2 production were reduced, suggesting that Tec also functions in TCR signaling (137).

As described earlier, optimal activation of naïve T cells requires not only TCR signals but also costimulatory signals from CD28. Biochemical studies have implicated Itk in CD28 signaling. These studies showed that, following CD28 ligation of Jurkat cells, Itk associates with the CD28 intracellular tail and becomes phosphorylated (138). This interaction between Itk and CD28 has been shown to require functional Lck activity (79, 139). Additionally, the interaction has been mapped to the proline rich region of

CD28 and the SH3 domain of Itk (140). Additionally, Itk has been shown to be capable of phosphorylating all tyrosine residues of the CD28 intracellular tail (141). Together, these data suggest that Itk may act as a positive regulator in CD28 signaling. However, a functional study using primary T cells from *Itk*$^{-/-}$ mice failed to substantiate the conclusions from these biochemical studies (142). On the contrary, this study showed hyperresponsiveness of *Itk*$^{-/-}$ T cells to anti-CD28 stimulation; these cells proliferated to a much higher extent when compared to T cells from littermate controls. Although this study did not examine the biochemical mechanisms of the hyperresponsiveness, the results led the investigators to conclude that Itk is a *negative* regulator of CD28 signaling (142). However, based on our knowledge of TCR and CD28 signaling, it is difficult to reconcile such opposing roles for Itk in the TCR and the CD28 signaling pathways. The role of Itk in CD28 costimulation signaling will be analyzed and discussed in detail in Chapter II.

In T cells, Itk, Rlk, and Tec are expressed at different levels. Experiments using quantitative real-time PCR have determined that Itk mRNA is present at the highest level, Rlk mRNA levels two- to threefold lower and Tec mRNA at a level close to 100-fold lower than that of Itk (143). This hierarchy of expression may imply their relative importance in T cells. Indeed, a comparison among all the single deficient mouse lines reveals that the loss of Itk results in the most severe defects on T cell development and function. Further, the loss of Rlk has moderate defects and the loss of Tec has no obvious defect on T cell development and function (115-117, 136). However, a single

deficiency of any of these kinases, and even double-deficiency of both Itk and Rlk, does not eliminate TCR signaling; instead, all TCR signaling aspects are reduced. These effects are in striking contrast to those of mutations in more TCR-proximal molecules such as Zap-70. These observations have generated the notion that Tec kinases modulate or amplify the efficiency of downstream signaling (144, 145). However, functional redundancy appears to exist among Tec family kinases. For instance, the defects observed in *Itk* and *Rlk* double deficient T cells are more severe than those of *Itk* single deficient T cells (117). Similarly, the defects in *Btk* and *Tec* double deficient B cells are more severe than those in *Btk* single deficient B cells (136).

Work presented in this thesis

To further understand the role of the Tec family kinases in T cell activation, three different approaches have been taken to address different questions. First, as presented in Chapter II, we performed a thorough analysis of CD28-mediated signaling events and functional responses with purified naïve T cells from *Itk*$^{-/-}$ mice in a highly controlled stimulation system. Data from these set of studies definitively demonstrate that CD28 costimulation functions efficiently in naïve CD4^{+} T cells in the absence of Itk. Second, in order to further study the functions of Tec kinases in mice that are functionally deficient in all three Tec kinases, we generated transgenic mouse lines expressing a kinase-dead (KD) mutant of *Tec* on the *Itk*$^{-/-}$*Rlk*$^{-/-}$ background. These experiments are presented in Chapter III. Intriguingly, the expression of the transgene was detected at the mRNA level, but not at the protein level. Although we did not obtain our expected mouse model for further studies, the results hint at the potential importance of the Tec kinases in T cell development and/or survival. Finally, to further understand the function of the Tec family kinase Itk and to identify potential transcriptional targets of Itk, we used microarray technology to compare global gene expression profiles of naïve and stimulated *Itk*$^{-/-}$ versus *Itk*$^{+/-}$ CD4^{+} T cells. As presented in Chapter IV, this analysis resulted in a short list of differentially expressed genes in *Itk*$^{-/-}$ versus *Itk*$^{+/-}$ CD4 T cells, providing a starting point for further studies of Itk in T cell function.

Overall, these studies contribute to the general knowledge of the role of the Tec family kinases in CD4^{+} T cell activation. Using these three different approaches, we

were able to reveal some unexpected aspects of this family of kinases in T cells and to clarify some existing confusion about the role of Itk in CD28 costimulation signaling. The data also indicate the potential importance of selection pressure in T cell development and homeostasis.

CHAPTER II

THE ROLE OF ITK IN CD28 COSTIMULATORY SIGNALING

Introduction

As described above, two signals are required for the optimal activation of naive T cells, one from the TCR and the second from a co-stimulatory receptor. In the absence of the co-stimulatory signal, TCR engagement often leads to T cell death or non-responsiveness (49). On naïve T cells, the CD28 receptor provides the primary costimulatory signal following interaction with CD80 or CD86 (B7.1 or B7.2, respectively) on antigen presenting cells (reviewed in (53, 146)). Functionally, CD28 costimulation enhances the survival, cell cycle progression, energy metabolism, and cytokine production by activated T cells. Although tremendous effort has been directed at elucidating the signaling pathway(s) initiated by CD28 stimulation, the detailed mechanism by which CD28 costimulation operates has not yet been completely determined, in part due to the difficulty of distinguishing the TCR- versus the CD28-mediated signals in primary T cells.

The Tec family tyrosine kinase Itk has previously been implicated in CD28 signaling. While Itk is primarily associated with TCR signaling (143, 147, 148), a number of biochemical studies have demonstrated an interaction between Itk and CD28. Specifically, Itk co-immunoprecipitates with CD28 from Jurkat tumor cells, and in addition, is tyrosine-phosphorylated in response to CD28 crosslinking (138, 140). *In vitro* studies using recombinant proteins indicate that Itk binding to CD28 depends on the activity of the Src-family tyrosine kinase, Lck (79), a conclusion that was reinforced by data from Lck-deficient Jurkat T cells (139). Structure-function analysis of CD28 further

demonstrated that the SH3 domain of Itk binds to proline-rich sequences in the CD28 cytoplasmic tail, an interaction that has been suggested to enhance Itk kinase activity (140). Finally, Itk has been shown to phosphorylate all four tyrosine residues of the CD28 cytoplasmic tail in *in vitro* kinase assays (141), providing additional evidence for a positive role of Itk in CD28 signaling.

To date, only a single study has addressed the role of Itk in CD28 signaling in primary T cells. Surprisingly, this study concluded that Itk is a negative regulator of CD28 signaling. This latter conclusion was based on the finding that $CD4^+$ T cells from $Itk^{-/-}$ mice showed enhanced proliferative responses to CD28 costimulatory signals compared to cells from wild type mice (142). One complication of this initial study is the fact that $Itk^{-/-}$ mice have a greatly increased population of previously-activated/memory $CD4^+$ T cells compared to controls. This altered subset distribution of naïve versus memory $CD4^+$ T cells in $Itk^{-/-}$ mice might have skewed the responses of these cells to TCR plus CD28 stimulation, independently of a role for Itk in CD28 signaling. Because of this concern, we chose to re-address the role of Itk in CD28 signaling using a panel of assays that assess CD28 signaling in the presence, as well as the absence, of TCR stimulation. Overall, the data demonstrate that Itk is not a negative regulator of CD28 costimulatory activity; in contrast, to the best of our knowledge, all aspects of CD28 signaling are intact in the absence of Itk.

Results

Increased numbers of memory phenotype CD4$^+$ T cells in Itk$^{-/-}$ mice

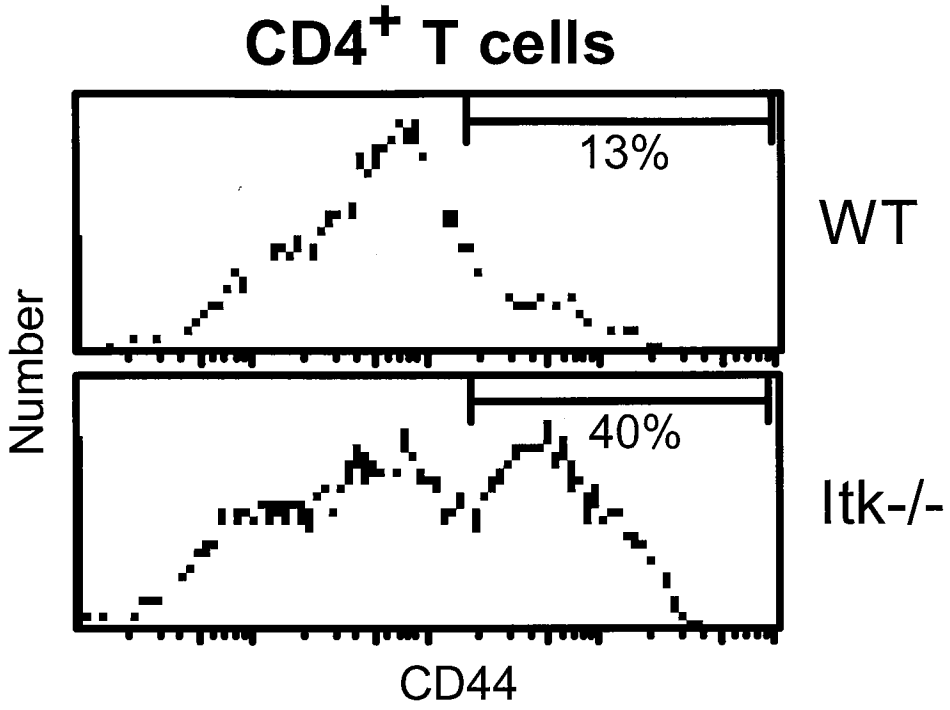
Previous studies have documented that *Itk*$^{-/-}$ mice have a modest defect in positive selection, resulting in a ~two-fold reduction in the total numbers of CD4$^+$ T cells in the spleen and lymph nodes of *Itk*$^{-/-}$ mice compared to controls (115, 116, 120). Surprisingly, however, the population of CD4$^+$ T cells in *Itk*$^{-/-}$ mice is highly enriched for cells with a previously-activated or memory phenotype (CD4$^+$CD44^{hi}CD62L^{lo}). As shown in Figure 2-1A, we routinely find a 3-5-fold increase in the proportion of CD4$^+$ T cells expressing high levels of CD44 in lymph nodes of *Itk*$^{-/-}$ mice compared to wild type C57BL/10 mice. In addition, an increased number of CD4$^+$CD44^{hi} T cells is detectable in *Itk*$^{-/-}$ mice as early as four weeks after birth. When CD4$^+$CD44^{hi} T cells were analyzed for CD69 and CD25 expression, fewer cells from *Itk*$^{-/-}$ mice compared to controls expressed these early activation markers, suggesting that these cells have not been recently activated (Figure 2-1B). Overall, these data indicate that, while total T cell numbers are reduced in the *Itk*$^{-/-}$ mice, the proportion of cells with a memory phenotype are actually increased. Although it is not clear why these cells accumulate in *Itk*$^{-/-}$ mice, one possibility relates to a modest defect in activation-induced cell death (AICD), as previously described (135).

Figure 2-1 *Itk*$^{-/-}$ mice have increased activated/memory-like CD4^{+} T cells

A. Lymph node cells from C57BL/10 (WT) and *Itk*$^{-/-}$ mice were stained with anti-CD4 and anti-CD44 antibodies. Histograms show CD44 staining on gated CD4^{+} T cells. The numbers on each histogram indicate the percentage of CD44^{hi} cells in the CD4^{+} population. Data shown are representative of five experiments.

B. Lymph node cells from WT and *Itk*$^{-/-}$ mice were stained with antibodies to CD4, CD44, and CD69 or CD25. Mean percentages ± SD of CD69^{+} or CD25^{+} cells among the CD4^{+}CD44^{hi} population are indicated for 2 WT and 6 *Itk*$^{-/-}$ mice analyzed.

A.



B.

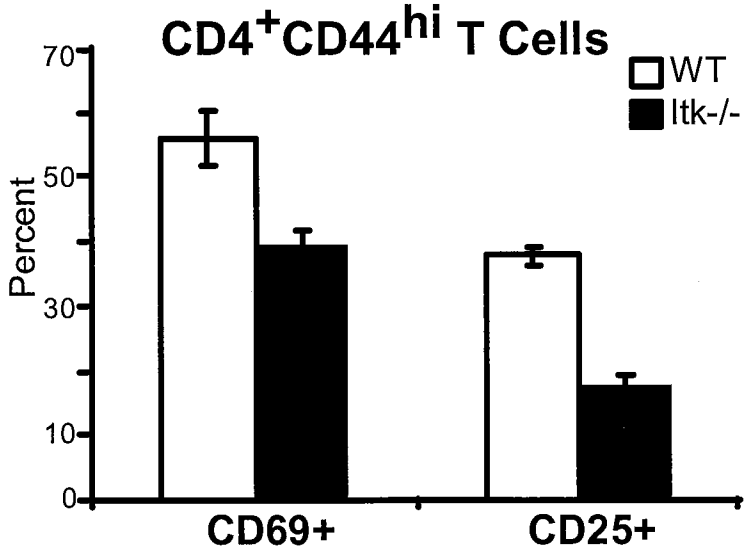


Figure 2-1 ***Itk*$^{-/-}$ mice have increased activated/memory-like CD4^{+} T cells**

Naïve Itk$^{-/-}$ CD4^{+} T cells are not hyperresponsive to CD28 costimulation

A previous study described increased responsiveness of *Itk*$^{-/-}$ CD4^{+} T cells compared to wild type CD4^{+} T cells following stimulation through CD28, leading to the conclusion that Itk is a negative regulator of CD28 signaling (142). Consistent with these earlier data, we also observe that when equal numbers of total CD4^{+} T cells from *Itk*$^{-/-}$ mice or wild type control mice are stimulated with PMA plus anti-CD28-coated beads, the proliferative response of the *Itk*$^{-/-}$ CD4^{+} T cells is significantly higher than that of the control cells (Figure 2-2A). However, since the CD4^{+} T cell population from *Itk*$^{-/-}$ mice contains an increased proportion of memory phenotype cells, we reasoned that the increased responsiveness of these cells might be attributable to this altered subset distribution. To test this possibility, we repeated this experiment using highly purified naïve CD4^{+} (CD44^{low}) T cells. As shown in Figure 2-2B, purified naïve *Itk*$^{-/-}$ CD4^{+} T cells are not hyperresponsive to CD28 costimulation. To further support the idea that increased proportions of activated CD4 T cells may increase the overall response of total CD4 T cells upon stimulation, purified *Itk*$^{-/-}$ CD44^{high} CD4 T cells proliferate much more vigorously than naïve CD4 T cells stimulated with PMA plus anti-CD28 (Figure 2-2C).

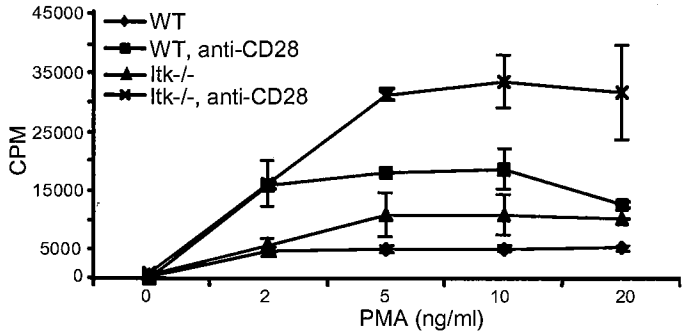
Figure 2-2 Purified naïve *Itk*$^{-/-}$ CD4^{+} T cells are not hyperresponsive to CD28 stimulation

A. Total CD4^{+} T cells were from WT and *Itk*$^{-/-}$ mice were stimulated with the indicated concentrations of PMA in the presence of anti-CD28 antibody-coated beads ("anti-CD28") or isotype control antibody-coated beads. Cell proliferation was measured by ^{3}H-thymidine incorporation at 72h.

B. Sorted naïve CD4^{+} T cells from WT and *Itk*$^{-/-}$ mice were stimulated with the indicated concentrations of PMA in the presence of anti-CD28 antibody-coated beads ("anti-CD28") or isotype control antibody-coated beads. Cell proliferation was measured by ^{3}H-thymidine incorporation at 72h. Data shown are representative of three experiments.

C. Sorted naïve (CD44^{low}) and activated (CD44^{high}) CD4 T cells from *Itk*$^{-/-}$ mice were stimulated with the indicated concentrations of PMA in the presence of anti-CD28 antibody-coated beads. Cell proliferation was measured by ^{3}H-thymidine incorporation at 72h.

A.



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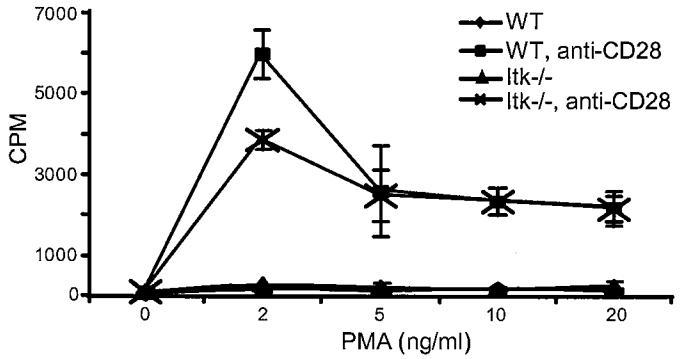


C.

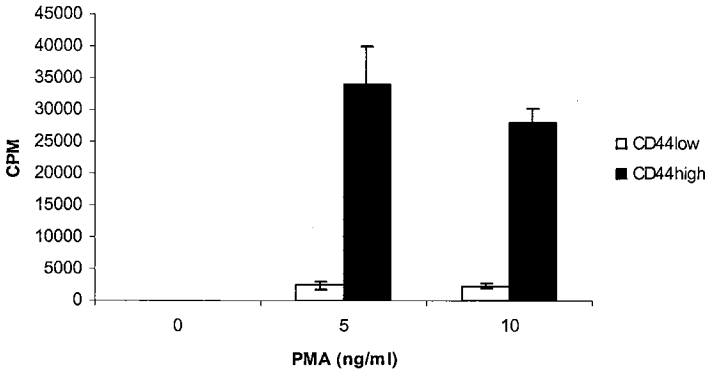


Figure 2-2 Purified naïve *Itk*$^{-/-}$ CD4^{+} T cells are not hyperresponsive to CD28 stimulation

These findings re-opened the question of the role of Itk in CD28 signaling and function, and suggested that further investigation of this issue was warranted. Therefore, we proceeded to examine the responses of *Itk*$^{-/-}$ T cells to stimulation through the TCR plus CD28, using conditions in which T cell activation is stringently dependent on CD28 costimulation. For these experiments, purified *Itk*$^{-/-}$ and wild type naïve CD4^{+} T cells were stimulated with antibody-coated latex beads. When stimulated with beads coated with anti-CD28 antibody alone, or anti-CD3 antibody alone, neither population of T cells exhibited any proliferative response. In contrast, when cells were stimulated with beads coated with a mixture of anti-CD3 plus anti-CD28 antibodies (1:9 ratio), both populations of cells proliferated robustly. While the response of the *Itk*$^{-/-}$ cells was reduced compared to that of the wild type T cells, this response still represents a ~300-fold enhancement over the response to anti-CD3 antibody alone (Figure 2-3A). Based on these data, we conclude that CD28 costimulatory activity functions quite efficiently in naïve *Itk*$^{-/-}$ CD4^{+} T cells.

To substantiate these findings using *bona fide* MHC/peptide stimulation in the presence or absence of B7.1 (CD80), we examined purified naïve CD4+ T cells from *Rag2*$^{-/-}$*5C.C7TgItk*$^{-/-}$ and *Rag2*$^{-/-}$*5C.C7TgItk*$^{+/-}$ mice. The 5C.C7 TCR recognizes moth cytochrome *c* residues 87-103 (MCC) peptide presented on MHC class II IE^{k} molecules (149). T cells were stimulated with CHO-IE^{k} cells or CHO-IE^{k}/B7.1 cells as APCs in the presence of varying concentrations of the MCC peptide, and proliferative responses were measured. At each given peptide concentration, both *Itk*$^{+/-}$ as well as *Itk*$^{-/-}$ CD4^{+} T cells

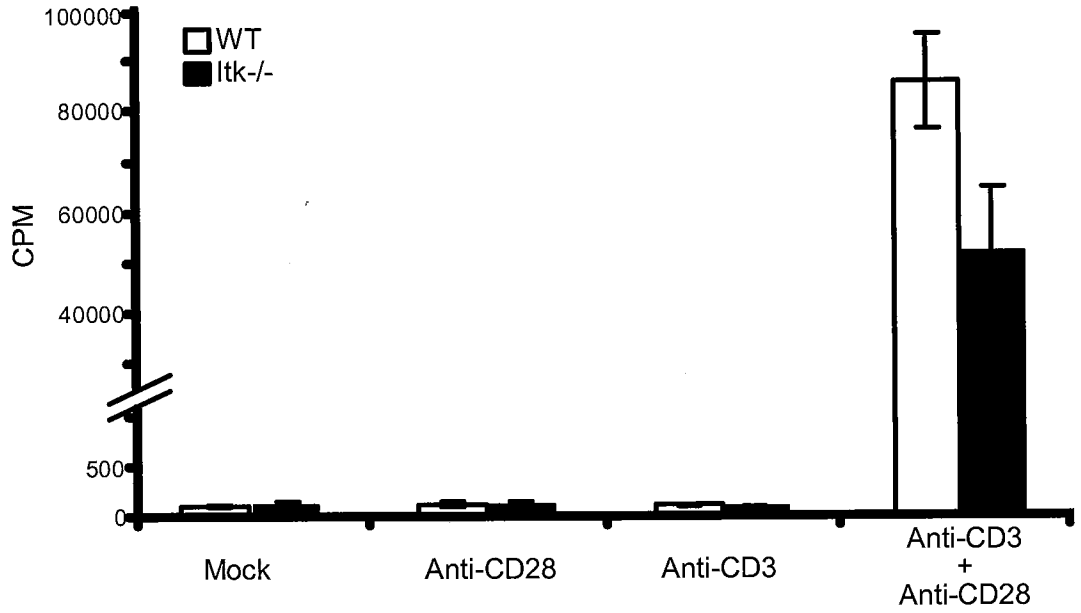
show a similar degree of increased responsiveness to stimulation with APCs expressing B7.1 compared to APCs that lack B7.1 (Figure 2-3B). These data confirm the conclusion that CD28 costimulation functions effectively in the absence of Itk.

Figure 2-3 CD28 costimulation functions efficiently in the absence of Itk

A. Sorted naïve $CD4^+$ T cells ($CD44^{lo}$) from WT and *Itk*$^{-/-}$ mice were stimulated with antibody-coated beads as indicated. Cell proliferation was measured by ^{3}H-thymidine incorporation 72h after stimulation. Mock, cells incubated with isotype control antibody-coated beads alone. Data shown are representative of three experiments.

B. Purified $CD4^+$ T cells from *Rag2*$^{-/-}$*5C.C7Tg Itk*$^{-/-}$ and *Rag2*$^{-/-}$*5C.C7Tg Itk*$^{+/-}$ mice were stimulated with mitomycinC-treated CHO-IE^k (IE^k) or CHO-IE^k/B7.1 (IE^k+B7.1) cells and the indicated concentrations of MCC peptide. Cell proliferation was measured by ^{3}H-thymidine incorporation 72h after stimulation.

A.



B.

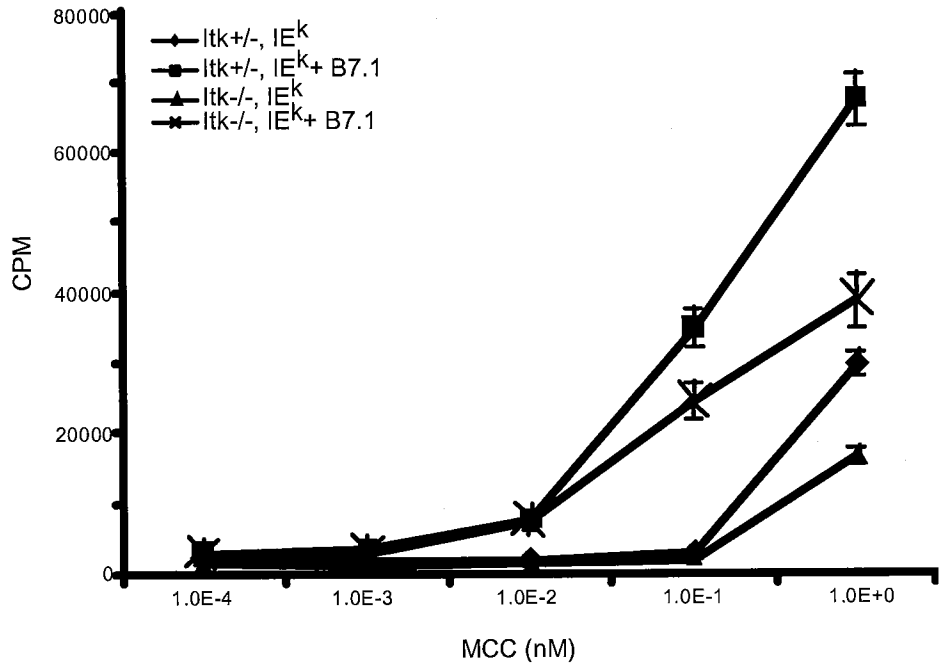


Figure 2-3 CD28 costimulation functions efficiently in the absence of Itk

CD28 costimulation enhances gene expression in the absence of Itk

One function of CD28 costimulation is to enhance gene expression induced by TCR signaling. Among the genes most dramatically affected by CD28 costimulation are those encoding the cytokine IL-2, the survival factor Bcl-xL, and the effector molecule CD40L (73, 95, 97, 150). To assess whether CD28 costimulation leading to enhanced gene expression was functional in the absence of Itk, wild type and *Itk*$^{-/-}$ naïve CD4^{+} T cells were stimulated with a variety of conditions, and IL-2, Bcl-xL, and CD40L mRNA levels were measured by real-time quantitative PCR. Whereas each one of these genes showed a unique response to the stimulation conditions tested, they all exhibited enhanced mRNA levels following CD28 costimulation (Figure 2-4). In addition, this pattern was observed for both wild type and *Itk*$^{-/-}$ T cells. Interestingly, the increased levels of IL-2, Bcl-xL, and CD40L mRNA seen with CD28 plus CD3 stimulation, compared to CD3 stimulation alone, are completely abolished following addition of the PI3K inhibitor, LY294002 (Figure 2-4). This effect is also observed for the CD28 costimulatory effect on Bcl-xL mRNA levels in response to PMA stimulation. Together, these data demonstrate the effectiveness of CD28 costimulatory signals to enhance gene expression in the absence of Itk, and further, indicate the importance of PI3K in this activity.

Figure 2-4 CD28–mediated enhancement of gene expression functions efficiently in the absence of Itk

Naïve $CD4^+$ T cells were purified from *WT* and $Itk^{-/-}$ mice. Cells were stimulated with antibody-coated beads or PMA as indicated, in the presence or absence of the PI3K inhibitor LY294002 at 10μM. Six hours after stimulation, cells were harvested, total RNA was isolated, and cDNA was synthesized. The levels of IL-2 (A), Bcl-xL (B), and CD40L (C) mRNA were determined by real-time quantitative PCR. Data were normalized to the expression of GAPDH mRNA in each sample. Data shown are representative of three experiments. NS, non-stimulated.

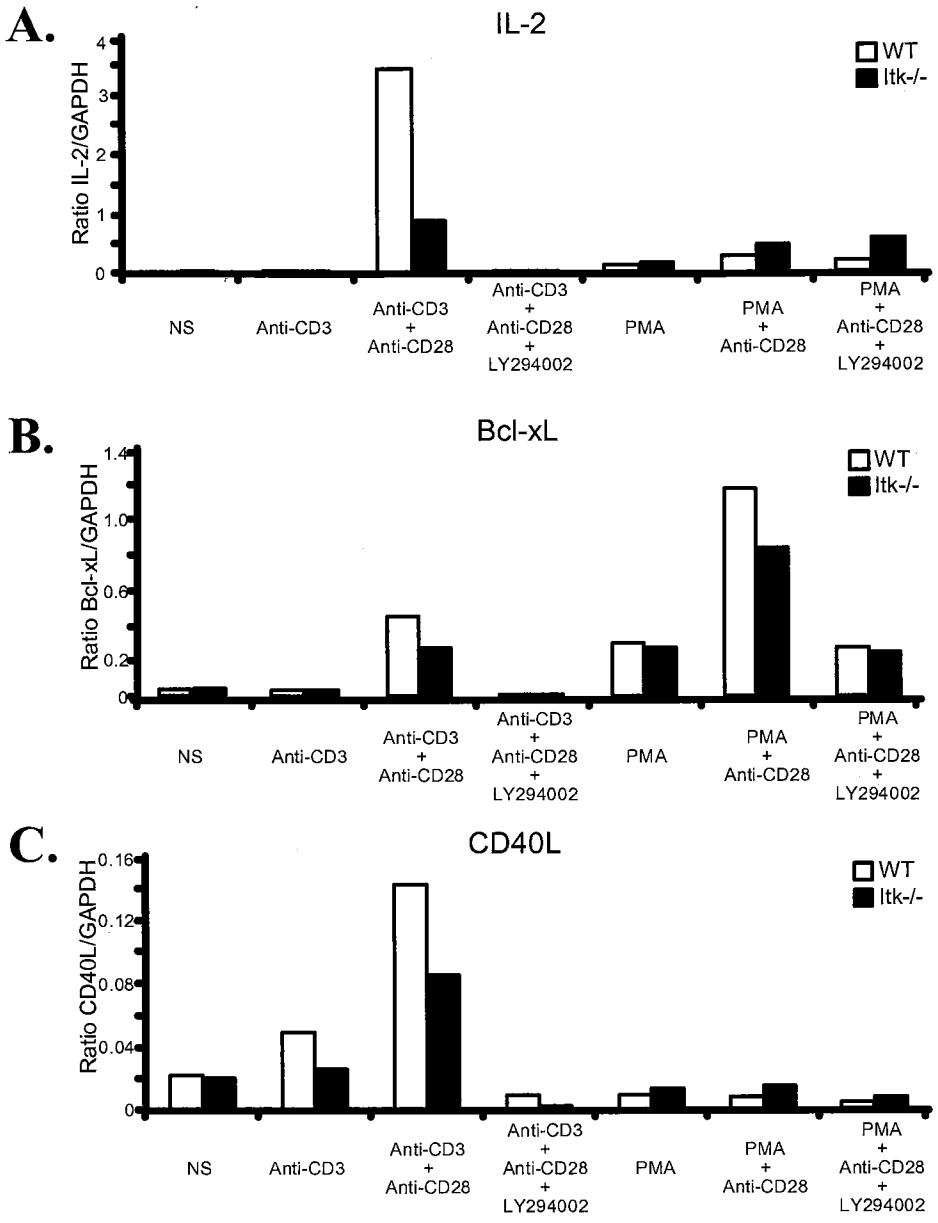


Figure 2-4 CD28–mediated enhancement of gene expression functions efficiently in the absence of Itk

CD28 costimulation activates NFκB in the absence of Itk

The transcription factor NFκB, is an important target of the CD28 costimulatory pathway (92). To assess the ability of CD28 costimulation to activate NFκB in the absence of Itk, we stimulated wild type and *Itk*$^{-/-}$ naïve CD4^{+} T cells with anti-CD3 antibody alone or in combination with anti-CD28 antibody. After 60 minutes, nuclear lysates were prepared from the cells, and levels of activated NFκB were examined by ELISA. As shown in Figure 2-5, anti-CD3 antibody stimulation is not sufficient to induce detectable NFκB activation in either cell type, whereas anti-CD3 plus anti-CD28 antibody stimulation induced significant levels of activated NFκB in both wild type and *Itk*$^{-/-}$ T cells (Figure 2-5). Although in these experiments we only measured the activation of p65 (RelA), we believe that other NFκB family members such as cRel would be activated similarly. These data confirm the ability of CD28 costimulatory signaling to activate the NFκB transcription factors in the absence of Itk.

Figure 2-5 CD28 costimulation activates NFκB in the absence of Itk

Naïve $CD4^+$ T cells were purified from WT and *Itk*$^{-/-}$ mice. Cells were stimulated with antibody-coated beads as indicated. One hour after stimulation, cells were harvested and nuclear lysates prepared. Activated NFκB p65 was measured by ELISA. Data shown are representative of three experiments. Mock, cells stimulated with isotype-control antibody-coated beads.

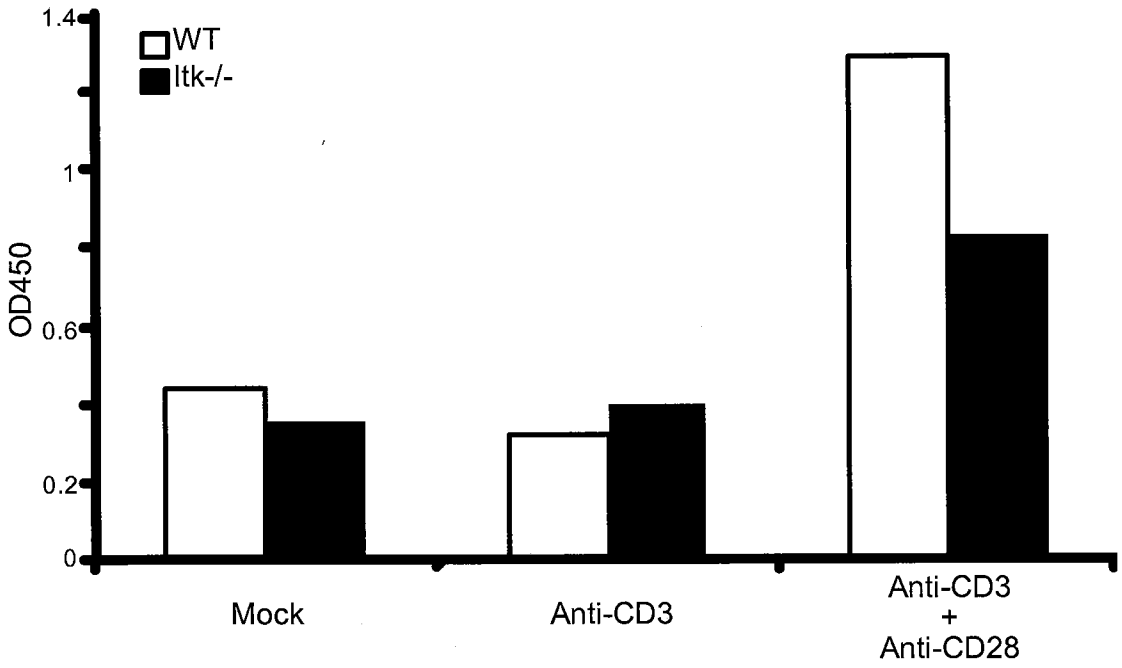


Figure 2-5 CD28 costimulation activates NFκB in the absence of Itk

CD28 costimulation does not enhance TCR-induced calcium mobilization in CD4$^+$ T cells

Calcium mobilization has also been implicated as a target of CD28 costimulation (151, 152). Specifically, when Acuto and colleagues examined intracellular biochemical events after CD28 engagement by expressing a tailless mutant of CD28 in Jurkat cells, PLCγ phosphorylation and calcium mobilization were found to be diminished in these cells after CD28 crosslinking. These defects could be partially rescued by overexpression of Itk. These data led to the conclusion that CD28 costimulation enhances calcium mobilization through Itk activation (152). To examine the potential role of CD28 in calcium mobilization, and to test whether Itk is involved, we performed calcium flux analysis and compared wild type and *Itk*$^{-/-}$ CD4$^+$ T cells, stimulated through TCR/CD3 or TCR/CD3 plus CD28. As shown in Figure 2-6, CD28 stimulation alone does not induce a calcium flux in wild type or *Itk*$^{-/-}$ CD4$^+$ T cells. In contrast, CD3 stimulation alone induced a robust calcium flux in wild type, but not *Itk*$^{-/-}$ CD4$^+$ T cells. This is consistent with previously published data (115). Importantly however, when CD4$^+$ T cells were stimulated by CD3 plus CD28 crosslinking, the calcium response was similar to that seen when cells were stimulated through CD3 alone. A serial titration of the amount of anti-CD3 was tested. Anti-CD28 did not enhance CD3-induced calcium mobilization at any dose. These data demonstrate that the CD28 costimulatory signal does not enhance TCR-induced calcium mobilization in primary T cells.

Figure 2-6 CD28 crosslinking does not enhance TCR-induced calcium mobilization

$CD4^+$ T cells from *WT* and *Itk*$^{-/-}$ mice were loaded with Ca^{++} indicator dyes. The cells were stimulated with anti-CD28 alone, anti-CD3 alone, or anti-CD3 plus anti-CD28 crosslinking. The calcium flux over time after stimulation was analyzed by FACS. Data shown are representative of a serial titration of anti-CD3 antibody.

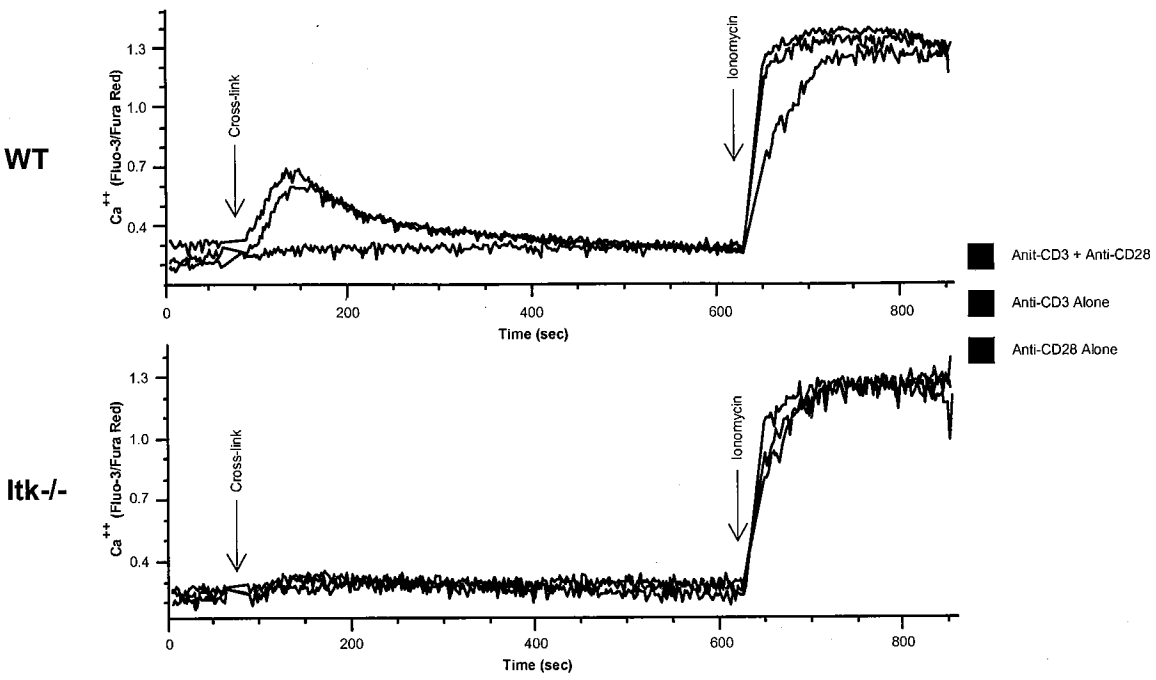


Figure 2-6 CD28 crosslinking does not enhance TCR-induced calcium mobilization

Stimulation of CD28 alone induces phosphorylation of Akt and GSK3β in the absence of Itk

Although signaling through CD28 alone does not lead to functional changes in T cells, several biochemical events can be detected following CD28 stimulation. One such signaling pathway is the activation of PI3K, leading to the phosphorylation and activation of the serine/threonine kinase, Akt, and the subsequent phosphorylation of GSK3β (90). To examine whether these events occurred normally in the absence of Itk, naïve $CD4^+$ T cells from wild type and $Itk^{-/-}$ mice were stimulated with anti-CD28 antibody alone, and Akt and GSK3β phosphorylation were detected with phospho-Akt- and phospho-GSK3β-specific antibodies. As shown in Figure 2-7, Akt and GSK3β were both phosphorylated comparably in wild type and $Itk^{-/-}$ $CD4^+$ T cells. These data demonstrate that the CD28-PI3K-Akt-GSK3β signaling pathway is intact in the absence of Itk, indicating that Itk is not required for CD28 signaling.

Figure 2-7 Stimulation of CD28 alone induces phosphorylation of Akt and GSK3β in the absence of Itk

Naïve $CD4^+$ T cells were purified from *WT* and $Itk^{-/-}$ mice. Cells were stimulated with plate-bound anti-CD28 antibody for the indicated times. Lysates were prepared and protein was resolved by SDS-PAGE. Akt phosphorylation (Ser473) and GSK3β phosphorylation (Ser9) were detected by immunoblotting with phospho-specific antibodies. Membranes were stripped and re-probed with antibodies to the p85 subunit of PI3K and GSK3β as loading controls. Data shown are representative of three experiments.

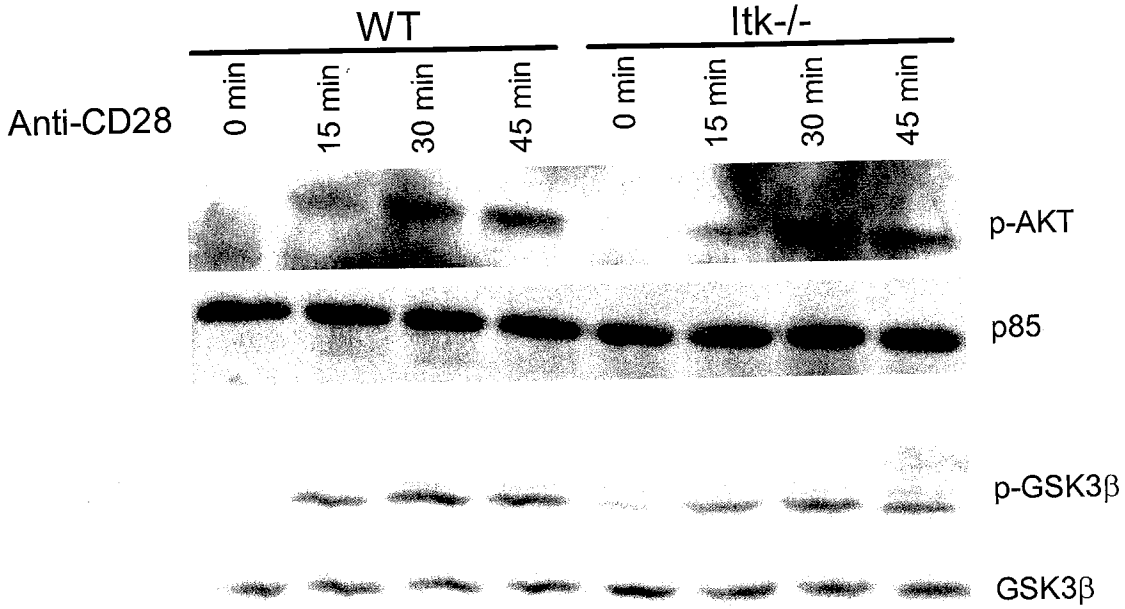


Figure 2-7 Stimulation of CD28 alone induces phosphorylation of Akt and GSK3β in the absence of Itk

Discussion

Based on previous studies, a number of independent lines of evidence had implicated Itk in CD28 signaling. For instance, several studies performed in Jurkat tumor cell lines or with recombinant proteins documented Itk binding to the CD28 cytoplasmic tail (79, 138, 139). In addition, Itk phosphorylation and activation has been demonstrated following CD28 stimulation of Jurkat T cells, and a peptide of the CD28 cytoplasmic tail was shown to activate Itk enzymatic activity *in vitro* (140). Finally, the CD28 cytoplasmic tail has also been shown to be a substrate of Itk kinase activity (141). These biochemical data were then complemented by a single study examining CD28 functional activity in unfractionated populations of *Itk*$^{-/-}$ CD4^{+} T cells (142). This latter study concluded that Itk has a negative role in CD28 signaling.

Using purified naïve CD4^{+} T cells and defined stimulation conditions we have examined in detail the requirement for Itk in CD28 signaling and in CD28-mediated costimulation. Due to the fact that Itk is required for optimal TCR signaling, it is difficult to ascertain whether CD28 costimulatory activity is equally effective in the presence versus the absence of Itk. Nonetheless, our data definitively demonstrate that CD28-mediated costimulation functions quite efficiently in the absence of Itk, and to a first approximation, is as effective in *Itk*$^{-/-}$ T cells as in wild type T cells. This conclusion is supported by the biochemical data showing that two measurable outcomes triggered by CD28 stimulation alone, namely the phosphorylation of Akt and GSK3β, are completely

independent of Itk. Taken together, these findings definitively demonstrate that Itk is not a negative regulator in CD28 costimulatory signaling. Instead, the data indicate that Itk is not required for CD28 signaling or function in naïve $CD4^+$ T cells.

CHAPTER III

GENERATION OF TEC KINASE-DEAD MUTANT TRANSGENIC MOUSE LINES

Introduction

At least three members of the Tec family of tyrosine kinases are expressed in T lymphocytes; they are Itk, Rlk and the prototypic member, Tec. Numerous studies have demonstrated the importance of Itk and Rlk in T cell activation and functional differentiation (reviewed in (147, 153, 154)). Data from biochemical and functional studies have indicated a role for Tec in TCR as well as CD28 signaling. First, Tec is tyrosine-phosphorylated and activated upon TCR or CD28 crosslinking (155, 156). Second, Tec protein levels in primary mouse T cells are up-regulated after TCR activation (157). Furthermore, overexpression of Tec in the Jurkat human T cell line enhanced NFAT activation and IL-2 and IL-4 promoter activities upon TCR and CD28 crosslinking (155, 156, 158) Finally, when Tec protein levels in primary T cells are decreased by using an antisense oligonucleotide, proliferation and IL-2 production are diminished upon TCR stimulation (137).

In spite of the established roles for Itk and Rlk, TCR signaling still functions in *Itk*$^{-/-}$ as well as *Itk*$^{-/-}$*Rlk*$^{-/-}$ T cells, but at reduced levels. For instance, PLCγ phosphorylation and Ca^{++} mobilization are reduced, instead of being completely abolished, upon TCR stimulation (115, 117). These data have led to the notion that Tec family kinases function as modulators or amplifiers that fine-tune TCR signaling (144, 145). This hypothesis was further supported by a set of studies where kinase-dead mutants of Lck or Itk were overexpressed in Jurkat cells (159). In this study, expression

of kinase-dead Lck fully abolished the calcium response upon T cell activation, but expression of kinase-dead Itk only reduced this response. However, one should be cautious in the interpretation of these data, as kinase activity may not be the only function of Tec family kinases. For instance, Itk may function as an adaptor protein to help establish the signaling complex through interactions with other signaling molecules, such as SLP-76, LAT, Vav1, and Grb2. Indeed, one very recent study has indicated a kinase-independent role for Itk. In this study, endogenous expression of Itk in Jurkat cells was knocked down using an RNAi approach. TCR-induced Vav localization and actin polarization were reduced in these cells. However, re-expression of either the wild type or a kinase-dead mutant of Itk rescued the defective Vav localization and actin polarization (133).

An alternative explanation of why T cells deficient for Itk or Itk/Rlk have only a reduction in TCR signaling could be functional redundancy among the Tec family kinases. For instance, T cells from *Rlk*$^{-/-}$ mice have no detectable defects in TCR signaling (117, 136), but T cells from *Itk*$^{-/-}$*Rlk*$^{-/-}$ mice manifested more severe defects than those from *Itk*$^{-/-}$ mice (117). These data suggested that Itk can compensate for Rlk function in *Rlk*$^{-/-}$ T cells. Furthermore, overexpression of Rlk can partially restore the defects of *Itk*$^{-/-}$ T cells (160). Similarly, T cells and B cells from *Tec*$^{-/-}$ mice do not exhibit detectable defects, but B cells from *Btk*$^{-/-}$*Tec*$^{-/-}$ mice manifested more severe defects than from *Btk*$^{-/-}$ cells (136). All these data suggest that functional redundancy

among Tec family kinases exists. To date, there is no report on the phenotype of Itk and Tec double deficient or Itk, Rlk and Tec triple deficient mice.

To further understand the function of the Tec family kinases in T cells, one ideal approach would be to generate a mouse line, in which all three Tec family members are deficient. However, the genes for *Rlk* and *Tec* are linked very closely on the same chromosome in both human and mouse. In human, both *Rlk* and *Tec* are mapped to the 4p12 locus (110, 161). In mouse, *Rlk* is mapped to 40.0cM on chromosome 5, and *Tec* is mapped to 41.0cM on the same chromosome (162-164). In addition, on mouse chromosome 5, there is merely 72,884bp between the *Rlk* and *Tec* genes. This makes it virtually impossible to generate $Rlk^{-/-}Tec^{-/-}$ mouse line by crossing $Rlk^{-/-}$ mice with $Tec^{-/-}$ mice. Furthermore, of the 72,884bp between the two genes on mouse chromosome 5, there are other genes, some of which may encode indispensable proteins, such as gamma-aminobutyric acid (GABA-A) receptor, and nicotinic cholinergic receptor alpha polypeptide 9. Thus an approach that would delete the whole locus in between the two genes is not appropriate. To circumvent this technical difficulty, we generated transgenic mouse lines, in which a kinase-dead (KD) mutant of Tec (TecKD) would be expressed in T lymphocytes on the $Itk^{-/-}Rlk^{-/-}$ double-deficient background. We expected that this overexpressed mutant protein would behave as a dominant negative competitor for the endogenous wild type Tec, and inhibit its function. We reasoned that, with this genetic strategy, we could learn more about the functions of Tec in T cells, especially the kinase dependent functions.

Results

Generation of constructs

The FLAG-tagged Tec K397E mutant (TecKD) cDNA was generated previously by site-directed mutagenesis, where the conserved lysine397 was replaced by glutamate so that the kinase cannot bind its substrate ATP. This cDNA has been successfully expressed in Jurkat cells (155). In order to obtain constitutive expression of the TecKD transgene, both in the thymus and in the periphery, we elected to use the vector pTLC. This vector contains the mouse *lck* gene proximal promoter, which drives the expression at early stages in developing thymocytes, and a human *CD2* gene 3'-locus control region (hCD2 3'-LCR), which ensures the expression in peripheral T cells (165, 166). Using this vector, Weih and colleagues successfully expressed a Nur77 transgene in both thymocytes and peripheral T cells (167).

We were also interested in controlling the expression of the transgene *in vivo*, to facilitate studying the functions of Tec in different stages in T cell development and activation. To this end, we decided to generate a second set of transgenic mice, in which the expression of the transgene could be induced. In addition, this second set of transgenic mice would also serve as a backup, in case the constitutive overexpression of the transgene might elicit tremendous defects through development. In order to obtain inducible expression of TecKD in the T cell lineage *in vivo*, we chose to use the tetracycline-responsive gene induction system (TetOn) that has been described previously (168, 169). Using this system, Zamoyska and colleagues successfully controlled the

expression of an Lck transgene on the *Lck*$^{-/-}$ background (169). The structures of the constructs are shown in Figure 3-1.

Figure 3-1 The constructs for generating TecKD transgenic mice

The upper panel shows the construct of TLC-TecKD, in which the 1.9kb FLAG-tagged TecKD cDNA was cloned into the vector under the control of the *lck* proximal promoter. The human CD2 3' locus control region (hCD2 3'-LCR) ensures the expression of transgene in the periphery as well as in the thymus. Lower panel shows the construct of TRE-TecKD, in which the 1.9kb FLAG-tagged TecKD cDNA was placed under the control of tetracycline responsive element (TRE) and the minimal human CMV promoter. The TRE promoter is activated by the binding of the inducer protein, rtTA, in the presence of tetracycline or a related antibiotic, doxycycline. The inner restriction sites (in italics) indicate where the cDNA was cloned in to the vectors; the outer restriction sites indicate how the transgenes were cut out of the vectors.

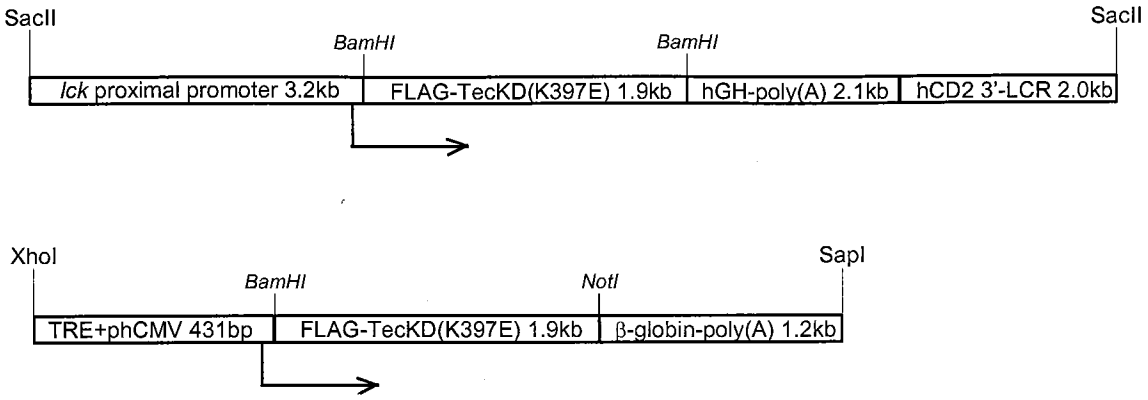


Figure 3-1 The constructs for generating TecKD transgenic mice

Founder lines and genotyping

Traditionally, to make transgenic mice, the transgene fragments are injected into fertilized eggs of a mixed background. This usually results in large numbers of fertilized eggs and pups for screening. In our experiments, to make all mice on the same genetic background while saving backcross steps, we generated transgenic mice directly on the $Itk^{-/-}Rlk^{-/-}$ background. To this end, three-week old female $Itk^{-/-}Rlk^{-/-}$ mice were superovulated and bred to adult male $Itk^{-/-}Rlk^{-/-}$ mice. The resulting fertilized eggs should all be $Itk^{-/-}Rlk^{-/-}$. The transgene fragments were micro-injected directly into these fertilized $Itk^{-/-}Rlk^{-/-}$ eggs. Therefore, all transgenic mice are on $Itk^{-/-}Rlk^{-/-}$ double-deficient background.

With the TLC-TecKD transgenic construct, five out of 53 pups were detected to be transgene positive by Southern Blot (Figure 3-2). All Southern Blot positive samples were PCR positive and *vice versa*, confirming that PCR can be used for routine genotyping tests. Three of the founders (#9, #12 and #53) were males and two (#11 and #37) were female. The mice were generally phenotypically normal. Over time, however, the mice, with the exception of line #9, were much heavier than wild type controls of the same age. When bred to $Itk^{-/-}Rlk^{-/-}$ mice, the two female founders (#11 and #37) failed to produce offspring. Of the progeny of line #9, all males were transgene positive and all females were transgene negative, indicating that the transgene was integrated in the Y chromosome.

With the TRE-TecKD transgenic construct, eight out of 44 pups were detected to be transgene positive by Southern Blot (Figure 3-3). Similarly, the PCR results matched Southern Blot results. When the founders were crossed to the inducer transgenic line *Itk*$^{-/-}$*Rlk*$^{-/-}$*rtTAtg* and fed doxycycline, the progeny from all lines were generally phenotypically normal, and did not manifest the "obesity' phenotype.

Figure 3-2 Genotype of the founders of TLC-TecKD transgenic mice

Genomic DNA from the tails was digested with BamHI and resolved on a 1% agarose gel. After transfer to nylon membrane, the samples were detected with ^{32}P labeled probe corresponding to the 1.9kb TecKD cDNA.

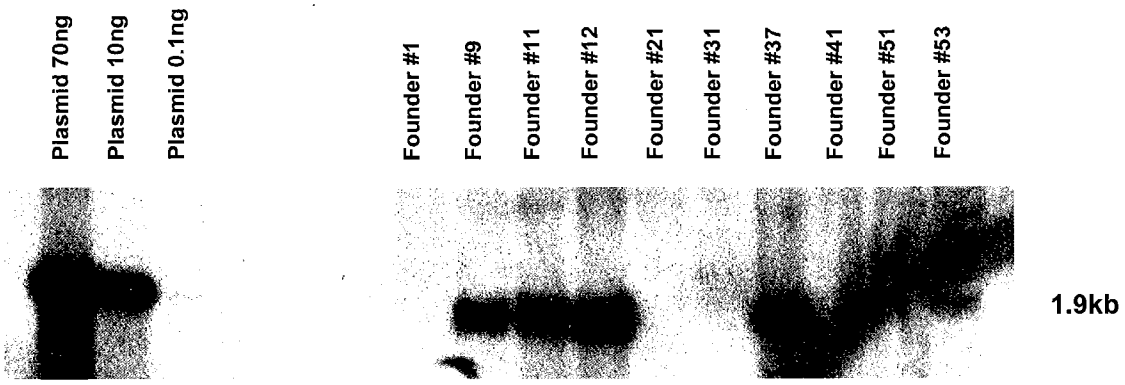


Figure 3-2 Genotype of the founders of TLC-TecKD transgenic mice

Figure 3-3 Genotype of the founders of TRE-TecKD transgenic mice

The upper panel: Genomic DNA from the tails was digested with EcoRI and resolved on a 1% agarose gel. After transfer to nylon membrane, the samples were detected with ^{32}P labeled probe corresponding to the 1.9kb TecKD cDNA. The lower panel: Genomic DNA samples were subjected to PCR with primers annealing to TRE promoter and Tec cDNA.

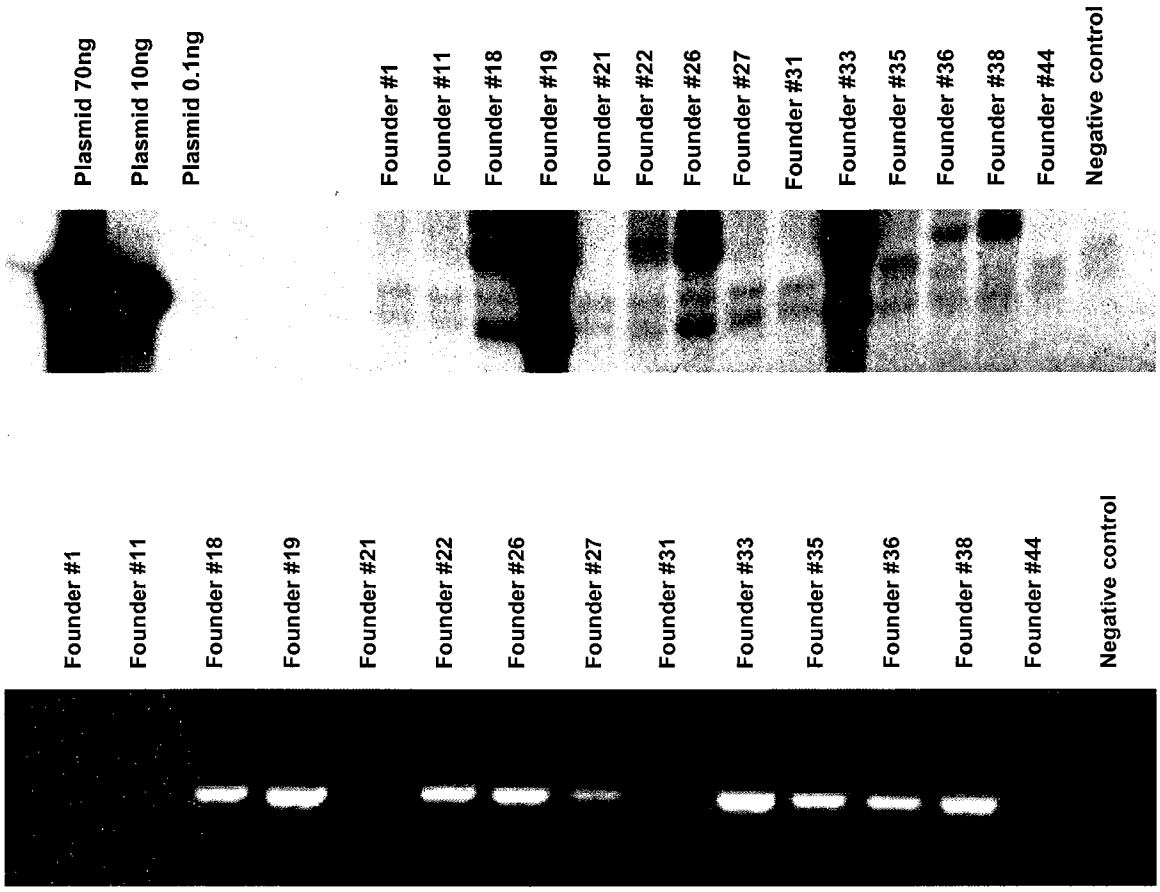


Figure 3-3 Genotype of the founders of TRE-TecKD transgenic mice

The expression of the transgenes

The mRNA expression of the TLC-TecKD transgenes was detected in thymocytes, lymphocytes and splenocytes from progeny of all TLC-TecKD lines and the founders by RT-PCR (Figure 3-4). Similarly, when the TRE-TecKD founders were crossed to the inducer transgenic line *Itk*$^{-/-}$*Rlk*$^{-/-}$*rtTAtg* (see Materials and Methods in Chapter VI) and fed with doxycycline, mRNA expression of the TRE-TecKD transgene was also detected (Figure 3-5). Further, full-length mRNA can be detected by RT-PCR with primers correlating to the 5' end of the FLAG tag and 3' end of Tec. These data further confirmed that the transgene constructs were the correct ones.

Because a FLAG tag (eight amino acid residues) was added to the N-terminus of TecKD, the molecular weight of the mutant protein is expected to be 0.8kDa larger than that of the wild type protein. Thus the protein was expected to be detected by Western Blotting as a slightly shifted band compared to the wild type Tec protein. However, when Western Blotting was performed with an antibody to Tec, no such shifted band was detected, and the signal from the wild type Tec band was not increased in the TecKD positive samples, where mRNA for the transgene was detected by RT-PCR (Figure 3-6). When an antibody to FLAG was used, no specific signal could be detected (data not shown). Exhaustive analysis has been performed with a variety of gel concentrations, cell lysate amounts, and film exposure periods. Furthermore, when anti-FLAG antibody was used in an immunoprecipitation assay, no signal was detected from the immunoprecipitate (data not shown). The same results were obtained for both TLC-

TecKD and the inducible TRE-TecKD transgenic lines. These data indicate that there is no or very little transgenic TecKD protein expressed in the cells.

Figure 3-4 mRNA expression of the TLC-TecKD transgene

Total RNA was isolated from thymi and spleens of TLC-TecKD transgenic mice, transgenic negative littermates (Tg-), and the parental *Itk*$^{-/-}$*Rlk*$^{-/-}$ mice. RT-PCR was performed for the transgene FLAG-Tec (upper panel), and HPRT control (lower panel). As expected, the transgene was detected in the thymus as well as in the periphery.

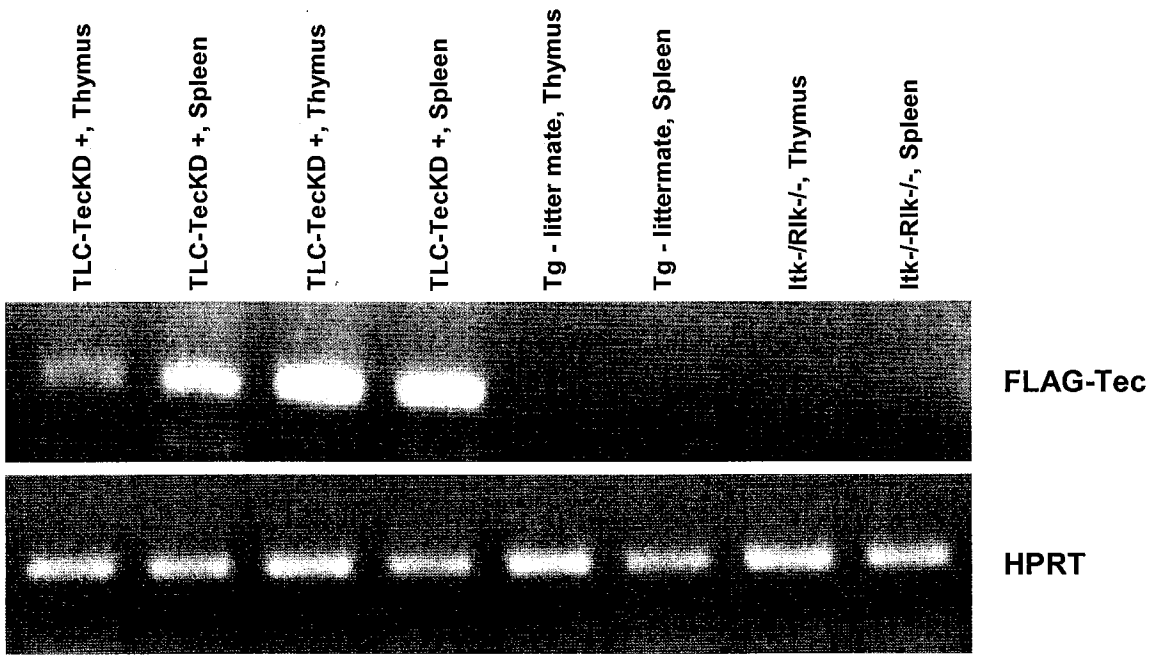


Figure 3-4 mRNA expression of the TLC-TecKD transgene

Figure 3-5 mRNA expression of the TRE-TecKD transgene

The TRE-TecKD transgenic mice were crossed to the inducer rtTA Tg line and fed doxycycline. Total RNA was isolated from transgenic positive mice (TRE-TecKD), transgenic negative littermate (Tg-), and the parental $Itk^{-/-}Rlk^{-/-}$ mice. RT-PCR was performed for the transgene FLAG-Tec (upper panel), and HPRT control (lower panel).

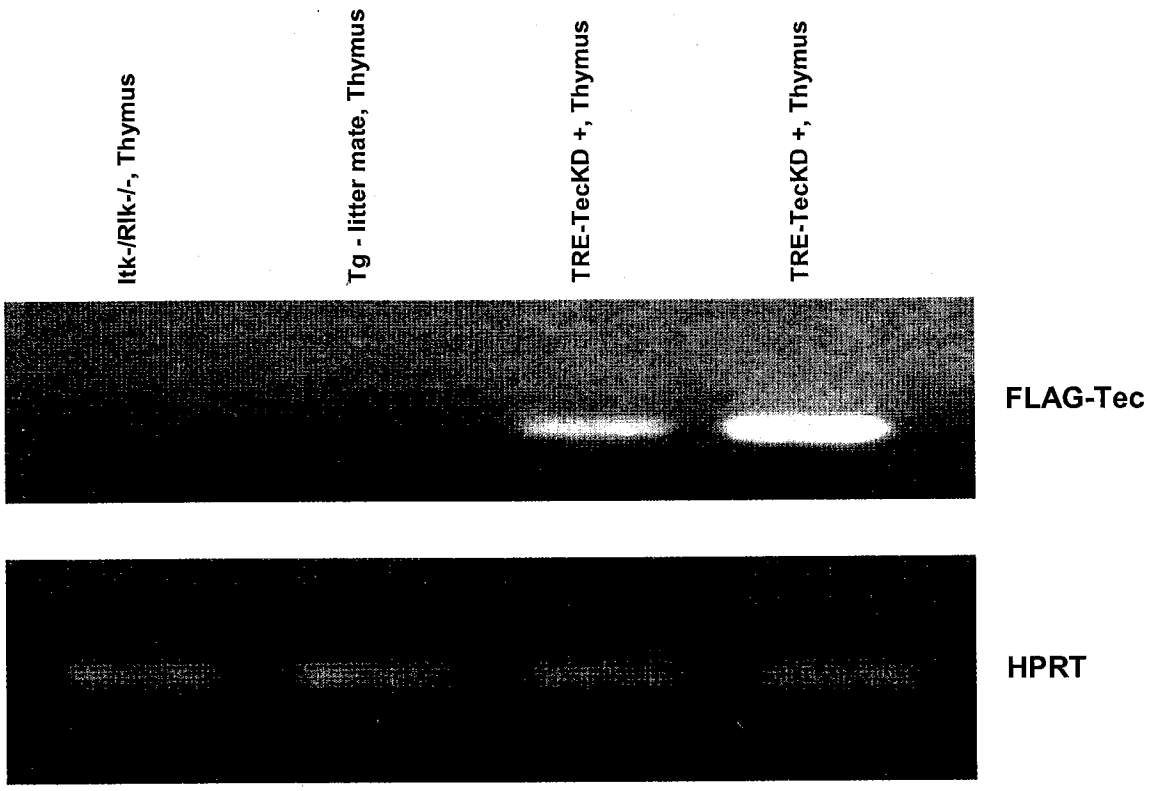


Figure 3-5 mRNA expression of the TRE-TecKD transgene

Figure 3-6 Protein expression of TecKD transgene by Western Blotting

Thymocytes and splenocytes from TLC-TecKD transgenic mice, transgenic negative littermate, and the parental *Itk*$^{-/-}$*Rlk*$^{-/-}$ mice were lysed. Equal amounts of cell lysate protein was resolved through SDS-PAGE. After transfer to PVDF, the samples were probed with anti-Tec antibody (upper panel). The membrane was stripped and re-probed with anti-PI3K P85 antibody as loading control (lower panel). No overexpression of the Tec protein can be detected. No band with increased MW correlating FLAG-Tec was detected. When the samples were probed with anti-FLAG antibody, no specific signal was detected (not shown here). The same results were obtained when TRE-TecKD transgenic mice were crossed to the inducer rtTA line and fed doxycyline (not shown here).

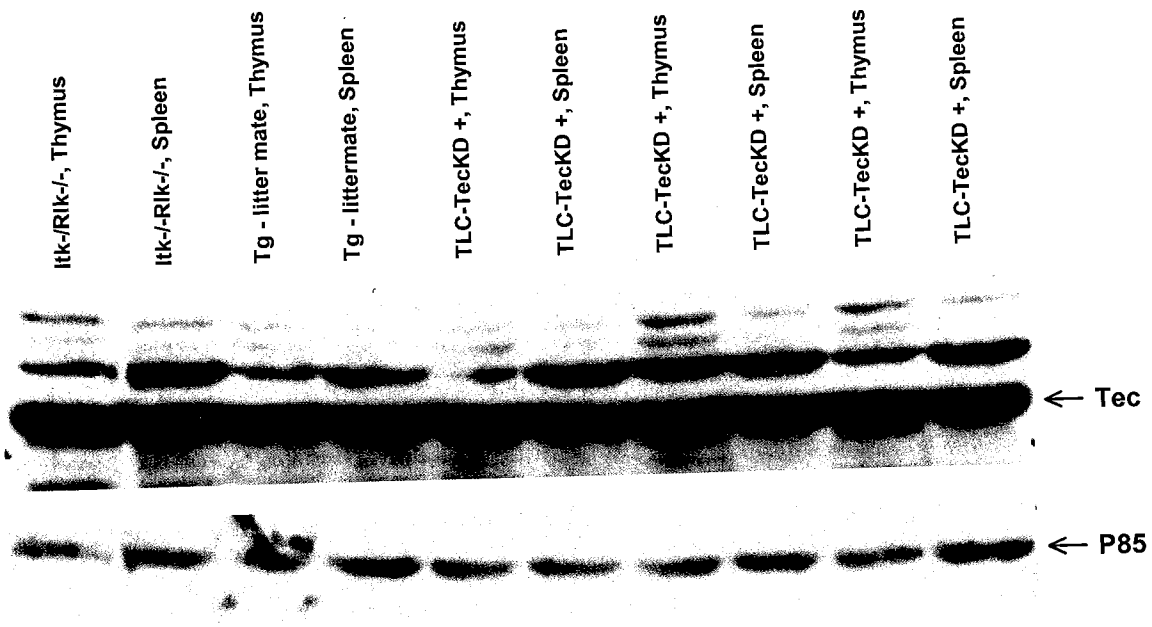


Figure 3-6 Protein expression of TecKD transgene by Western Blotting

Phenotype of TecKD transgenic mice

As mentioned above, the transgenic mice expressing TecKD mRNA were generally normal and bred well, with the exception of two female TLC-TecKD founders that did not generate any litters. The cellularity of thymi, lymph nodes, and spleens from the transgenic mice were very comparable with that of the parental $Itk^{-/-}Rlk^{-/-}$ mouse line (Figure 3-7). When thymocytes and lymphocytes from TecKD transgenic mice were analyzed by flow cytometry, the cell populations and percentages were found to be very comparable to those from the parental $Itk^{-/-}Rlk^{-/-}$ mouse line (Figure 3-8, 3-9).

Figure 3-7 Thymus and lymph node cellularity from TecKD transgenic mice

The TRE-TecKD mice were crossed to the inducer rtTA transgenic line and fed doxycycline water. Cell numbers of the thymi and lymph nodes from both TecKD transgenic lines are not significantly different from those in the parental *Itk*$^{-/-}$*Rlk*$^{-/-}$ mice. Data shown are representative of three separate experiments.

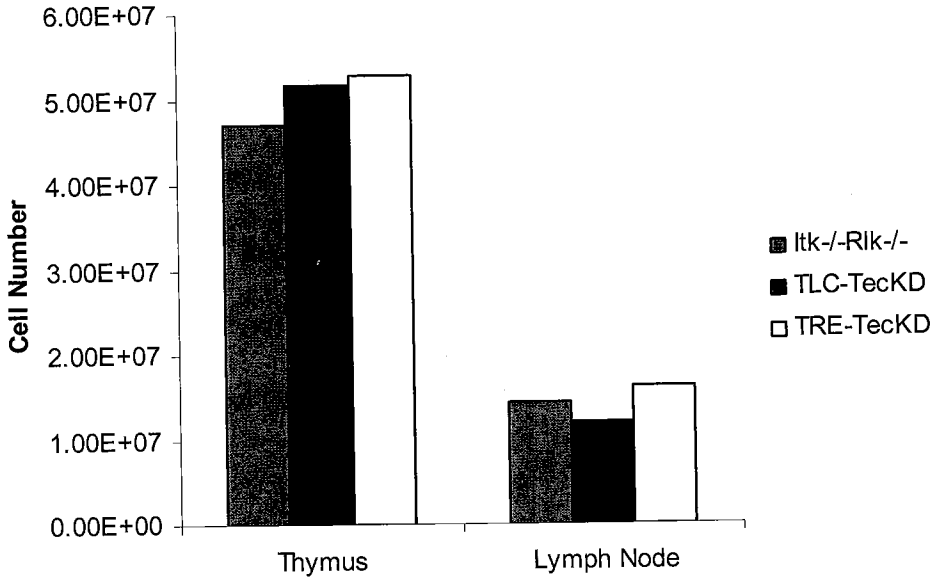


Figure 3-7 Thymus and lymph node cellularity from TecKD transgenic mice

Figure 3-8 Flow cytometric analysis of thymus and lymph node from TLC-TecKD transgenic mice

Thymocytes and lymph node cells from the TLC-Tec transgenic mice (*TLC-TecKD Tg*) and the parental $Itk^{-/-}Rlk^{-/-}$ mice were stained for CD4 and CD8 and analyzed by FACS. The data show no significant difference between the two genotypes. Data shown are representative of three separate experiments.

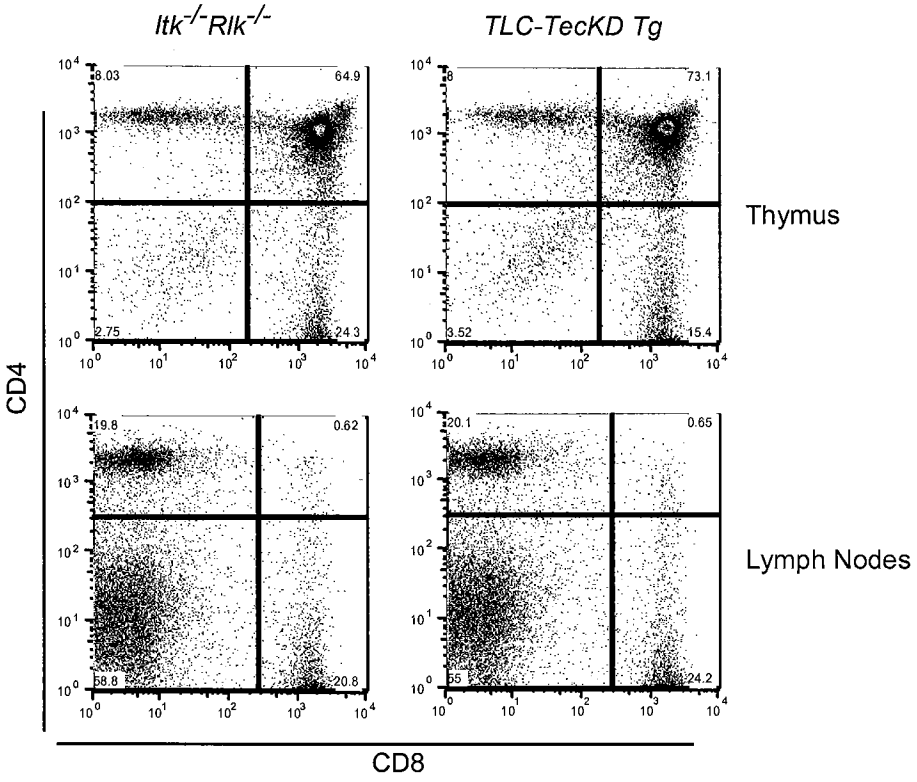


Figure 3-8 Flow cytometric analysis of thymus and lymph node from TLC-TecKD transgenic mice

Figure 3-9 Flow cytometric analysis of thymus and lymph nodes from TRE-TecKD transgenic mice

TRE-TecKD transgenic mice were crossed to the inducer rtTA transgenic mice (*TRE-TecKDTg x rtTATg*). The mice were fed doxycycline water. Thymocytes and lymph node cells from these mice and the parental $Itk^{-/-}Rlk^{-/-}$ mice were stained for CD4 and CD8 and analyzed by FACS. The data show no significant difference between the two genotypes. Data shown are representative of three separate experiments.

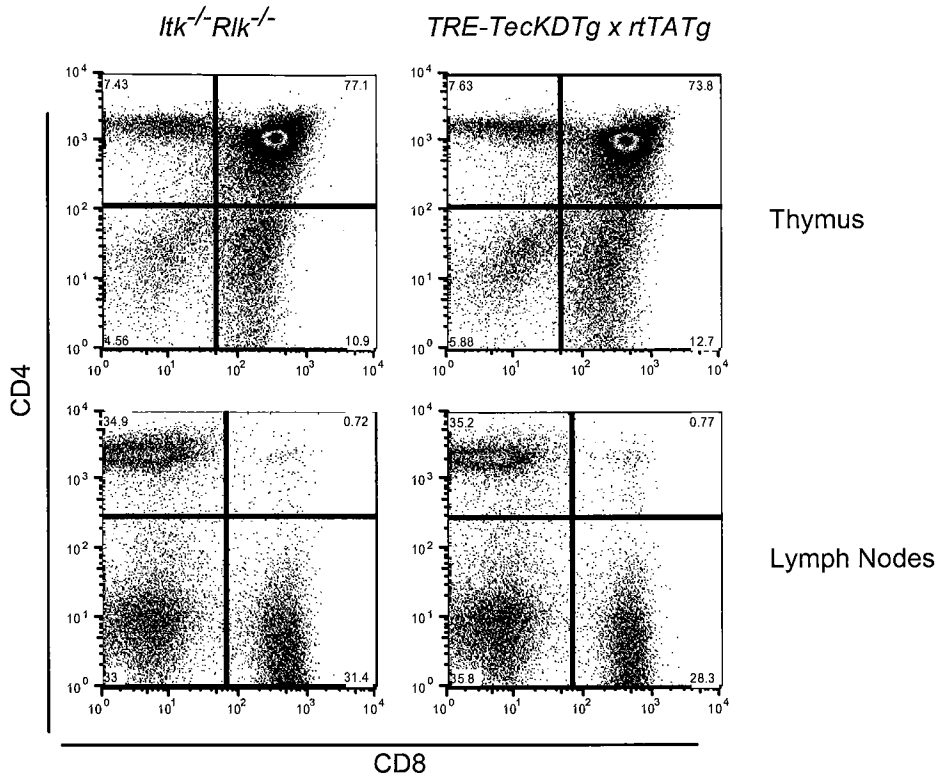


Figure 3-9 Flow cytometric analysis of thymus and lymph nodes from TRE-TecKD transgenic mice

Discussion

In an effort to elucidate the function of Tec, and other Tec family kinases in T cell development and function, we generated transgenic mouse lines expressing kinase-dead Tec on $Itk^{-/-}Rlk^{-/-}$ double-deficient background. Although the expression of the transgene was clearly detected at the mRNA level, no expression of the transgenic protein could be detected by Western Blotting. The reason(s) for this is not clear. Nonetheless, we can rule out some possible technical issues. First, the expression of mRNA can be detected. This indicates the proper activation of the promoters and correct junctions between the cDNA and promoters. Second, after initial analysis of the transgenic mice, the constructs were again sequenced again and found to be correct (data not shown). Thus, it is most likely that the problem occurred at or post translation. It is not very likely that the FLAG tagged protein is unstable, because the same cDNA, has been expressed at high levels in Jurkat cells under the control of other promoters and acted as a dominant negative competitor (155, 156). There are some other technical issues that we did not rule out. For instance, efficient initiation of translation may not be started, or the translation may be stopped pre-maturely, due to mutation(s) or damage(s) of the transgenic fragment before microinjection into the fertilized eggs. To test these, the TLC-TecKD construct can be transfected into Jurkat cells. If TecKD protein can be expressed in Jurkat cells, the problem would not have been due to the construct. Moreover, the sequence of the expressed TecKD message from the transgenic mice could be analyzed and verified.

Successful expression of the TecKD cDNA in a cell line but not in transgenic mouse models led us to the hypothesis that the loss of protein expression of the transgene may be due to selection/survival pressure on the T cells. For instance, it is possible that normal expression and function of the Tec family members Itk, Rlk, and Tec are critical for T cell development in the thymus and survival in the periphery. The loss of Itk and Rlk could be partially compensated by Tec. However, when all three members are deficient, it is possible that the T cells were unable to mature in the thymus or could not survive in the periphery. Thus, only those cells, in which the TecKD transgenic protein expression was somehow suppressed, could mature, migrate to and survive in the periphery. One caveat of this explanation is that this "suppression" process would have to function very efficiently, as thymus and lymph node cellularities in the transgenic mice are comparable to those in the parental mouse line. Nonetheless, this hypothesis is in agreement with the notion that there is functional redundancy among Tec family kinases. In support of this hypothesis, efforts in expressing the TecKD mutant in bone marrow cells with a retroviral delivery system also did not yield protein expression (W.-C. Yang, L. Atherly and L.J. Berg, unpublished observations). Therefore, enforced expression of a dominant negative competitor has not been a successful approach to study the function of Tec in primary T cells. A few experiments could be done to test this hypothesis. First, the *TLC-TecKDTg* or *TRE-TecKDTgxrtTATg* mice could be bred to *WT* background to examine if the TecKD transgenic protein can be expressed in T cells. Second, the *TRE-TecKDTg* mice could be crossed to mice that express the *rtTA* transgene under the control of non-T cell-specific promoters, *e.g.* the *actin* gene promoter, to test if the TecKD

transgenic protein can be expressed in non-T cells. Similarly, the TecKD-encoding retroviral vector could be transfected into bone marrow cells from *WT* mice. Under these conditions, the other Tec family kinases, *i.e.* Itk and Rlk, are expressed normally in T cells; hence there should be no selection pressure against the expression of TecKD cDNA. If under these conditions, the overexpression of the TecKD protein would support the above hypothesis. On the other hand, if under these non-pressure conditions, the expression of the TecKD protein still can not be detected, it would likely be due to some technical issues, *e.g.* mutation(s) in the cDNA constructs.

CHAPTER IV

GLOBAL GENE EXPRESSION PROFILING OF ITK DEFICIENT $CD4^+$ T CELLS

Introduction

The functions of a cell rely on the proteins it contains, which is controlled mainly by the transcription of the genes encoding them. Thus, the functions of an activated T cell depend primarily on *de novo* gene expression upon stimulation. The established role for Itk, a Tec family kinase, in TCR signaling is to positively regulate PLCγ, hence activating the downstream calcium-dependent and DAG-dependent pathways (reviewed in (153)). Therefore, positive selection, which requires TCR signaling, during T cell development in the thymus is impaired in the absence of Itk (120). Additionally, upon stimulation, peripheral mature T cells proliferate and produce IL-2 to diminished levels in the absence of Itk (115, 116). It has been shown that upon stimulation, the activation of transcription factors that are important for T cell function is defective in $Itk^{-/-}$ T cells, compared to wild type T cells. For instance, when $Itk^{-/-}$ T cells are stimulated through TCR or TCR plus CD28, both NFAT and NFκB activation are impaired compared to wild type controls (121, 170). Moreover, the activation of MAP kinases, ERK and JNK, is defective in $Itk^{-/-}$ T cells upon stimulation (134, 135, 144). In addition, it was recently reported that c-Jun phosphorylation was diminished when Itk deficient T cells were stimulated with a super antigen, SEB (171). These data suggest that the activation of the AP1 transcription factor is also defective in $Itk^{-/-}$ T cells. Therefore, the global gene expression profile in $Itk^{-/-}$ T cells may be altered.

The transcription factors NFAT, NFκB and AP1 control the expression of a variety of genes. However, there are other transcription regulators that may be regulated by Itk. Furthermore, Itk may directly function in the nucleus. It was reported that Itk can localize to the nucleus after TCR stimulation by phosphorylating and interacting with the nuclear importin Rch1α (172). In an recent *in vitro* study, it was shown that Itk can directly phosphorylate the Th1 specific transcription factor, T-bet, promoting an interaction between T-bet and GATA3 that reduces GATA3 DNA binding and represses Th2 development (173). These data imply that Itk may have a function in the nucleus. To further understand the functions of Itk in T cell development and T cell activation and to identify potential downstream targets of Itk, we decided to compare global gene expression profiles between $Itk^{-/-}$ and wild type $CD4^+$ T cells, at naïve and activated states, using the Affymetrix GeneChip microarray MOE430A containing 22,600 probe sets, which can be used to analyze over 14,000 well-characterized mouse genes.

Since positive selection during T cell development in the thymus is impaired in the absence of Itk (120), TCR repertoires in $Itk^{-/-}$ mice may be skewed such that positively selected T cells may express TCRs with increased affinities than those from wild type mice. These possibly biased T cell repertoires in $Itk^{-/-}$ mice could potentially complicate the analysis. To circumvent this possible complication, we chose to compare $CD4^+$ T cells from *5C.C7TgRag*$^{-/-}$*Itk*$^{+/-}$ ($Itk^{+/-}$ or *WT*) and *5C.C7TgRag*$^{-/-}$*Itk*$^{-/-}$ ($Itk^{-/-}$) mice. All $CD4^+$ T cells from these mice should express the same 5C.C7 TCR homogeneously. To stimulate the cells, we chose to use anti-CD3 plus anti-CD28 coated latex beads, a

condition that has been shown to fully activate naïve $CD4^+$ T cells (170). Data from replicate experiments were statistically analyzed with Bioconductor packages (174) and dChip (175).

Results and discussion

Quality assessments

For comparison of non-stimulated (NS) naïve $CD4^+$ T cells, five separate experiments were performed for both *5C.C7TgRag*$^{-/-}$*Itk*$^{+/-}$ and *5C.C7TgRag*$^{-/-}$*Itk*$^{-/-}$ genotypes. For comparison of stimulated $CD4^+$ T cells, $CD4^+$ T cells were stimulated and cultured for 6 or 36 hours. Three separate experiments were performed for each genotype at each time point. Thus data from a total of 22 arrays were analyzed.

The data quality of the 22 arrays was assessed, using the Affy and AffyPLM packages from Bioconductor. The parameters chosen are Relative Log Expression (RLE), Normalized Unscaled Standard Errors (NUSE), and RNA Degradation Plot. RLE values are calculated for each probe set by comparing the expression value on each array against the median expression value for that probe set across all arrays. Under the assumption that the expression of most genes does not change across arrays, ideally the majority of the RLE values should be close to 0 (zero). An array that has RLE values far higher or lower than zero indicates that the array is of low quality. For NUSE, the standard error estimates for each gene on each array are standardized across arrays so that the median standard error for each gene is 1 (one) across all arrays. This process accounts for variability differences among genes. An array that has elevated NUSE relative to the other arrays is typically of low quality. RNA Degradation Plot compares intensities of probe sets of each gene from the 3' end to the 5' end. RNA degradation

patterns are expected to be similar across all the arrays. An array that has reduced 3' end intensities compared to the other arrays is of low quality.

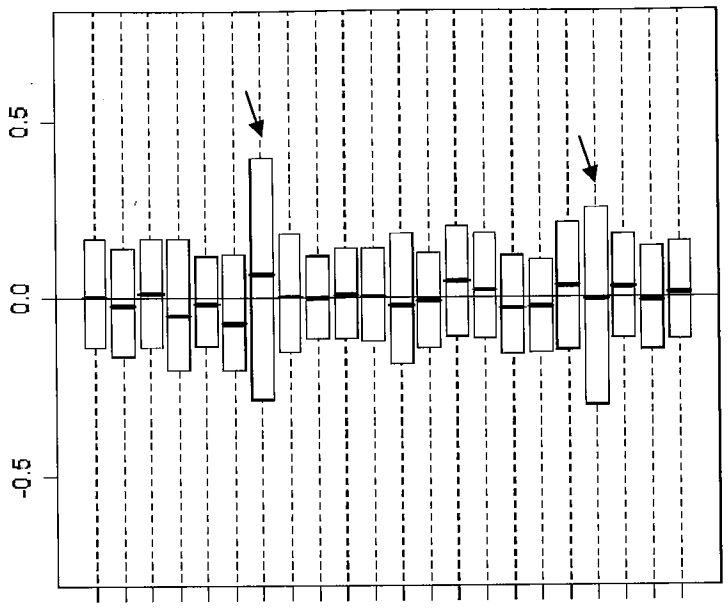
These quality analyses flagged out two arrays with lower quality than all others. One of these was from non-stimulated *Itk*$^{-/-}$ cells, while the other was from *Itk*$^{-/-}$ cells that had been stimulated for 6 hours. Both of these samples had extended ranges of RLE (Figure 4-1A), increased NUSE (Figure 4-2A), and reduced 3' intensities (Figure 4-3 A), compared to all other samples. Thus, these two arrays were excluded from further analysis. The RLE, NUSE and RNA degradation plots of these 20 arrays are shown in Figures 4-1B, 4-2B, and 4-3B, respectively. These twenty arrays include: 5 arrays of non-stimulated *Itk*$^{+/-}$ cells, 4 arrays of non-stimulated *Itk*$^{-/-}$ cells, 3 arrays of 6-hour stimulated *Itk*$^{+/-}$ cells, 3 arrays of 6-hour stimulated *Itk*$^{-/-}$ cells, 3 arrays of 36-hour stimulated *Itk*$^{+/-}$ cells, and 2 arrays of 36-hour stimulated *Itk*$^{-/-}$ cells. Further statistical analyses were performed by using the AffylmGUI package from Bioconductor and dChip program.

Figure 4-1 RLE plots

A. RLE plot for all 22 arrays. The two arrays that are indicated with arrows have extended RLE ranges and thus are of low quality. These two arrays were excluded from further analysis.

B. RLE plot for the remaining 20 arrays. The RLE ranges of these 20 arrays are similar, and the values are close to 0.

A.



B.

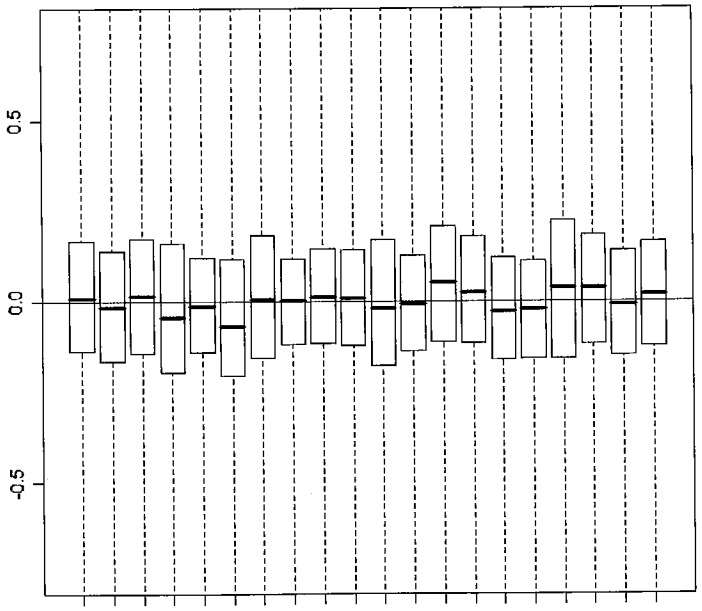


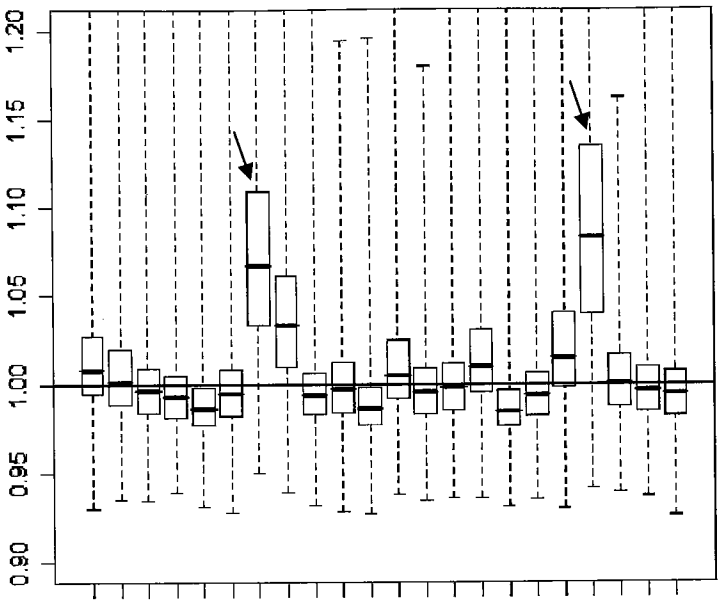
Figure 4-1 RLE plots

Figure 4-2 NUSE plots

A. NUSE plot for all 22 arrays. The two arrays that are indicated with arrows have increased values and thus are of low quality. These two arrays were excluded from further analysis.

B. NUSE plot for the remaining 20 arrays. The NUSE ranges of these 20 arrays are similar, and the values are within 1±0.05.

A.



B.

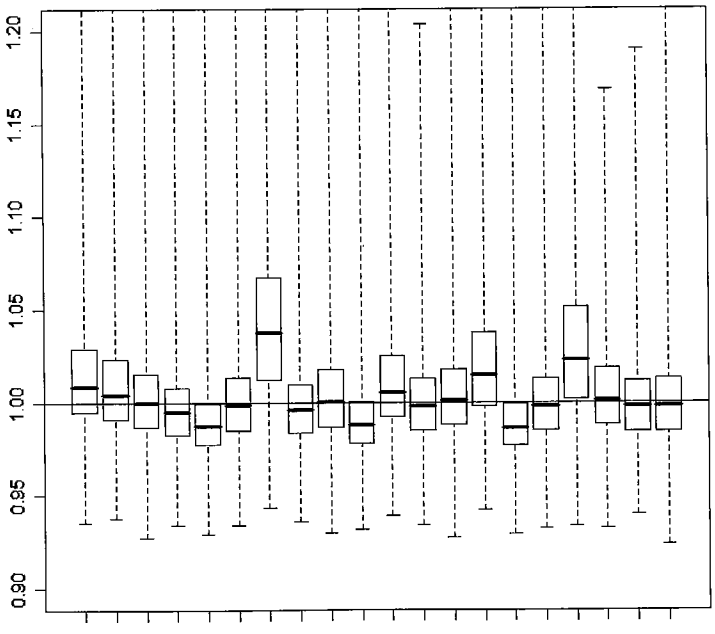


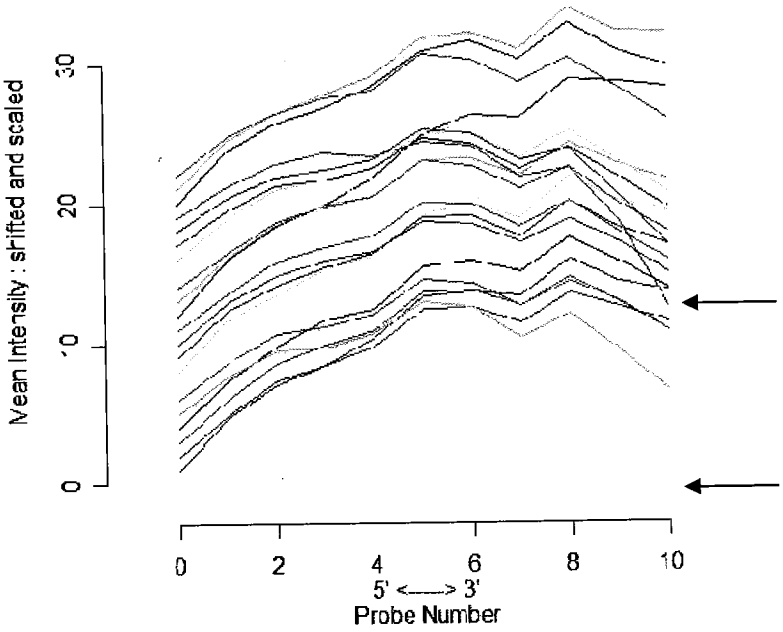
Figure 4-2 NUSE plots

Figure 4-3 RNA degradation plots

A. RNA degradation plot for all 22 arrays. The two arrays that are indicated with arrows have decreased 3' intensities and thus are of low quality. These two arrays were excluded from further analysis.

B. The RNA degradation curves of the remaining 20 arrays are similar and roughly parallel.

A.



B.

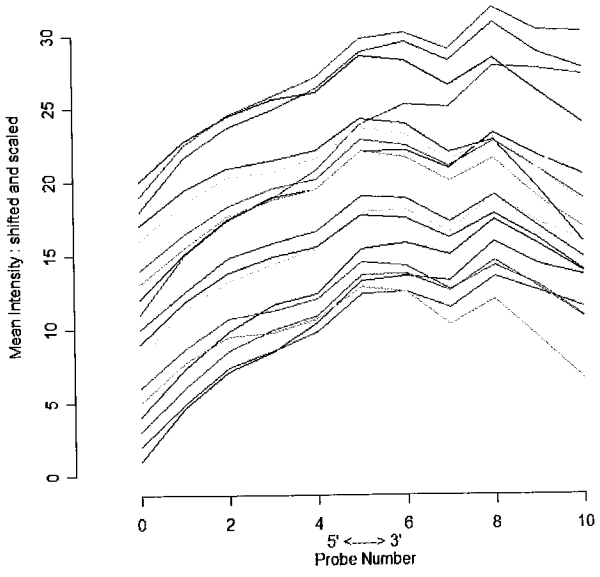


Figure 4-3 RNA degradation plots

Patterns of the expression of Tec family kinases

First, we were interested in comparing the expression levels of all T cell-expressed Tec family kinases in *Itk*$^{+/-}$ CD4^{+} T cells, Itk, Rlk, and Tec. As shown in Figure 4-4A, the signals of all three Tec family kinases in naïve *Itk*$^{+/-}$ CD4^{+} T cells have a hierarchy of *Itk>Rlk>Tec*. These data are consistent with previous quantitative Real-Time PCR data generated in our lab (143). Thus, this result can serve as another layer of quality control. Interestingly, as shown in Figure 4-4B, the kinetics of the expression of these kinases over the course of stimulation are different. The expression of *Itk* is slightly up-regulated at 6-hour of stimulation, but down-regulated after an extended stimulation of 36-hour. However, the expression of both *Tec* and *Rlk* is down-regulated after stimulation (Figure 4-4B).

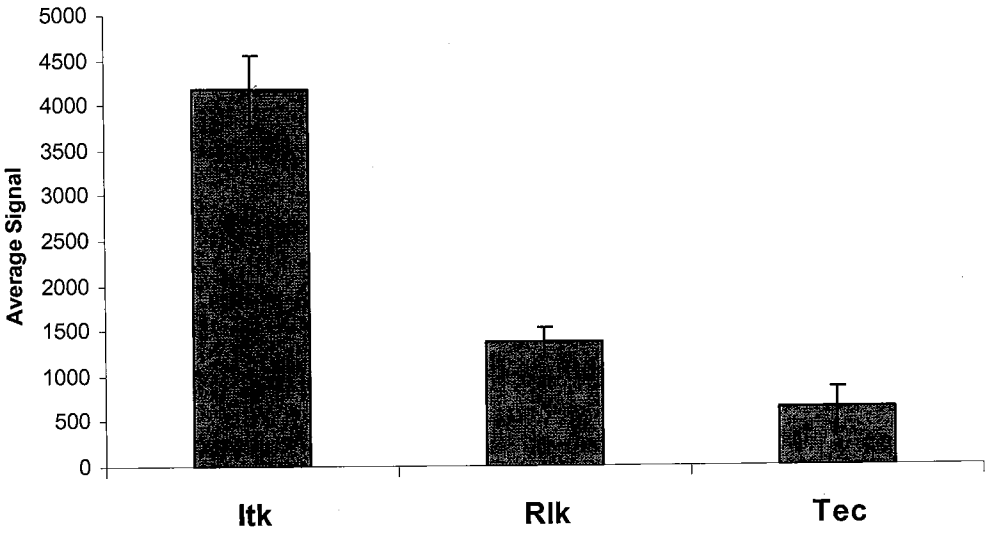
The expression patterns of *Tec* and *Rlk* in *Itk*$^{-/-}$ CD4^{+} T cells are similar to *Itk*$^{+/-}$ CD4^{+} T cells (Figure 4-5). These results indicate that while other Tec family kinases, i.e. *Tec* and *Rlk*, may compensate for the loss of Itk in *Itk*$^{-/-}$ T cells, the possible compensation is not through increasing the transcription levels of these kinases.

Figure 4-4 The expression patterns of Tec family kinases in *Itk*$^{+/-}$ CD4^{+} T cells

A. Average signals and standard deviations of *Itk*, *Rlk*, and *Tec* from naïve *Itk*$^{+/-}$ (WT) CD4^{+} T cells were calculated from 5 replicate samples and plotted. The transcription levels of these Tec family kinases demonstrate the hierarchy of *Itk>Rlk>Tec*.

B. A heat map plot of the expression of Itk, Rlk, and Tec in naïve and stimulated *Itk*$^{+/-}$ (WT) CD4^{+} T cells was generated in the dChip program. White represents the average signal across all samples; blue represents signals lower than the average and the darker the color the lower the signal; red represents signals higher than the average and the darker the color the higher the signal. The expression of both *Rlk* and *Tec* is down-regulated after 6-hour (WT06.x) and 36-hour (WT36.x) stimulation. The expression of *Itk*, however, is slightly up-regulated after 6-hour stimulation (WT06.x) and is down-regulated after 36-hour stimulation (WT36.x). WT0.x: replicates of non-stimulated *Itk*$^{+/-}$ samples.

A.



B.

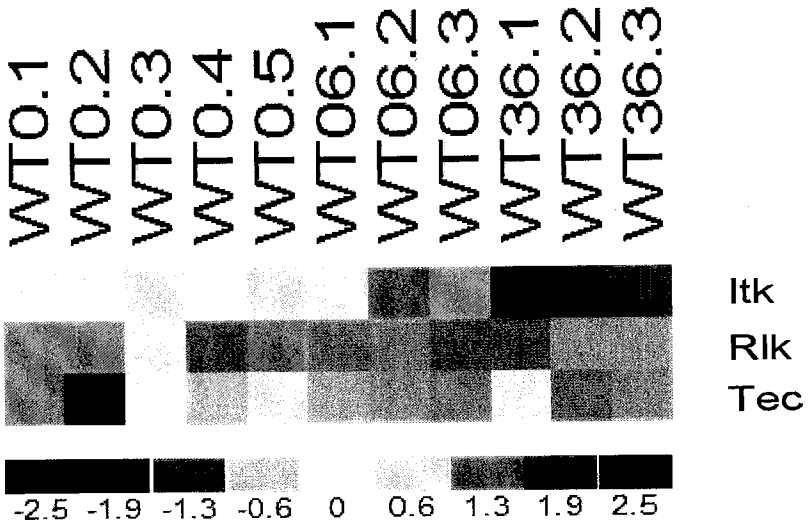


Figure 4-4 The expression patterns of Tec family kinases in *Itk*$^{+/-}$ CD4^{+} T cells

Figure 4-5 The expression patterns of *Rlk* and *Tec* in $Itk^{+/-}$ and $Itk^{-/-}$ $CD4^+$ T cells

A heat map plot of the expression of *Rlk* and *Tec* in $Itk^{+/-}$ (WT) and $Itk^{-/-}$ (KO) $CD4^+$ T cells was generated in the dChip program. White represents the average signal across all samples; blue represents signals lower than the average and the darker the color the lower the signal; red represents signals higher than the average and the darker the color the higher the signal. The expression of both *Rlk* and *Tec* is similar in naïve $Itk^{-/-}$ (KO0.x) $CD4^+$ T cells and in naïve $Itk^{+/-}$ (WT0.x) $CD4^+$ T cells. After 6-hour (KO06.x and WT06.x) and 36-hour (KO36.x and WT36.x) stimulation with anti-CD3 plus anti-CD28 coated beads, the expression of *Rlk* and *Tec* is decreased in $CD4^+$ T cells from both genotypes.

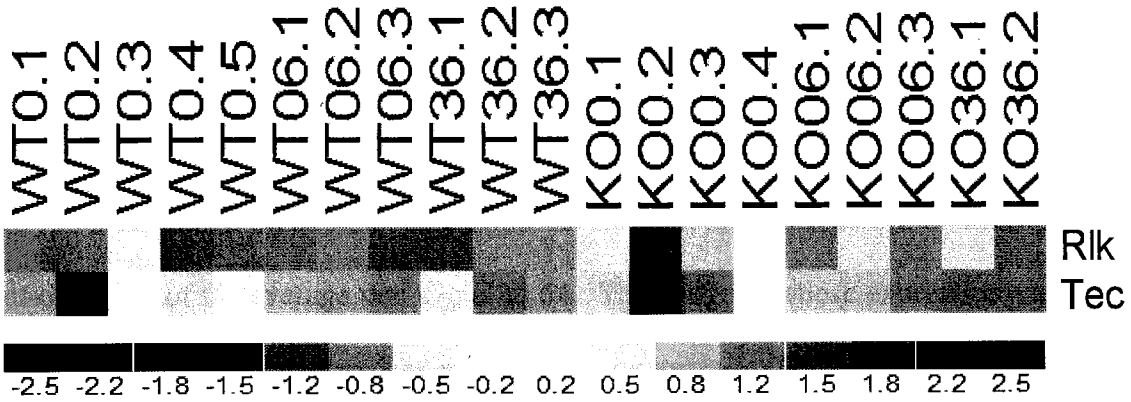


Figure 4-5 The expression patterns of *Rlk* and *Tec* in $Itk^{+/-}$ and $Itk^{-/-}$ $CD4^{+}$ T cells

Differences in global gene expression of naïve Itk$^{+/-}$ and Itk$^{-/-}$ CD4^{+} T cells

We compared global gene expression profiles of naïve CD4^{+} T cells from *Itk*$^{+/-}$ and *Itk*$^{-/-}$ mice. The data from non-stimulated samples were thus pooled into two groups according to the genotypes; average expression value for each gene was calculated across all samples. Statistical analysis was then performed using the Bioconductor package AffylmGUI. Based on overall noise signal levels from the raw data, we arbitrarily set the cut-off value of the average raw signal to 64. Thus, a gene whose expression value was lower than 64 was considered as noise or not expressed. The criteria for determining significance was that the change in expression should have p-values of lower than or equal to 0.05 and should have B-statistic values higher than zero. B-statistic is another guide of significance; the value is the log-odds that the gene is differentially expressed. From these analyses, we found approximately 189 genes differentially expressed in *Itk*$^{-/-}$ CD4^{+} T cells when compared to *Itk*$^{+/-}$ CD4^{+} T cells. In general, the changes were moderate, as few of these genes had fold-changes of higher than 3. More surprisingly, however, among these genes, we found only 6 genes whose expression was reduced in *Itk*$^{-/-}$ CD4^{+} T cells. On the other hand, the majority of the "up-regulated" genes were those that are expressed in other cell types, especially monocytes and dendritic cells. We noticed that, although the CD4^{+} T cell preparations from both *Itk*$^{+/-}$ mice and *Itk*$^{-/-}$ mice are 95% pure (in terms of CD4 staining), the CD4^{+} T cell preparations from *Itk*$^{-/-}$ mice were consistently less pure (in terms of TCR or CD3 staining). Based on this fact, we reasoned that most, if not all, of the "up-regulated" genes were likely to be the result of contaminations from other cell types. With this in mind, we disregarded the "up-

regulated" genes, and instead, focused on the genes that are "down-regulated" in naïve *Itk*$^{-/-}$ CD4^{+} T cells (Table 4-1, Figure 4-6).

Among the genes that are down-regulated in naïve *Itk*$^{-/-}$ CD4^{+} T cells, three are cell membrane proteins including CD5, CD6, and integral membrane protein 2A (ITM2A) (Table 4-1). CD5 and CD6 are glycosylated membrane proteins of the scavenger receptor family. During T cell development in the thymus, CD5 expression is regulated according to the TCR signal strength received by developing thymocytes (176). Studies with CD5 deficient mice suggested that CD5 acts as a negative regulator of TCR signaling (177, 178). Available information on CD6 and ITM2A is limited but indicates that CD6 and ITM2A are expressed with a similar pattern to CD5 (179-181). These studies imply that all three proteins may function in a similar way. In one set of studies, Lucas *et al* demonstrated that CD5 expression levels are lower on *Itk*$^{-/-}$ DP thymocytes and *Itk*$^{-/-}$ 5C.C7 TCR-transgenic CD4 SP thymocytes (120). Since we did not detect differential expression-pattern changes between *Itk*$^{-/-}$ and *Itk*$^{+/-}$ samples after T cell stimulation (see below), we consider the lower expression levels of CD5, CD6, and ITM2A in naïve *Itk*$^{-/-}$ CD4^{+} T cells to be carryovers from development in the thymus. Interestingly, CD5 has been implicated in transducing a negative signal to stimulated T cells (178, 182). Therefore, the lower CD5 expression on naïve *Itk*$^{-/-}$ CD4^{+} T cells may enable these cells to be more easily activated *in vivo*.

We found that two transcription factors, Sox4 and Tox, are down-regulated in naïve *Itk*$^{-/-}$ T cells (Table 4-1). Both Sox4 and Tox are members of the high mobility group (HMG) family of transcription factors. Sox4 is expressed in T and B cells in adult animals. The *Sox4*$^{-/-}$ mice die *in utero* due to a defect in development of the heart (183). When fetal liver cells from *Sox4*$^{-/-}$ mice were used to reconstitute irradiated wild-type mice, B cell development was found to be blocked at the pro-B stage, demonstrating a critical role of this transcription factor in B cell development (183). When fetal liver cells from *Sox4*$^{-/-}$ mice were mixed with those from wild-type mice and injected into thymi of irradiated mice, the *Sox4*$^{-/-}$ cells displayed a disadvantage in competing with the wild-type cells (184). This suggests that Sox4 may play a subtle role in T cell development. In an *in vitro* system, Sox4 was shown to be able to bind to and transactivate the human *CD2* gene enhancer (185). On the other hand, Tox was identified from a gene array analysis comparing DP thymocytes cultured with or without PMA and ionomycin (186). Studies with Tox transgenic mice suggested that it may play a role in CD8 lineage differentiation (186, 187). To date, there are no reported studies suggesting possible function(s) of Sox4 and Tox in peripheral T cells. We did not detect differential regulation of these two genes between *Itk*$^{-/-}$ and *Itk*$^{+/-}$ samples after T cell stimulation, suggesting that the expression of these two transcription factors may not be targets of Itk in peripheral T cell activation. However, it will still be interesting to examine the expression patterns of Sox4 and Tox in different stages of developing *Itk*$^{-/-}$ thymocytes.

Finally, we found a possible negative signaling regulator, TRB2, whose expression is down-regulated in naïve *Itk*$^{-/-}$ CD4^{+} T cells (Table 4-1). TRB2 is a mammalian homologue of the *Drosophila* gene *Tribbles*. Tribbles is crucial in *Drosophila* gastrulation, because it inhibits cell cycle progression by inducing degradation of the CDC25 activator, String (188-190). There are three TRB proteins identified in mammals, TRB1, TRB2 and TRB3, all of which share a central Trb domain. The Trb domain is homologous to protein serine/threonine kinase, but lacks the active lysine site. Thus, Trb is predicted to be kinase-dead (190, 191). Working in different systems, a few groups implicated TRB proteins in various signaling pathways (192-197). In liver cells, TRB2 and TRB3 can be coimmunoprecipitated with Akt, and can inhibit the phosphorylation of Akt induced by insulin signaling at both S473 and T308 sites (192). In 293 cells, overexpressed TRB3 was found to down-regulate TNF-induced NFκB activation by inhibiting the phosphorylation of p65 (195). In Hela cells and NIH3T3 cells, overexpressed TRB1 was found to interact with the MAP kinase kinase MEKK-1 and inhibit its activity. Thus, overexpression of TRB1 can reduce AP-1 transcription factor activity (193). Together, these studies implicate the TRB proteins in a few different pathways, and they all indicate that TRB proteins function as negative regulators. The expression of TRB2 in naïve *Itk*$^{-/-}$ CD4^{+} T cells is lower than naïve *Itk*$^{+/-}$ CD4^{+} T cells (Table4-1). Although at this point we do not know the protein levels of TRBs in the cells, it is tempting to speculate that lower levels of negative regulators in naïve *Itk*$^{-/-}$ CD4^{+} T cells could partially compensate for the signaling defects in *Itk*$^{-/-}$ CD4^{+} T cells, at least at the early stage of activation. Moreover, reduced expression levels of

negative regulators in naïve *Itk*$^{-/-}$ CD4^{+} T cells could, again, be the result of selection during thymocyte development. Further studies will be needed to determine the protein levels of TRBs in developing as well as mature peripheral CD4^{+} T cells.

Overall, we found very few genes that are differentially expressed in naïve *Itk*$^{-/-}$ CD4^{+} T cells compared to naïve *Itk*$^{+/-}$ CD4^{+} T cells. Further, the magnitudes of the differences are very low; none of them is over 2-fold downregulated (Table 4-1). These data indicate that the naïve *Itk*$^{-/-}$ and *Itk*$^{+/-}$ CD4^{+} T cells are almost identical in terms of the basal levels of gene expression in the resting state.

Table 4-1 Down-regulated genes in naïve *Itk*$^{-/-}$ vs. *Itk*$^{-/-}$ CD4^{+} T cells

Accession Number	*Gene Symbol*	*Name*	**Ratio*	*P. Value*
Cell surface Markers				
U12434.1	Cd6	CD6 antigen	0.56	0.00077
NM_007650.1	Cd5	CD5 antigen	0.49	0.00079
BI966443	Itm2a	integral membrane protein 2A	0.52	0.039
Transcription Factors				
BE952590	Sox4	SRY-box containing gene 4	0.55	0.0045
AF472514.1	Tox	Thymocyte selection-associated HMG box gene	0.70	0.026
Intracellular Signaling Molecule				
BC027159	Trib2 (Trb2)	tribbles homolog 2 (Drosophila)	0.63	0.038

Global gene expression was compared between naïve *5C.C7Rag*$^{-/-}$*Itk*$^{+/-}$ (WT) and *5C.C7Rag*$^{-/-}$*Itk*$^{-/-}$ (KO) CD4^{+} T cells. The results are from statistical analyses of 5 WT and 4 KO samples, using Bioconductor package AffylmGUI.

* Ratio = (KO signal/WT signal)

Figure 4-6 Down-regulated genes in naïve *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells

A heat map plot of the genes whose expression is decreased in naïve *Itk*$^{-/-}$ CD4^{+} T cells was generated with the dChip program. White represents the average signal across all samples; blue represents signals lower than the average and the darker the color the lower the signal; red represents signals higher than the average and the darker the color the higher the signal. WT0.x: replicates of non-stimulated *Itk*$^{+/-}$ samples; KO0.x: replicates of non-stimulated *Itk*$^{-/-}$ samples.

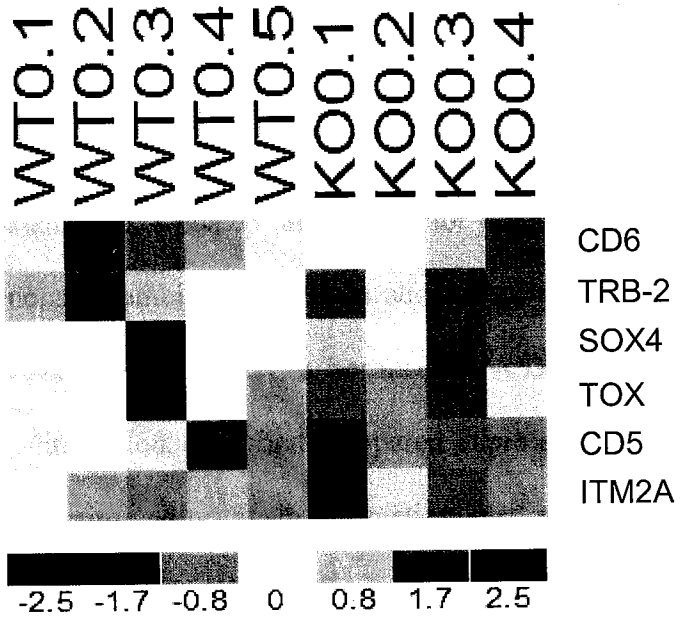


Figure 4-6 Down-regulated genes in naïve *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells

Differences in global gene expression of stimulated $Itk^{+/-}$ and $Itk^{-/-}$ $CD4^+$ T cells

We compared global gene expression profiles of $Itk^{+/-}$ and $Itk^{-/-}$ $CD4^+$ T cells stimulated with anti-CD3 plus anti-CD28. In these experiments, we stimulated the cells with anti-CD3 plus anti-CD28 coated latex beads for 6 and 36 hours. For the 36-hour stimulation, exogenous recombinant IL-2 was added in the culture to rule out possible secondary effects caused by a lack of IL-2, because $Itk^{-/-}$ $CD4^+$ T cells have defects in IL-2 production upon stimulation. We first compared expression profiles from stimulated samples (6- or 36-hour time point) against non-stimulated samples (0 time point) within each genotype. Then we compared the expression-changes between the two genotypes. This analysis would allow us to identify genes whose expression is regulated by both stimulation and the presence of Itk.

Interestingly, the expression-regulation pattern after stimulation from $Itk^{-/-}$ samples is almost identical to that from $Itk^{+/-}$ controls. For example, if a gene's expression is up-regulated upon stimulation in the $Itk^{+/-}$ samples, it is also up-regulated in the $Itk^{-/-}$ samples, and *vice versa*. The criteria for determining significance was that the change should have p-values of lower than or equal to 0.1 and should have B-statistic values of higher than zero. The significantly differentially regulated genes are listed in Table 4-2 and Table 4-3 for the 6-hour and 36-hour stimulation time points, respectively; heat maps depicting the expression patterns of these genes from both genotypes are shown in Figures 4-7 and 4-8, respectively. It is interesting to note that there is no overlap between these two lists. Intriguingly, closer examination of the data revealed that

the genes that are differentially regulated at 6-hour of stimulation are transiently up- or down-regulated in both genotypes. It will be interesting to compare expression profiles at more time points after stimulation.

After 6 hours of stimulation, the expression of the regulatory subunit of PI3K, p85, is down-regulated in both *Itk*$^{+/-}$ and *Itk*$^{-/-}$ CD4^{+} T cells (Table 4-1). This could be one mechanism by which activating signals are contracted, as p85 is required for PI3K activation. However, the magnitude of the downregulation is lower in *Itk*$^{-/-}$ CD4^{+} T cells than in *Itk*$^{+/-}$ CD4^{+} T cells. This result may indicate a delay in the signal contraction in *Itk*$^{-/-}$ CD4^{+} T cells and allow PI3K signals to continue in stimulated *Itk*$^{-/-}$ CD4^{+} T cells for a longer period of time. The continued stimulating signals may in turn allow *Itk*$^{-/-}$ CD4^{+} T cells to maintain wild type levels of gene expression by extended stimulation. However, not all the differentially regulated genes are expressed with the same pattern. For instance, the magnitude of down-regulation of the negative regulator, TRB2, is lower in *Itk*$^{-/-}$ CD4^{+} T cells compared to *Itk*$^{+/-}$ control cells. In addition, there are a few genes that are up-regulated in both genotypes, such as IL-22 and GM-CSF. Intriguingly, the magnitudes of up-regulation of these genes are increased in the absence of Itk (Table 4-2). The functional relevance of this is not clear, since this increase was transient and did not continue when the cells were stimulated longer. At 6-hour of stimulation with anti-CD3 plus anti-CD28, we consistently find reduced levels of *IL-2* expression in *Itk*$^{-/-}$ CD4^{+} T cells compared to WT CD4^{+} T cells, by quantitative real-time RT-PCR analysis [Chapter II and (170)]. To our surprise, this GeneChip analysis failed to identify such

defect in *Itk*$^{-/-}$ CD4^{+} T cells. Since the CD4 T cell preparations from *Itk*$^{-/-}$ mice are less pure than from control mice, signals from other contaminating cell types may have complicated the analysis. In addition, the stimulation conditions may have been too strong to reveal defects in *Itk*$^{-/-}$ CD4^{+} T cells. It is also possible that the GeneChip array analysis may not sensitive enough to detect defects in Itk deficient T cells. Therefore, this analysis may have missed genes whose expression is defective in *Itk*$^{-/-}$ T cells upon TCR plus CD28 stimulation, but can not be detected by this method.

Interestingly, at the 36-hour time point of stimulation, we found a set of differentially regulated genes that are in one pathway. After 36 hours of stimulation, IL-4 expression is up-regulated (about 3-fold) in *Itk*$^{-/-}$ CD4^{+} T cells while the change in expression of IL-4 in *Itk*$^{+/-}$ CD4^{+} T cells is negligible at this time point. At the same time point, GATA3, the IL-4 specific transcription factor (198), is down-regulated (about 2-fold) in *Itk*$^{+/-}$ CD4^{+} T cells, but it changes little in *Itk*$^{-/-}$ CD4^{+} T cells. Furthermore, Rog (repressor of GATA), which has been shown to interact with GATA3 and suppress the latter's transcription activity (199), is up-regulated in both stimulated *Itk*$^{+/-}$ and *Itk*$^{-/-}$ CD4^{+} T cells, but the magnitude of up-regulation in *Itk*$^{-/-}$ CD4^{+} T cells is lower than that in *Itk*$^{+/-}$ CD4^{+} T cells (Table 4-3). It is tempting to speculate that the differentially regulated Rog and GATA3 may allow higher IL-4 expression in *Itk*$^{-/-}$ CD4^{+} T cells at this time point. These results suggest that higher proportion of *Itk*$^{-/-}$ CD4^{+} T cells may be proceeding to Th2 differentiation. In line with this, the expression of CCR8, which is preferentially

expressed in Th2 cells (200), is up-regulated to a higher extent in *Itk*$^{-/-}$ CD4^{+} T cells than in *Itk*$^{+/-}$ CD4^{+} T cells stimulated for 36 hours.

In this analysis, we have obtained very short lists of genes that are differentially expressed and differentially regulated in *Itk*$^{-/-}$ CD4^{+} T cells compared to *Itk*$^{+/-}$ CD4^{+} T cells. The patterns of the expression of these genes are complicated. This suggests that Itk may function in regulating a variety of pathways. One possible complication is that Tec and Rlk could partially compensate for the function of Itk in *Itk*$^{-/-}$ T cells, although their message levels are down-regulated upon stimulation. However, since this analysis did not reveal the defect of *IL-2* expression, which is consistently identified by other methods, in *Itk*$^{-/-}$ CD4^{+} T cells, this analysis may have missed some other genes.

Table 4-2 Differentially regulated genes in *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells after 6-hour stimulation

Accession Number	*Gene Symbol*	*Name*	*#Changes in KO Samples*	*¶Changes in WT Samples*	**Ratio*	*P. Value*
Cell Surface Markers						
AI385482	Tnfrsf5	Tumor necrosis factor receptor superfamily, member 5 (CD40)	6.23	2.48	2.53	0.058
Cytokines or Chemokines						
X03019.1	Csf2	Colony stimulating factor 2 (granulocyte-macrophage) (GM-CSF)	14.1	4.53	3.12	0.086
BC012658.1	Ccl22	Chemokine (C-C motif) ligand 22	19.56	4.79	3.97	0.086
AJ249492.1	Iltifb (Il22)	Interleukin 10-related T cell-derived inducible factor beta (Interleukin 22)	83.29	23.59	3.53	0.026
Transcription Factors						
NM_030887.1	Jundm2	Jun dimerization protein 2	3.43	1.58	4.72	0.036
AJ252157.1	Foxo1	Forkhead box O1	0.60	0.31	1.93	0.054
Intracellular Signaling Molecules						
BC027159	Trib2 (Trb2)	Tribbles homolog 2 (Drosophila)	0.44	0.19	2.25	0.086
NM_010215.1	Il4i1	Interleukin 4 induced 1	7.21	2.48	2.91	0.023
M60651.1	Pik3r1	PI3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	0.68	0.38	1.79	0.054

Global gene expression was compared between 6-hour stimulated and naïve *5C.C7Rag*$^{-/-}$ *Itk*$^{+/-}$ (WT) as well as between 6-hour stimulated and naïve *5C.C7Rag*$^{-/-}$*Itk*$^{-/-}$ (KO) CD4^{+} T cells. The differences between stimulated versus naïve KO samples were then compared to those between stimulated versus naïve WT samples. The results are from statistical analyses using Bioconductor package AffylmGUI.

Changes in KO samples= (KO 6Hr signal/KO 0Hr signal)

¶ Changes in WT samples= (WT 6Hr signal/WT 0Hr signal)

* Ratio= (KO 6Hr signal/KO 0Hr signal)/(WT 6Hr signal/WT 0Hr signal)]

Figure 4-7 Differentially regulated genes in *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells after 6-hour stimulation

A heat map plot of the genes whose expression is differentially regulated in *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells after 6-hour stimulation was generated with the dChip program. White represents the average signal across all samples; blue represents signals lower than the average and the darker the color the lower the signal; red represents signals higher than the average and the darker the color the higher the signal. WT0.x: replicates of non-stimulated *Itk*$^{+/-}$ samples; WT06.x: replicates of 6-hour stimulated *Itk*$^{+/-}$ samples; KO0.x: replicates of non-stimulated *Itk*$^{-/-}$ samples; KO06.x: replicates of 6-hour stimulated *Itk*$^{-/-}$ samples.

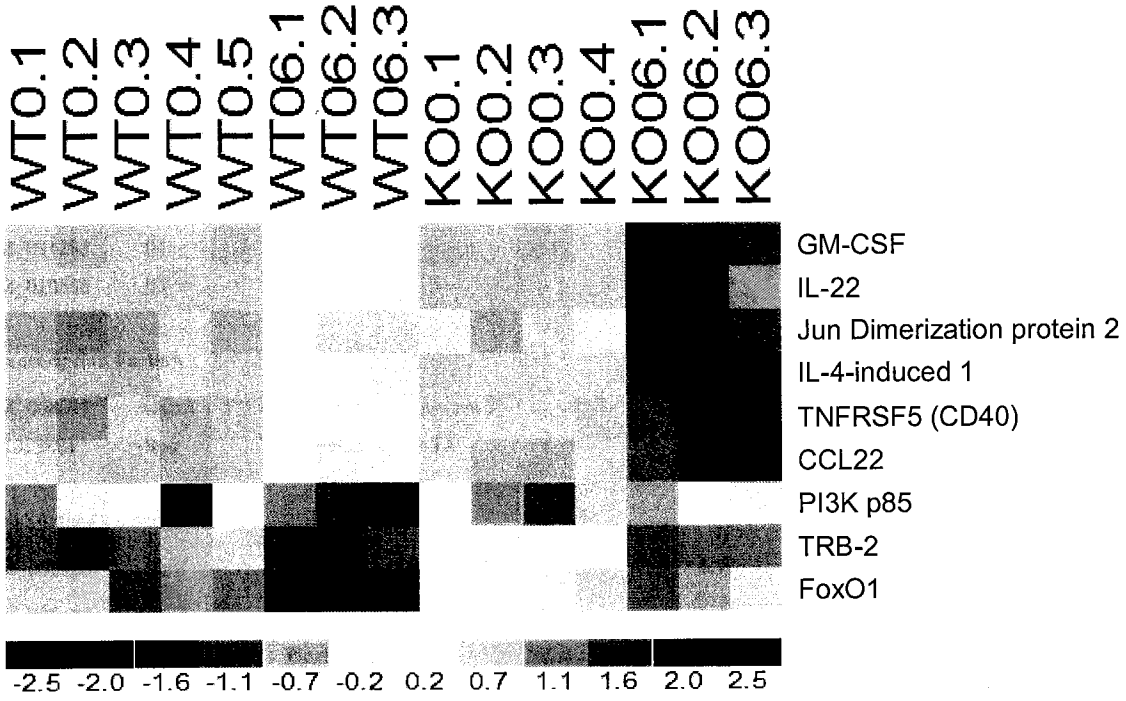


Figure 4-7 Differentially regulated genes in *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells after 6-hour stimulation

Table 4-3 Differentially expressed genes in *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells after 36-hour stimulation

Accession Number	*Gene Symbol*	*Name*	*#Changes in KO Samples*	*¶Changes in WT Samples*	**Ratio*	*P. Value*
Cytokines						
NM_021283	Il4	Interleukin 4	2.99	1.14	2.62	0.038
NM_010556	Il3	Interleukin 3	1.85	7.89	0.23	0.082
Transcription Factors						
NM_008091	Gata3	GATA binding protein 3	1.18	0.47	2.53	0.038
AK015881	Rog	Repressor of GATA	1.41	2.75	0.51	0.082
Cell Surface Markers						
NM_007720	Ccr8	Chemokine (C-C motif) receptor 8	6.77	2.69	2.51	0.038
NM_008479	Lag3	Lymphocyte-activation gene 3	1.46	5.50	0.27	0.08
BB476707	Plxnc1	Plexin C1 (CD232)	0.91	2.79	0.33	0.017
Metabolism related						
BC022959	Acsl6	Acyl-CoA synthetase long-chain family member 6	1.60	7.31	0.22	0.002
NM_022888	Folr4	Folate receptor 4 (delta)	4.32	1.77	2.45	0.038

Global gene expression was compared between 36-hour stimulated and naïve *5C.C7Rag*$^{-/-}$ *Itk*$^{+/-}$ (WT) as well as between 36-hour stimulated and naïve *5C.C7Rag*$^{-/-}$*Itk*$^{-/-}$ (KO) CD4^{+} T cells. The differences between stimulated versus naïve KO samples were then compared to those between stimulated versus naïve WT samples. The results are from statistical analyses using Bioconductor package AffylmGUI.

Changes in KO samples= (KO 36Hr signal/KO 0Hr signal)

¶ Changes in WT samples= (WT 36Hr signal/WT 0Hr signal)

* Ratio= (KO 36Hr signal/KO 0Hr signal)/(WT 36Hr signal/WT 0Hr signal)

Figure 4-8 Differentially regulated genes in *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells after 36-hour stimulation

A heat map plot of the genes whose expression is differentially regulated in *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells after 36-hour stimulation was generated with dChip program. White represents the average signal across all samples; blue represents signals lower than the average and the darker the color the lower the signal; red represents signals higher than the average and the darker the color the higher the signal. WT0.x: replicates of non-stimulated *Itk*$^{+/-}$ samples; WT36.x: replicates of 36-hour stimulated *Itk*$^{+/-}$ samples; KO0.x: replicates of non-stimulated *Itk*$^{-/-}$ samples; KO36.x: replicates of 36-hour stimulated *Itk*$^{-/-}$ samples.

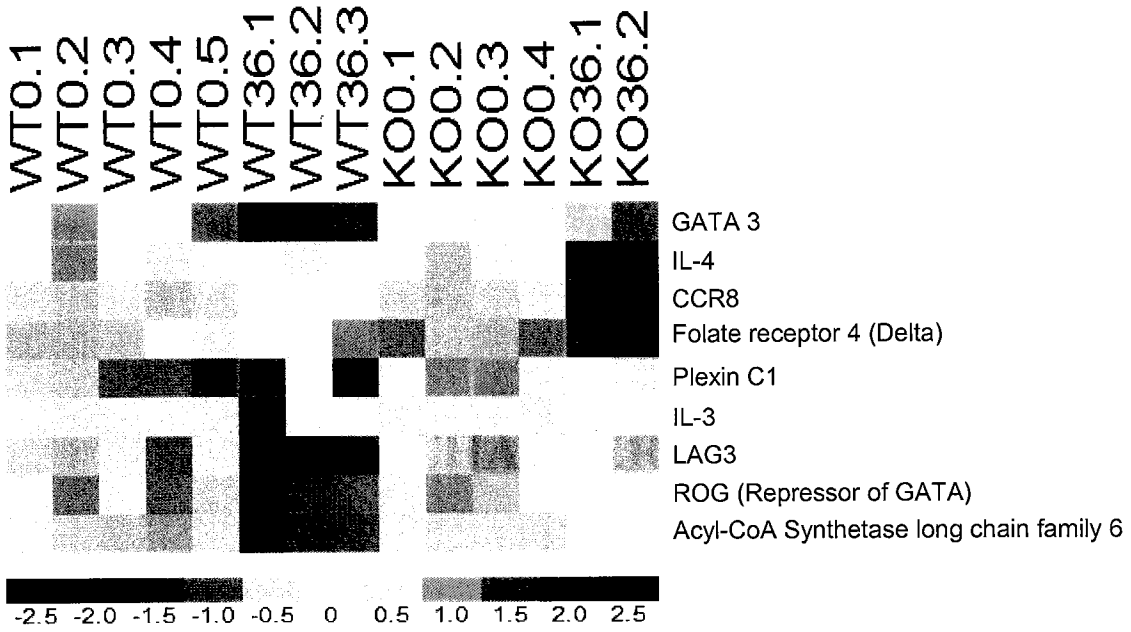


Figure 4-8 Differentially regulated genes in *Itk*$^{-/-}$ vs. *Itk*$^{+/-}$ CD4^{+} T cells after 36-hour stimulation

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

The data presented in this thesis have addressed the role of the Tec family kinases in $CD4^+$ T cell activation by using three different approaches. We were able to reveal some unexpected aspects of this family of kinases in T cells and to clarify some existing controversy about the putative role for Itk in CD28 costimulation signaling. The data also hint at the potential importance of selection pressure in T cell development and homeostasis. These studies contribute to the overall knowledge of the role of the Tec family kinases in $CD4^+$ T cell activation.

The role of Tec family kinases in CD28 costimulation

Numerous studies have established that the Tec kinases are critical for TCR signaling by activating PLCγ1. However, their role in other pathways has been controversial. Intense efforts to elucidate the CD28 costimulatory signaling pathway involved biochemical studies performed in Jurkat cell lines and implicated one of these family members, Itk, in positively regulating CD28 signaling. These biochemical studies relied on at least one of the following conditions: the use of Jurkat cells or other cell lines, the overexpression of proteins, and the use of anti-CD28 antibody for stimulation. First, the Jurkat cell line and its derivatives have been widely and successfully used in investigating the details in signaling in T cells (201). However, potential defects in this cell line may complicate the interpretation of the results. For instance, the phosphatase PTEN is defective in Jurkat cells, which results in accumulation of PIP_3 on the plasma membrane and constitutive localization of Itk at the membrane (202). Since PI3K and its downstream signaling pathways are important for CD28 signaling, Jurkat cells receive

constitutive "costimulation" intracellularly. Second, overexpression strategies make studying protein function convenient; however, overexpressed protein may skew the equilibrium of intermolecular interactions, leading to non-physiological associations. Finally, use of anti-CD28 antibodies to crosslink CD28 molecules assumes that antibody binding to CD28 mimics the interaction between B7 molecules and CD28. In light of a recent analysis comparing different anti-CD28 antibody clones and mapping the binding sites on the CD28 molecule (203), this assumption may not necessarily be true. For instance, while the anti-mouse CD28 antibody clone 37.51 binds close to the MYPPPY motif where B7 binds, other antibody clones, such as anti-human CD28 clone 5.11A1, bind to a different motif, the C"D loop (203). Binding to different sites may induce distinct conformational changes of the CD28 intracellular tail (61), and may induce a mitogenic effect by directly activating NFκB (203). Therefore, in studying CD28 signaling by antibody stimulation methods, one must be careful in selecting antibodies for stimulation and making conclusions based on these results.

On the other hand, another study examined the response of total primary $CD4^+$ T cells from *Itk*$^{-/-}$ mice by measuring T cell proliferation after anti-CD3 plus anti-CD28 stimulation. These data showed that total $CD4^+$ T cells from *Itk*$^{-/-}$ mice proliferated much more vigorously than $CD4^+$ T cells from litter-mate controls. These data led the investigators to conclude that Itk may act as a negative regulator in CD28 costimulatory signaling (142). This conclusion strikingly contradicted that of the biochemical studies in Jurkat cells. However, we now know that the proportion of memory-like $CD4^+$ T cells is

consistently 3-5 fold higher in *Itk*$^{-/-}$ mice than that of wild type mice and thus this conclusion is based on the response of non-equivalent cell populations. To confirm that this is the case, we decided to re-examine the role of Itk in CD28 signaling using highly purified naïve CD4^{+} T cells and a well-controlled stimulation system. As described in Chapter II, results from our functional analyses show that naïve CD4^{+} T cells from *Itk*$^{-/-}$ mice are not hyperresponsive to anti-CD3 plus anti-CD28 stimulation or MHC/peptide plus B7.1 stimulation. Therefore, Itk is not a negative regulator in the CD28 costimulatory signaling pathway. Furthermore, CD28 costimulation functions very efficiently in naïve *Itk*$^{-/-}$ CD4^{+} T cells. Moreover, the PI3K-Akt-GSK3 pathway, the only biochemically detectable signaling event upon CD28 ligation alone, is normal in naïve *Itk*$^{-/-}$ CD4^{+} T cells. These data indicate that Itk is not essential in CD28 signaling. We believe that this study helps to clarify the controversies in the literature about the role of Itk in CD28 costimulation.

The PI3K-Akt pathway has been implicated as being the most important transducer of CD28 costimulatory signals (90, 204, 205). Our data re-affirm the crucial role of PI3K in CD28 costimulation signaling, since CD28 costimulation effects are completely abolished when naïve CD4^{+} T cells are treated with the PI3K inhibitor LY294002 (Figure 2-4).

Importantly, our studies identified a previously unappreciated defect in NFκB activation in *Itk*$^{-/-}$ CD4^{+} T cells stimulated with anti-CD3 plus anti-CD28 antibodies

(Figure 2-5). Since NFκB activity is indispensable for T cell activation and effector function, elucidation of the mechanisms by which Itk regulates the NFκB pathway is crucial. One possible mechanism through which Itk may mediate NFκB activation is through PKCθ. PKCθ is a calcium-independent, DAG-dependent member of the PKC family. PKCθ is primarily expressed in T cells and is uniquely recruited into the immunological synapse formed during T cell activation (206, 207). The importance of PKCθ in TCR/CD28-induced NFκB activation has been demonstrated by *PKCθ*$^{-/-}$ T cells. In these cells, TCR/CD28-induced activation of NFκB, but not NFAT, is dramatically impaired. Strikingly, the activation of NFκB in the absence of PKCθ is specifically impaired in response to TCR ligation, since the activation of NFκB is normal in response to TNF treatment in *PKCθ*$^{-/-}$ T cells (208).

One mechanism by which Itk may regulate PKCθ is through the PLCγ1 product DAG. Another possible mechanism may be via an Itk-PKCθ interaction. Itk and Tec have been shown to be able to associate with PKCθ in an *in vitro* system (209). It is possible that by directly interacting with PKCθ, Itk facilitates the recruitment of PKCθ to the membrane, a critical step for its function (210). Alternatively, Itk may indirectly regulate the localization of PKCθ through regulating TCR-induced actin polymerization and polarization. Since a role for Itk is implicated in actin polymerization and polarization (133, 211, 212), it is possible that in the absence of Itk, defective actin polymerization and polarization may affect localization of some proteins to the rafts upon

TCR/CD28 stimulation, including PKCθ. Therefore, it will be interesting to examine IKK activation, PKCθ localization and activation after CD3/CD28 stimulation in *Itk*$^{-/-}$ T cells.

In the absence of Itk, CD28 stimulation efficiently enhances T cell responses induced by TCR signaling. However, the magnitude of the response from *Itk*$^{-/-}$ T cells is still lower than that from wild type T cells. Since Itk is required for optimal TCR signaling, we reason that this decreased response from *Itk*$^{-/-}$ T cells is likely due to the TCR signaling defects in these cells. Because of the difficulty in dissecting the CD28 and TCR signals functionally, it is hard to ascertain whether CD28 costimulatory activity is equally effective in the presence or absence of Itk. It is still possible that other T cell Tec family kinases, Tec and Rlk, may partially compensate for the loss of Itk, although we did not detect increased expression levels of Tec or Rlk in *Itk*$^{-/-}$ T cells. For instance, Tec has been shown to be capable of associating with the CD28 intracellular tail via the Tec SH3 domain and the proline rich regions of CD28 (155). This binding pattern is similar to the binding between Itk and CD28 (140). Moreover, Tec can be activated by CD28 stimulation, and activated Tec can then phosphorylate the RasGAP associated adaptor protein, p62^{Dok} (Dok1) (155). Finally, overexpressed Tec can enhance transcription of IL-2 and IL-4 reporter constructs in response to anti-CD28 plus PMA stimulation (155, 156). These data indicate that Tec may also play a role in CD28 costimulatory signaling. Whether these data from experiments with cell lines truly reflect a physiological role for Tec in CD28 costimulation awaits the examination of responses

of naïve *Tec*$^{-/-}$ and *Tec*$^{-/-}$*Itk*$^{-/-}$ T cells to CD28 costimulation. To date, there is no report implicating Rlk in CD28 signaling. Nonetheless, it will be interesting to examine CD28 signaling and CD28 costimulatory activity in naïve T cells that are deficient for all three Tec family kinases.

TecKD transgenic mice

In an effort to elucidate the function of Tec family kinases in T cell development and function, we generated transgenic mouse lines expressing kinase-dead Tec on the *Itk*$^{-/-}$*Rlk*$^{-/-}$ double-deficient background, hoping to obtain mice functionally lacking all three Tec kinases. The expression of the transgene was clearly detected at the message level, as shown in Figures 3-4 and 3-5. However, expression of the transgenic protein could not be detected. Although we cannot rule out possible technical issues, it is tempting to speculate that the loss of protein expression of the TecKD transgene may be due to a selection/survival pressure on the TecKD transgenic T cells. For instance, in the TecKD transgenic *Itk*$^{-/-}$*Rlk*$^{-/-}$ mice, if the Tec family kinases are essential for T cell development and/or survival, the survival pressure may select for those T cells in which the transgenic kinase-dead Tec protein is not expressed and/or the transgenic protein is rapidly degraded.

Data from some of our other studies also prompted us to think that there might be some selection or compensational mechanisms functioning in T cells. In those studies, transgenic mice were generated that express the phosphatase PTEN under the control of

the proximal *lck* promoter. Expression of the transgenic protein was high in thymocytes. It was expected that overexpressed PTEN would decrease the amount of PIP_3 in the plasma membrane. However, contrary to this, the PIP_3 levels in the thymocytes from these mice were much higher than those from wild type control mice (W-C Yang, C-R Li and LJ Berg, unpublished data). These data imply that in the thymocytes from the PTEN transgenic mice, some mechanisms may have developed to compensate for the overexpressed PTEN by increasing PI3K activity, since signaling down-stream of PIP_3 is important for cell survival and cell cycle progression. The development of these mechanisms may have been the result of a selection pressure for survival. Surprisingly, selection is very efficient in both the PTEN transgenic mice and the TecKD transgenic mice, as the thymic cellularity of the PTEN transgenic mice and the TecKD transgenic mice are similar to those of their littermate controls.

If such selection mechanisms truly exist and function efficiently in T cells, strategies involving expression-based disruption of Tec would not be successful on the $Itk^{-/-}Rlk^{-/-}$ background. Therefore, an $Itk^{-/-}Rlk^{-/-}Tec^{-/-}$ mouse line is still needed for these studies. As mentioned earlier, the *Tec* and *Rlk* genes are linked closely on the same chromosome, making it very difficult to generate a *Tec Rlk* double-mutant allele with conventional targeting methods. This problem might be solved by using a recently developed BAC- (bacterial artificial chromosome) based targeting technique (213). This method would save large amounts of time and resources necessary to achieve such double mutations by conventional targeting techniques.

Global gene expression profiling of $Itk^{+/-}$ and $Itk^{-/-}$ $CD4^+$ T cells

In an effort to identify potential transcriptional targets of Itk, the major Tec family kinase expressed in T cells, we performed a gene expression profile analysis on $Itk^{+/-}$ and $Itk^{-/-}$ $CD4^+$ T cells. To our surprise, this set of analyses resulted in a very short list of genes that are differentially expressed in $Itk^{-/-}$ versus $Itk^{+/-}$ $CD4^+$ T cells. When comparing non-stimulated naïve $Itk^{-/-}$ $CD4^+$ and $Itk^{+/-}$ $CD4^+$ T cells, we disregarded all "up-regulated" genes, because the majority of these may be the result of the $CD4^+$ T cells from $Itk^{-/-}$ mice being less pure than those from $Itk^{+/-}$ mice. We understand that by doing this we may be missing some *bona fide* up-regulated genes in $Itk^{-/-}$ T cells. Even then, the small number of down-regulated genes in naïve $Itk^{-/-}$ $CD4^+$ T cells suggests that, in terms of the basal level of gene transcription, the $Itk^{-/-}$ $CD4^+$ and $Itk^{+/-}$ $CD4^+$ T cells are very similar. In addition, comparison of the non-stimulated naïve $CD4^+$ $Itk^{-/-}$ and $CD4^+$ $Itk^{+/-}$ T cells suggests that the few down-regulated genes are the result of altered TCR signaling during development of $Itk^{-/-}$ $CD4^+$ T cells.

Furthermore, when comparing stimulated $Itk^{+/-}$ $CD4^+$ and $Itk^{-/-}$ $CD4^+$ T cells, we did not identify many differentially regulated genes. Surprisingly, this analysis even failed to identify genes, such as *IL-2* and *Bcl-xL*, whose expression is always found by quantitative real-time PCR to be lower in $Itk^{-/-}$ $CD4^+$ T cells compared to *WT* $CD4^+$ T cells stimulated under the same anti-CD3 plus anti-CD28 condition. This could be due to the conditions of the antibody stimulation, which may have been too strong to reveal defects in $Itk^{-/-}$ $CD4^+$ T cells. It is also possible that the GeneChip array analysis may not

sensitive enough to detect defects in Itk deficient T cells. Therefore, this analysis may have missed genes whose expression is defective in *Itk*$^{-/-}$ T cells upon TCR plus CD28 stimulation, but can not be detected by this method. Moreover, when the cells were stimulated for 36 hours, exogenous IL-2 was added to the culture, to rule out possible defects in *Itk*$^{-/-}$ CD4^{+} T cells that are secondary to their reduced ability to produce IL-2 upon stimulation. This added IL-2 may have in turn masked some defects in *Itk*$^{-/-}$ CD4^{+} T cells, because there are shared signaling pathways between IL-2 and TCR/CD28, such as the PI3K-Akt pathway. With this in mind, it will be interesting to compare expression profiles from *Itk*$^{+/-}$ CD4^{+} and *Itk*$^{-/-}$ CD4^{+} T cells that are stimulated with various conditions, *e.g.* titration of anti-CD3 and anti-CD28 antibodies, for various periods of time. Again, possible functional compensation from Rlk and Tec may also contribute to the results. Nonetheless, we have found several interesting genes, whose expression is regulated both by TCR/CD28 stimulation and by Itk. These differentially regulated genes not only re-emphasize the importance of Itk in TCR signaling but also provide clues or a new starting point for further investigation of the functions of this Tec family kinase. The first step in these efforts will be to verify these findings by other methods, such as quantitative real-time RT-PCR. If the differential expression of these genes can be verified, the next step will be to examine whether these differentially expressed genes result in differential protein levels in the cells. In addition, the complex expression patterns of these genes imply that Itk may participate in regulating several different pathways at different stages of T cell activation.

The implications of functions of Tec kinases in T cells and other cell types

Compelling evidence has indicated that the Tec family kinase Itk plays an important role in Th2 effector differentiation. When wild type naïve $CD4^+$ T cells are stimulated under Th2-skewing conditions, Itk expression levels are increased (134). When naïve *Itk*$^{-/-}$ $CD4^+$ T cells are stimulated they produced reduced amounts of IL-4 than wild type $CD4^+$ T cells (121, 134). Furthermore, under certain conditions, e.g. stimulation with altered peptide ligand, where wild type $CD4^+$ T cells produce IL-4, *Itk*$^{-/-}$ $CD4^+$ T cells preferentially produce IFNγ (134). More importantly, *Itk*$^{-/-}$ mice cannot mount protective Th2 responses to infections with pathogens that are used to evaluate effective Th2 responses, such as in the *Schistosoma mansoni* and *Leishmania major in vivo* models (119, 121). Consistent with a role for Itk in Th2 differentiation, when *Itk*$^{-/-}$ mice were subjected to allergic asthma induction, these mice had decreased production of Th2 cytokines such as IL-5 and IL-13, reduced T cell infiltration in the lung and reduced mucus production (214). The mechanisms by which Itk may promote Th2 differentiation may involve the regulation of T-bet, a Th1 specific transcription factor. Itk has been shown to directly phosphorylate T-bet, and may inhibit its expression (134, 173).

Taken together, these findings not only established a role for Itk in Th2 differentiation and related allergic response but also indicate that Itk is a promising therapeutic target. To support this idea, expression levels or polymorphisms of the Tec family kinase Itk has been correlated with human allergic disorders. For instance, increased expression of Itk has been detected in peripheral blood T cells from patients

with atopic dermatitis (215). Moreover, it was recently found that certain polymorphisms at the 5' end of the *Itk* gene are related to human atopy (216). Therefore, successful control of the functions of Itk *in vivo* may help to treat human allergic disorders such as asthma and atopy.

In designing therapeutic agents, it should be noted that kinase activity may not be the only function of Tec family kinases. Indeed, a kinase-independent function of Itk has been recently demonstrated in studies where the endogenous expression of Itk in Jurkat cells was eliminated using an RNAi-based approach. TCR-induced Vav localization and actin polarization were reduced in these cells, but were rescued by re-expression of a kinase-dead mutant of Itk (133). These data indicate that the Tec kinases may function not only as kinases but also as adaptor molecules that integrate the signaling complexes. Thus, more attention is needed to dissect the functions of individual domains of Tec kinases in T cell activation. A better understanding of the functions of different domains of the Tec kinases will certainly assist in designing of therapeutic agents that target members of this family of kinases. To this end, genetically targeted mouse models will be needed that express physiological (endogenous) levels of Tec kinases with mutations in different domains.

The use of primary T cells from genetically manipulated animals, such as the $Itk^{-/-}$ or the $Itk^{-/-}Rlk^{-/-}$ mouse lines, has successfully provided substantial knowledge on the genes and/or their products. However, there are two potential difficulties in assessing

data from these experiments in general. One of these is the inability to distinguish between the direct effects of the gene of interest at the developmental stage of the cells under investigation versus the effects imposed by the gene on the cells through development up to that point. Specifically, the peripheral T cells from *Itk*$^{-/-}$ mice have developed in the absence of Itk throughout the developmental stages in the thymus. When these cells are stimulated and compared to wild type peripheral T cells that have developed in the presence of Itk and stimulated under the same conditions, it is difficult to rule out the possible developmental effects that the lack of Itk has had on the peripheral *Itk*$^{-/-}$ T cells.

The other difficulty is that a single gene may be expressed in a variety of cells types that can affect one another. For instance, in addition to T cells, Itk is also expressed in mast cells, NK cells, and possibly others. Possible defects in these cells may have extrinsic effects on T cells in an *Itk*$^{-/-}$ mouse. Indeed, when *Itk*$^{-/-}$ mast cells are stimulated through the IgE receptor, they produce dramatically increased amounts of IL-4, IL-13 and IL-6 when compared to wild type mast cells, implying that Itk may play a negative role in mast cell activation (Y Kosaka and LJ Berg, personal communication). Increased levels of these cytokines may drive the overall environment of the *Itk*$^{-/-}$ mice to the Th2 type. In support of this idea, serum from *Itk*$^{-/-}$ mice contains higher levels of IgE, an indication of a Th2-skewed environment [(119), Y Kosaka and LJ Berg, personal communication]. This environment in *Itk*$^{-/-}$ mice is strikingly contrary to what one would predict based on the role of Itk in Th2 differentiation. T cells that develop and survive in such a

dysregulated cytokine environment, however, may have been re-wired to fit in that environment. Therefore, when these cells are stimulated and compared to cells from a wild type (un-skewed) environment and stimulated under the same conditions, it is difficult to distinguish an intrinsic effect of the gene of interest (*Itk* in this case) and the extrinsic effect from the environment.

Moreover, there may also be an increase of other cell types in $Itk^{-/-}$ mice, such as $CD4^+$ non-T cells, since the $CD4^+$ T cell preparations from $Itk^{-/-}$ mice are consistently less pure (in terms of CD3 or TCR staining) than those from $Itk^{+/-}$ or wild type mice. It is not clear why and how the numbers of other types of cells should increase. However, it is possible that Itk may also be expressed in other cell types, such as dendritic cells, macrophages and granulocytes. The loss of Itk may then affect the development and/or function of these cells, which may in turn affect yet other cell types such as T cells. This is a yet poorly explored field that may prove to be an important and fruitful one. Results from these studies will not only help to explain the phenotypes of $Itk^{-/-}$ mice, but also further our understanding of how this family of kinases functions in different cell type and aid in a more rational design of therapeutic agents. Therefore, a better understanding of the functions of the Tec family kinases in different cell types will require cell lineage-specific as well as developmental or activation stage-specific manipulation of these kinases.

CHAPTER VI

MATERIALS AND METHODS

Mice

The *Itk*$^{-/-}$ mouse line was generated previously in the lab (115) and has been backcrossed to C57BL/10 (B10) mice (Charles River Laboratories, Wilmington, MA) for over ten generations. The *Itk*$^{-/-}$ mice were also crossed to the *Rag2*$^{-/-}$ background and 5C.C7 TCR transgenic (*5C.C7Tg*). The *Itk*$^{-/-}$*Rlk*$^{-/-}$ mouse line was a generous gift from Dr. Pamela Schwartzberg (117). The hCD2-rtTA transgenic mouse line on the B10 background was a generous gift from Dr. Rose Zamoyska (169). This mouse line was crossed to *Itk*$^{-/-}$*Rlk*$^{-/-}$ mice to generate *Itk*$^{-/-}$*Rlk*$^{-/-}$*rtTATg*. All mice used were between 6 to 12 weeks old and maintained in a Specific-Pathogen-Free (SPF) facility.

Preparation of naïve CD4^{+} T cells

Lymph nodes and spleens were dissected from mice, and single-cell suspensions were prepared. After red blood cells were lysed, the cells were labeled with anti-CD4-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA). The labeled cells were then separated by Auto-MACS (Miltenyi Biotec, Auburn, CA) according to manufacturer's instructions. The enriched CD4^{+} cells were stained with anti-CD4-CyChrome (Cy) and anti-CD44-FITC (BD Pharmingen, San Diego, CA). CD4^{+}CD44^{low} (naïve) T cells were sorted on a Becton-Dickinson FACSVantage cell sorter. Purified populations were >95% CD4^{+}CD44^{low} cells. For some experiments, naïve CD4^{+} T cells were purified with anit-CD4-FITC (BD Pharmingen), anti-FITC MultiSort kit (Miltenyi Biotec) and anti-CD62L magnetic microbeads (Miltenyi Biotec), according to the manufacturer's instructions.

This purification strategy yielded 90% to 97% $CD4^+CD44^{low}$ T cells. The $CD4^+$ T cells from *Rag2*$^{-/-}$*5C.C7Tg Itk*$^{-/-}$ and *Rag2*$^{-/-}$*5C.C7TgItk*$^{+/-}$ mice are consistently >95% $CD44^{low}$. After purification, the cells were resuspended with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 3mM $_L$-Glutamine, 100 unit/ml penicillin, 100μg/ml streptomycin, 10mM HEPES, and 50μM 2-mercaptoethanol.

T cell stimulation

Purified naïve $CD4^+$ T cells were stimulated with antibody-coated latex beads or plates. Surfactant-free sulfate-charged 4.9μm white polystyrene latex beads (Interfacial Dynamics Corporation, Tualatin, Oregon) were coated with the following combinations of monoclonal antibodies: anti-CD3ε (clone 145 2C11, BD Pharmingen) at 0.5μg/ml plus a hamster IgG isotype control (eBioscience, San Diego, CA) at 4.5μg/ml; anit-CD3□at 0.5μg/ml plus anti-CD28 (clone 37.51, eBioscience) at 4.5μg/ml; anti-CD28 at 5μg/ml; or a hamster IgG isotype control at 5μg/ml. For antibody coating, latex beads were incubated at $1x10^7$/ml with the indicated combinations of antibodies at 37°C for 1.5 hours with rotation; after washing with PBS, the beads were resuspended in RPMI-1640 medium supplemented with 10% FBS. To coat plates with anti-CD28 antibody, plates were incubated at 37°C with anti-CD28 (clone 37.51, eBioscience) at 5μg/ml for 1.5 hours, and then stored at 4°C.

To stimulate naïve $CD4^+$ T cells, purified cells were incubated with an equal number of antibody-coated latex beads, or on anti-CD28 coated plates, at 37°C for

indicated period of time. Where indicated, PMA (Sigma, St. Louis, MO) was added at various concentrations.

T cell proliferation assay

To stimulate naïve $CD4^+$ T cells, $1x10^5$ purified cells were incubated with an equal number of antibody-coated latex beads. Where indicated, PMA (Sigma) was added at various concentrations. For stimulation of *Rag2$^{-/-}$5C.C7Tg* T cells, $1x10^5$ purified CD4+ T cells were incubated with varying concentrations of the moth cytochrome c (MCC) peptide residues 93-103 plus $1x10^5$ mitomycin C-treated CHO cells expressing mouse MHC class II IE^k (CHO-IE^k; (217)), or CHO cells expressing IE^k and mouse B7.1 (CHO-IE^k/B7.1; (135)). The culture volume was 200 µl. After incubation at 37°C for 48 hours, 1µCi of ^{3}H-Thymidine (NEN, Boston, MA) was added to each well, and the plates were incubated for 18 additional hours. The cells were then harvested on a Tomtec Harvester 96 (Orange, CT), and ^{3}H-Thymidine was quantified on a Trilux microbeta counter (PerkinElmer, Wellesley, MA). As a control, cells were also stimulated with 5ng/ml PMA and 375ng/ml ionomycin (Calbiochem, La Jolla, CA).

Western Blotting analysis

Following stimulation, the cells were lysed with lysis buffer containing 25mM Hepes (pH7.5), 150mM NaCl, 1mM EDTA, 1% Triton x-100, 1mM Na_3VO_4, and

protease inhibitor cocktail (Roche, Indianapolis, IN). Forty micrograms of lysate protein from each sample was resolved by SDS-PAGE, transferred to Immobilon-P PVDF membrane (Millipore, Billerica, MA), and then probed with antibodies to phospho-AKT or phospho-GSK3β (Cell Signaling Technology, Beverly, MA). After stripping, the membranes were re-probed with antibodies to total AKT (Cell Signaling Technology), GSK3 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), or p85 subunit of PI-3-kinase (Upstate Biotechnology, Lake Placid, NY). Antibody binding was detected by chemiluminescence using the ECL kit (Amersham Biosciences, Piscataway, NJ). To detect Tec transgene expression, antibodies to mouse Tec (Upstate Biotechnology) and to FLAG (Sigma) were used.

Quantitative real-time PCR

T cells were stimulated as described above for 6 hours; total RNA was isolated from stimulated cells with RNeasy kit (Qiagen, Valencia, CA). After treatment with DNAse I (Ambion Inc, Austin, TX), 1μg of total RNA was used to synthesize cDNA with SuperScript first strand cDNA synthesis kit (Invitrogen, San Diego, CA). Quantitative Real-Time PCR was performed on an i-Cycler (BioRad, Hercules, CA), using SYBR Green PCR core reagents (ABI, Foster City, CA). The primer sequences are as the followings:

IL2 F 5'CCTGAGCAGGATGGAGAATTAC3',

IL2 R 5'TCCAGAACATGCCGCAGAG3';

BclxL F 5'ATTGGTGAGTCGGATTGC3',

BclxL R 5'CACAGTCATGCCCGTCAG3';

CD40L F 5'GATCCTCAAATTGCAGCAC3',

CD40L R 5'CCGATTAGAGCAGAAGGTG3';

GAPDH F 5'ATGTGTCCGTCGTGGATCTGA3',

GAPDH R 5'CCTGCTTCACCACCTTCTTGAT3'.

Preparation of nuclear extracts and detection of activated NFκB (p65)

After stimulation, the cells were harvested and washed with PBS. The cells were then resuspended with 200μl Buffer A (10mM HEPES pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, and protease inhibitor cocktail). The samples were incubated on ice for 15 minutes, and 12.5μl of 10% NP40 was added. After vigorous vortexing, the samples were briefly centrifuged. The supernatant was removed and the pellets were washed once with Buffer A. Fifty microliters of Buffer C (20mM HEPES pH7.9, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, and protease inhibitor cocktail) was added to each sample. The samples were incubated on ice for one hour, with vigorous vortexing every 5 minutes. The samples were then centrifuged at maximum speed for 15 minutes. The supernatant containing nuclear protein was collected and protein concentration was determined by Bradford assay (BioRad, Hercules, CA) according to manufacturer's instructions. Five micrograms of nuclear protein was subjected to NFκB (P65) functional ELISA using BD TransFactor NFκB P65

kit (BD Biosciences, Palo Alto, CA), according to the manufacturer's instructions. Briefly, 5μg of nuclear protein, mixed with blocking solution to a volume of 50 μl, was added into a well pre-coated with NFκB binding oligonucleotides (sequence GGGGTATTTCC). The binding of activated NFκB from the nuclear extract to the oligonucleotides was detected with an anti-p65 antibody, HRP-conjugated secondary antibody, and HRP substrate TMB. The signals were read on an EMax precision microplate reader (Molecular Devices Corporation, Sunnyvale, CA)

FACS analysis

Cells were stained with the indicated antibodies in HBSS supplemented with 3% FCS for 30 min at 4°C. Cells were then washed and analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA). Data were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA) or FlowJo software (Tree Star Inc, Ashland, OR). The antibodies used were anti-CD4-CyChrome (Cy), anti-CD4-PE, anti-CD69-FITC, anti-CD44-FITC, anti-CD44-Cy, anti-CD25-FITC, and anti-CD62L-PE, anti-CD8-FITC, anti-TCRβ-FITC and anti-TCRγδ-FITC (BD Pharmingen, San Diego, CA).

Calcium flux analysis

CD4+ T cells were resuspended in RPMI-1640 containing 3% FBS and calcium indicator dyes Fluo-3 (Molecular Probes Inc, Eugene, OR) at 3μg/ml and Fura-Red (Molecular Probes Inc) at 5μg/ml M. Cells were incubated at 37°C in the dark for 45

minutes, washed twice with RPMI-1640 containing 3% FBS, and incubated with the same medium for 30 minutes in the dark. The cells were then stained with Biotin-conjugated anti-CD3 (clone 145 2c11, eBioscience) at 2μg/ml or 5μg/ml with or without 10μg/ml of Biotin-conjugated anti-CD28 (clone 37.51, eBioscience). The cells were washed twice with HBSS, resuspended with HBSS at 10^7/ml, and kept on ice in the dark until analysis. Prior to analysis, $4X10^6$ cells (in 300μl) were added to 500μl HBSS and warmed to 37°C. Flow cytometric analysis was performed at 37°C on a BD FACSCalibur (BD Biosciences). Cells were collected for 30 seconds prior to stimulation, and 5 minutes after addition of 5μg/ml streptavidin (Pierce Biotechnology Inc, Rockford, IL). Finally, 5μg/ml ionomycin was added, and cells were collected for additional 1.5 minutes. The results were analyzed with FlowJo software.

Generation of constructs for TecKD transgenic mice

A point mutation, K397E, in the mouse Tec cDNA was introduced by site-directed mutagenesis; a FLAG tag was added to the 5' end of the cDNA. This mutant version of Tec cDNA is called TecKD, since the mutation disrupts the binding of ATP and hence the activity of the kinase (158). The vector pTLC, which contains a 3.2kb mouse *lck* proximal promoter, a BamHI cloning site and a human CD2 3'-LCR, was a kind gift of Dr. Falk Weih (167). The vector pTRE2 was purchased from BD Biosciences Clontech (Palo Alto, CA).

To generate a construct in which the expression of TecKD is driven by the *lck* promoter, the TecKD cDNA was first cut out from pMSCV vector with XhoI and HindIII, and sub-cloned in to pBluescript (pBS). The plasmid pBS-TecKD was then digested with BamHI and the insert was cloned into pTLC after the *lck* promoter. The resulting plasmid is named pTLC-TecKD. DNA sequencing was performed at every cloning step to verify correct junctions, direction and sequences.

To generate a construct in which the expression of TecKD can be induced by tetracycline or doxycycline, the pBS-TecKD plasmid was digested with BamHI and NotI, and then cloned into pTRE2 after the tetO/CMVmin promoter. The resulting plasmid was named pTRE-TecKD. DNA sequencing was performed to verify correct junctions, direction and sequence.

The plasmids were expanded in E. *coli.*, and purified with a plasmid Maxi-prep kit (Qiagen). To remove the vector, plasmids pTLC-TecKD was digested with SacII and plasmid pTRE-TecKD was digested with the combination of XhoI and SapI. Finally, the transgenic fragments TLC-TecKD and TRE-TecKD were purified by gel-extraction.

Generation of TecKD transgenic mouse lines

Three-week old female $Itk^{-/-}Rlk^{-/-}$ mice were superovulated and bred to adult male $Itk^{-/-}Rlk^{-/-}$ mice. Thus, the resulting fertilized eggs should all be $Itk^{-/-}Rlk^{-/-}$. The transgene

fragments were micro-injected directly into these fertilized *Itk*$^{-/-}$*Rlk*$^{-/-}$ eggs. Therefore, all transgenic mice are on *Itk*$^{-/-}$*Rlk*$^{-/-}$ double-deficient background. The micro-injection was performed by the University of Massachusetts Medical School Transgenic Animal Modeling Core. Founders were screened by both Southern Blot and PCR on genomic DNA from tails.

Screening transgenic founder lines by Southern Blot

The 1.9kb FLAG-TecKD fragment was labeled with α^{32}PdCTP (NEN, Boston, MA), random hexamer primers, and the Klenow fragment of DNA polymerase I. The labeled probes were cleaned with MicroSpin G-25 columns (Amersham Biosciences, Piscataway, NJ). The tail DNA from founders and wild-type controls were digested with BamHI (for the TLC-TecKD lines) or XhoI plus SapI (for the TRE-TecKD lines). Digested tail DNA was resolved with a 1% agarose gel in TAE, and transferred to nylon membrane. The membranes were then probed with ^{32}P labeled FLAG-TecKD probes. After stringent wash, the signal was detected by autoradiography.

Genotyping TecKD transgenic mice by PCR

PCR was also utilized in the initial screening of the founders. After confirming that PCR results were the same as those from Southern Blotting, PCR was used routinely for genotyping progeny.

For genotyping the TLC-TecKD lines, the primers used were:

5'CTGTGAACTTGGTGCTTGAGGGCTC3' (on the *lck* promoter)

5'GACAGGGAATGACACCATCATCG3' (on Tec cDNA)

For genotyping the TRE-TecKD lines, the primers used were:

5'CGCCTGGAGACGCCATC3' (on the "Tet-on" promoter)

5'GACAGGGAATGACACCATCATCG3' (on Tec cDNA)

Induction of TecKD transgene expression

To induce the expression of the transgene in the TRE-TecKD mouse lines, the mice were crossed to *Itk*$^{-/-}$*Rlk*$^{-/-}$*rtTATg* mice. The mice were fed drinking water containing doxycycline (Sigma) at 2mg/ml and 0.4% sucrose. Drinking water was prepared fresh and changed every two days.

Stimulation of CD4^{+} *T cells for microarray analysis*

CD4+ T cells were purified from *Rag2*$^{-/-}$*5C.C7Tg Itk*$^{-/-}$ and *Rag2*$^{-/-}$*5C.C7TgItk*$^{+/-}$ mice as described above. The cells were left unstimulated or stimulated for 6 hours or 36 hours with anti-CD3 plus anti-CD28 antibody-coated beads. The six-hour stimulation was the same as described above. For the 36-hour stimulation, 1X10^{7} CD4+ T cells were incubated with an equal number of antibody-coated beads, incubated at 37°C for 24 hours. Subsequently, recombinant mouse IL-2 (BD Pharmingen) was added to a final

concentration of 5ng/ml. The culture was continued at 37°C for 12 additional hours before harvest.

Isolation of total RNA and synthesis of cRNA for microarray analysis

After cell harvest, total RNA was extracted with TriZol reagent (Invitrogen) according to the manufacturer's instructions. The RNA samples were further purified with RNeasy kit (Qiagen, Valencia, CA). cDNA and cRNA synthesis were performed according to Affymetrix protocols. Briefly, 5-10μg of each total RNA sample was used to synthesize double-strand cDNA using cDNA synthesis kit (Invitrogen) with an oligo$(dT)_{24}$ primer containing a T7 promoter sequence added to the 3' end (Affymetrix, Santa Clara, CA). Subsequently, biotin-labeled cRNA was synthesized from the cDNA samples by *in vitro* transcription (IVT) using the ENZO^{TM} BioArray HighYield RNA Transcription Labeling kit (ENZO Life Sciences Inc, Farmingdale, NY). The labeled cRNA samples were purified using RNeasy kit (Qiagen), and then fragmented by mild alkaline treatment at 94°C for 35 minutes.

Microarray hybridization and scanning

Fragmented cRNA samples were used to prepare hybridization cocktail at a final concentration of 0.05μg/μl. A mixture of four control cRNAs from bacterial and phage genes was included in the hybridization cocktail (BioB, BioC, BioD and cre, at 1.5, 5, 2.5

and 100 pM respectively) to serve as hybridization controls. A biotin-labeled oligonucleotide, B2, was also included to the hybridization cocktail, which hybridized to unique features at the center and four corners of each chip to facilitate precise alignment and mapping of the probe sets. Hybridization and scanning were performed at University of Massachusetts Medical School Genomics Core Facility, according to Affymetrix protocols. Briefly, the sample cocktails were incubated at 99°C for 5 minutes, equilibrated to 45°C for 5 minutes, and clarified by centrifugation (14,000g) at room temperature for 5 minutes. Aliquots of each sample were hybridized to MOE430A GeneChip arrays (Affymetrix) at 45°C for 16 hours with rotation. The arrays were then washed, stained with R-phycoerythrin Streptavidin (Molecular Probes), washed again, and scanned by the GeneArray Scanner (Agilent Technologies). Prior to MOE430A hybridization, Test GeneChip arrays (Affymetrix) were hybridized to test sample integrity.

Microarray data analysis

Initial data analysis was performed by Affymetrix Microarray Suite (MAS) 5.0 software (Affymetrix). All further statistical analyses and quality assessment were performed by using Bioconductor version 1.6 (http://www.bioconductor.org) (174) in the R environment (http://www.r-project.org). Expression values were background corrected, normalized, and summarized by using the default settings of the Affy, AffyPLM and AffylmGUI packages from Bioconductor and the dChip program (175).

CHAPTER VII

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APENDIX

PUBLICATION

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Itk Is Not Essential for CD28 Signaling in Naive T Cells[1]

Cheng-Rui Li and Leslie J. Berg[2]

Itk, a member of the Tec family of tyrosine kinases, is critical for TCR signaling, leading to the activation of phospholipase Cγ1. Early biochemical studies performed in tumor cell lines also implicated Itk in CD28 signaling. These data were complemented by functional studies on primary $Itk^{-/-}$ T cells that suggested a negative role for Itk in CD28 signaling. In this report, we describe a thorough analysis of CD28-mediated responses in T cells lacking Itk. Using purified naive $CD4^+$ T cells from $Itk^{-/-}$ mice, we examine a range of responses dependent on CD28 costimulation. We also analyze Akt and glycogen synthase kinase-3β phosphorylation in response to stimulation of CD28 alone. Overall, these experiments demonstrate that CD28 signaling, as well as CD28-mediated costimulation of TCR signaling, function efficiently in the absence of Itk. These findings indicate that Itk is not essential for CD28 signaling in primary naive $CD4^+$ T cells. The Journal of Immunology, 2005, 174: 4475–4479.

Two signals are required for the optimal activation of naive T cells, one from the TCR and the second from a costimulatory receptor. On naive T cells, the CD28 receptor provides the primary costimulatory signal following interaction with CD80 or CD86 on APCs (for review, see Refs. 1 and 2). Functionally, CD28 costimulation enhances the survival, cell cycle progression, and cytokine production by activated T cells. Although tremendous effort has been directed at elucidating the signaling pathway(s) initiated by CD28 stimulation, the detailed mechanism by which CD28 costimulation operates has not yet been determined, in part due to the difficulty of distinguishing the TCR- vs the CD28-mediated signals in primary T cells.

The Tec family tyrosine kinase, Itk, has been previously implicated in CD28 signaling. Although Itk is primarily associated with TCR signaling (3–5), a number of biochemical studies have demonstrated an interaction between Itk and CD28. Specifically, Itk coimmunoprecipitates with CD28 from Jurkat tumor cells and, in addition, is tyrosine phosphorylated in response to CD28 cross-linking (6, 7). In vitro studies using recombinant proteins indicate that Itk binding to CD28 depends on the activity of the Src-family tyrosine kinase, p56lck (Lck) (8, 9). Structure-function analysis of CD28 additionally demonstrated that the Src homology 3 domain of Itk binds to proline-rich sequences in the CD28-cytoplasmic tail, an interaction that has been suggested to enhance Itk kinase activity (7). Finally, Itk has been shown to phosphorylate all four tyrosine residues of the CD28-cytoplasmic tail in in vitro kinase assays (10), providing additional evidence for a positive role of Itk in CD28 signaling.

To date, only a single study has addressed the role of Itk in CD28 signaling in primary T cells. Surprisingly, this study concluded that Itk is a negative regulator of CD28 signaling. This latter conclusion was based on the finding that $CD4^+$ T cells from $Itk^{-/-}$ mice showed enhanced proliferative responses to CD28 costimulatory signals compared with cells from wild-type (WT)[3] mice (11). One complication of this initial study is the fact that $Itk^{-/-}$ mice have a greatly increased population of previously activated/memory $CD4^+$ T cells compared with controls, potentially skewing the responses of these cells to TCR plus CD28 stimulation, independently of a role for Itk in CD28 signaling. Based on this concern, we chose to readdress the role of Itk in CD28 signaling using a panel of assays that assess CD28 signaling in the presence, as well as the absence, of TCR stimulation. Overall, our data demonstrate that Itk is not a negative regulator of CD28 costimulatory activity; in contrast, to the best of our knowledge, all aspects of CD28 signaling are intact in the absence of Itk.

Materials and Methods

Mice

The $Itk^{-/-}$ mouse line (12) was backcrossed to C57BL/10 for >10 generations. Where indicated, $Itk^{-/-}$ mice were crossed to 5C.C7 TCR-transgenic $Rag2^{-/-}$ mice (Taconic Farms). All mice used were 6–12 wk of age and maintained in a specific, pathogen-free facility, following review and approval by the Institutional Animal Care and Use Committee.

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[3] Abbreviations used in this paper: WT, wild type; MCC, moth cytochrome *c*; CHO, Chinese hamster ovary; GSK3β, glycogen synthase kinase-3β.

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Preparation of naive CD4$^+$ T cells

CD4$^+$ T cells were purified from lymph nodes and spleens as described previously (13). CD4$^+$CD44^{low} (naive) T cells were sorted on a BD Biosciences FACSVantage cell sorter. Purified populations were >95% CD4$^+$CD44^{low} cells. For some experiments, naive CD4$^+$ T cells (CD62L^{high}) were purified on an AutoMACS following labeling with anti-CD4-FITC (BD Pharmingen), anti-FITC MultiSort kit (Miltenyi Biotec), and anti-CD62L-magnetic microbeads (Miltenyi Biotec). This purification strategy yielded 90–97% pure CD4$^+$CD44^{low} T cells. The CD4$^+$ T cells from *Rag2$^{-/-}$5C.C7TgxItk$^{-/-}$* and *Rag2$^{-/-}$5C.C7TgxItk$^{+/-}$* mice are consistently >95% CD44^{low}.

T cell stimulations

Purified naive CD4$^+$ T cells were stimulated with surfactant-free, sulfate-charged, 4.9-μm white polystyrene latex beads (Interfacial Dynamics) coated with the following combinations of mAbs: anti-CD3ϵ (145-2C11; BD Pharmingen) at 0.5 μg/ml plus a hamster IgG isotype control (eBiosciences) at 4.5 μg/ml; anti-CD3ϵ at 0.5 μg/ml plus anti-CD28 (37.51; eBiosciences) at 4.5 μg/ml; anti-CD28 at 5 μg/ml; or a hamster IgG isotype control at 5 μg/ml. For Ab coating, latex beads were incubated at 1×10^7/ml with the indicated combinations of Abs at 37°C for 1.5 h with rotation. For biochemical assays, naive CD4$^+$ T cells were stimulated with anti-CD28 Ab-coated plates.

To stimulate naive CD4$^+$ T cells, 1×10^5 purified cells were incubated with an equal number of Ab-coated latex beads. Where indicated, PMA (Sigma-Aldrich) was added at various concentrations. For stimulation of *Rag2$^{-/-}$5C.C7Tg* cells, 1×10^5 purified CD4$^+$ T cells were incubated with varying concentrations of the moth cytochrome *c* (MCC) peptide (93-103) plus 1×10^5 mitomycin C-treated Chinese hamster ovary (CHO) cells expressing mouse MHC class II IE^k (CHO-IE^k; Ref. 14) or CHO cells expressing IE^k and mouse CD80 (CHO-IE^k/B7.1; Ref. 15). Proliferation was assessed by pulsing cells overnight with [^{3}H]thymidine (NEN).

Western blot analysis

Following stimulation, cells were lysed as described previously (16). Forty micrograms of protein for each sample were transferred to polyvinylidene difluoride membranes and probed with Abs to phospho-Akt or phospho-glycogen synthase kinase-3β (GSK3β) (Cell Signaling Technology). After stripping, the membranes were reprobed with Abs to total Akt (Cell Signaling Technology), GSK3β (Santa Cruz Biotechnology), or the p85 subunit of PI3K (Upstate Biotechnology).

Quantitative real-time PCR

T cells were stimulated for 6 h, and RNA and cDNA were prepared as described previously (15). Real-time quantitative PCR was performed on an i-Cycler (Bio-Rad). Primer sequences are available upon request.

Preparation of nuclear extracts and detection of activated NF-κB (p65)

After stimulation, cells were harvested, and nuclear lysates were prepared. Five micrograms of nuclear protein were subjected to the NFκB (P65) functional ELISA using the BD TransFactor NF-κB p65 kit (BD Biosciences). Signals were analyzed on an EMax precision microplate reader (Molecular Devices).

Flow cytometry

Cells were stained with the indicated Abs 30 min at 4°C, washed, and analyzed on a BD FACSCalibur (BD Biosciences). Data were analyzed using CellQuest software (BD Immunocytometry Systems). The Abs used were anti-CD4-CyChrome (Cy), anti-CD4-PE, anti-CD69-FITC, anti-CD44-FITC, anti-CD44-Cy, anti-CD25-FITC, and anti-CD62L-PE (BD Pharmingen).

Results and Discussion

Increased numbers of memory phenotype CD4$^+$ T cells in Itk$^{-/-}$ mice

Previous studies have documented that *Itk$^{-/-}$* mice have a modest defect in positive selection, resulting in an ~2-fold reduction in the total numbers of CD4$^+$ T cells in the spleens and lymph nodes of *Itk$^{-/-}$* mice compared with controls (12, 17–19). However, surprisingly, the population of CD4$^+$ T cells in *Itk$^{-/-}$* mice is highly enriched for cells with a previously activated or memory phenotype (CD4$^+$CD44^{high}CD62L^{low}). As shown in Fig. 1*A*, we routinely find an ~3-fold increase in the proportion of CD4$^+$ T cells expressing high levels of CD44 in lymph nodes of *Itk$^{-/-}$* mice compared with WT C57BL/10 mice. When CD4$^+$CD44^{high} T cells were analyzed for CD69

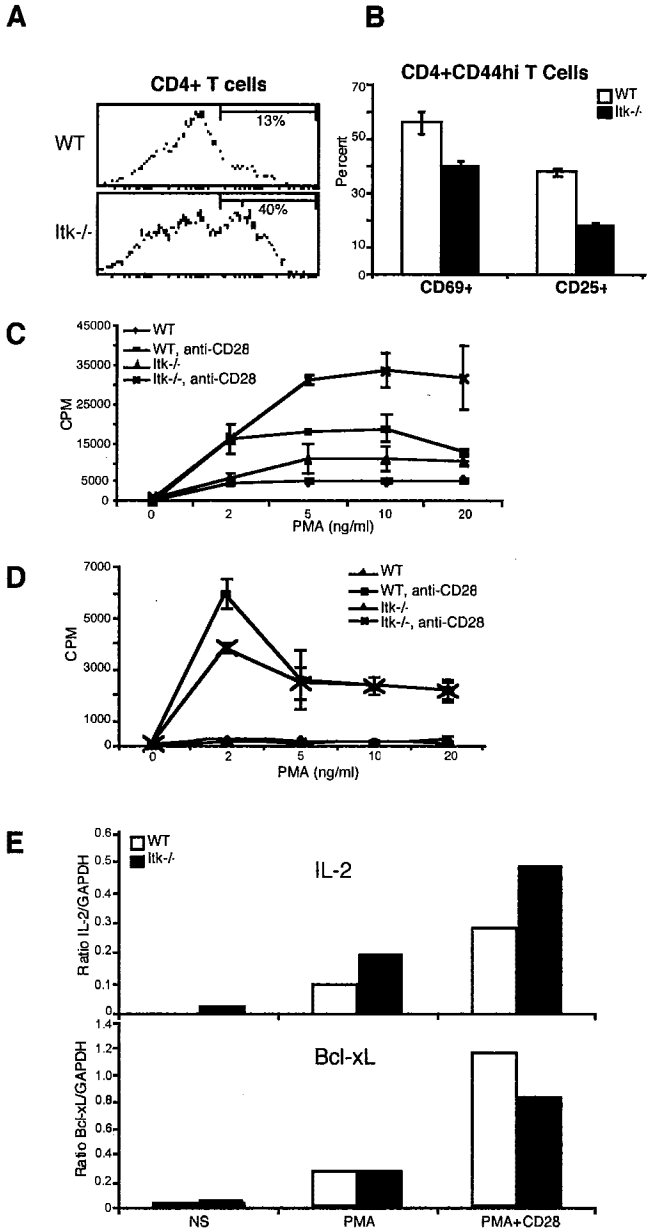

FIGURE 1. Naive *Itk$^{-/-}$* CD4$^+$ T cells are not hyperresponsive to CD28 stimulation. *A*, Lymph node cells from C57BL/10 (WT) and *Itk$^{-/-}$* mice were stained with anti-CD4 and anti-CD44 Abs. Histograms show CD44 staining on gated CD4$^+$ T cells; numbers indicate the percentage of CD44^{high} cells. Data shown are representative of five experiments. *B*, Lymph node cells from WT and *Itk$^{-/-}$* mice were stained with Abs to CD4, CD44, and CD69 or CD25. Mean percentages ± SD of CD69$^+$ or CD25$^+$ cells among the CD4$^+$CD44^{high} population are indicated for two WT and six *Itk$^{-/-}$* mice analyzed. *C*, Total CD4$^+$ T cells from WT and *Itk$^{-/-}$* mice were stimulated with the indicated concentrations of PMA in the presence of anti-CD28 or isotype control Ab-coated beads. Cell proliferation was measured by [^{3}H]thymidine incorporation at 72h. *D*, Sorted naive CD4$^+$ T cells from WT and *Itk$^{-/-}$* mice were stimulated with the indicated concentrations of PMA in the presence of anti-CD28 or isotype control Ab-coated beads. Cell proliferation was measured by [^{3}H]thymidine incorporation at 72h. Data shown are representative of three experiments. *E*, Naive CD4$^+$ T cells were purified from *WT* and *Itk$^{-/-}$* mice. Cells were stimulated for 6 h with 5 ng/ml PMA in the presence or absence of anti-CD28 Ab-coated beads. The levels of IL-2 and Bcl-xL mRNA were determined by real-time quantitative PCR. Data were normalized to the expression of GAPDH mRNA in each sample and are representative of two experiments. NS, nonstimulated.

and CD25 expression, fewer cells from $Itk^{-/-}$ mice compared with controls expressed these early activation markers, suggesting that these cells have not been recently activated (Fig. 1*B*). Overall, these data indicate that, although total T cell numbers are reduced in the $Itk^{-/-}$ mice (12, 17), the proportion of cells with a memory phenotype is actually increased.

Naive $Itk^{-/-}$ $CD4^+$ T cells are not hyperresponsive to CD28 costimulation

A previous study described increased responsiveness of $Itk^{-/-}$ $CD4^+$ T cells compared with WT $CD4^+$ T cells following stimulation through CD28, leading to the conclusion that Itk is a negative regulator of CD28 signaling (11). To bypass the TCR-signaling defect intrinsic to $Itk^{-/-}$ T cells, these experiments used PMA plus anti-CD28 Ab as a stimulus. Consistent with these earlier data, we also observe that when total $CD4^+$ T cells from $Itk^{-/-}$ mice or WT control mice are stimulated with PMA plus anti-CD28-coated beads, the response of the $Itk^{-/-}$ $CD4^+$ T cells is significantly higher than that of the control cells (Fig. 1*C*). However, because the $CD4^+$ T cell population from $Itk^{-/-}$ mice contains an increased proportion of memory phenotype cells, we reasoned that the increased responsiveness of these cells might be attributable to this altered subset distribution. To test this possibility, we repeated this experiment using highly purified naive $CD4^+$ $CD44^{low}$ T cells. As shown in Fig. 1, *D* and *E*, purified naive $Itk^{-/-}$ $CD4^+$ T cells are not hyperresponsive to PMA plus anti-CD28 Ab stimulation and, under these conditions, respond comparably to WT naive $CD4^+$ T cells.

These findings reopened the question of the role of Itk in CD28 signaling and function. Therefore, we proceeded to examine the responses of $Itk^{-/-}$ T cells to stimulation through the TCR plus CD28, using conditions in which T cell activation is dependent stringently on CD28 costimulation. For these experiments, purified $Itk^{-/-}$ and WT naive $CD4^+$ T cells were stimulated with Ab-coated latex beads. When stimulated with beads coated with anti-CD28 Ab alone or anti-CD3 Ab alone, neither population of T cells exhibited any proliferative response. In contrast, when cells were stimulated with beads coated with a mixture of anti-CD3 plus anti-CD28 Abs (1:9 ratio), both populations of cells proliferated robustly. Although the response of the $Itk^{-/-}$ cells was reduced compared with that of the WT T cells, this response still represents an ~300-fold enhancement over the response to anti-CD3 Ab alone. Based on these data, we conclude that CD28 costimulatory activity functions quite efficiently in naive $Itk^{-/-}$ $CD4^+$ T cells.

To substantiate these findings using bona fide MHC/peptide stimulation in the presence or absence of B7.1 (CD80), we examined purified naive $CD4^+$ T cells isolated from transgenic mice expressing the 5C.C7 TCR (5C.C7Tg). For these experiments, T cells from *Rag2*$^{-/-}$ *5C.C7Tg*$\times$*Itk*$^{-/-}$ and *Rag2*$^{-/-}$ *5C.C7Tg*$\times$*Itk*$^{+/-}$ mice were stimulated with CHO-IE^k cells or CHO-IE^k/B7.1 cells as APCs in the presence of varying concentrations of the MCC peptide. At each given peptide concentration, both $Itk^{+/-}$ as well as $Itk^{-/-}$ $CD4^+$ T cells show a similar degree of increased responsiveness to stimulation with APCs expressing B7.1 compared with APCs that lack B7.1 (Fig. 2*B*). These data confirm the conclusion that CD28 costimulation functions effectively in the absence of Itk.

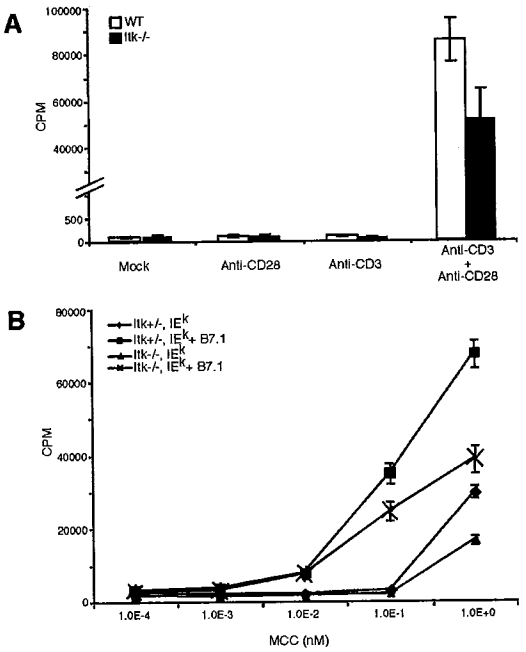

FIGURE 2. CD28 costimulation functions efficiently in the absence of Itk. *A*, Sorted naive $CD4^+$ T cells from WT and $Itk^{-/-}$ mice were stimulated with Ab-coated beads as indicated. Cell proliferation was measured 72 h after stimulation. Mock, cells incubated with isotype control Ab-coated beads alone. Data shown are representative of three experiments. *B*, Purified $CD4^+$ T cells from *Rag2*$^{-/-}$ *5C.C7Tg Itk*$^{-/-}$ and *Rag2*$^{-/-}$ *5C.C7Tg Itk*$^{+/-}$ mice were stimulated with CHO-IE^k (IE^k) or CHO-IE^k/B7.1 (IE^k+B7.1) cells and the indicated concentrations of MCC peptide. Cell proliferation was measured 72 h after stimulation.

CD28 costimulation enhances gene expression in the absence of Itk

One function of CD28 costimulation is to enhance gene expression induced by TCR signaling. Among the genes most dramatically affected by CD28 costimulation are those encoding the cytokine, IL-2, the survival factor, Bcl-xL, and the effector molecule, CD40L (20–23). To assess whether CD28 costimulation leading to enhanced gene expression is functional in the absence of Itk, WT, and $Itk^{-/-}$, naive $CD4^+$ T cells were stimulated, and IL-2, Bcl-xL, and CD40L mRNA levels were measured by real-time quantitative PCR. As shown in Fig. 3, each of these genes exhibited enhanced mRNA levels following CD28 costimulation in both WT and $Itk^{-/-}$ T cells. Interestingly, the activation-induced increases in IL-2, Bcl-xL, and CD40L mRNA were abolished completely following addition of the PI3K inhibitor, LY294002 (Fig. 3). Taken together, these data demonstrate the effectiveness of CD28 costimulatory signals to enhance gene expression in the absence of Itk.

CD28 costimulation activates NFκB in the absence of Itk

The transcription factor, NF-κB, is an important target of the CD28 costimulatory pathway (24). To assess the ability of CD28 costimulation to activate NF-κB in the absence of Itk, we stimulated WT and $Itk^{-/-}$ naive $CD4^+$ T cells with anti-CD3 Ab alone or in combination with anti-CD28 Ab. After 60 min, nuclear lysates were prepared from the cells, and levels of activated NF-κB were examined by ELISA. As can be seen in Fig. 4*A*, anti-CD3 Ab stimulation is not sufficient to induce detectable NF-κB activation in either cell type, whereas anti-CD3 plus anti-CD28 Ab stimulation induced significant levels

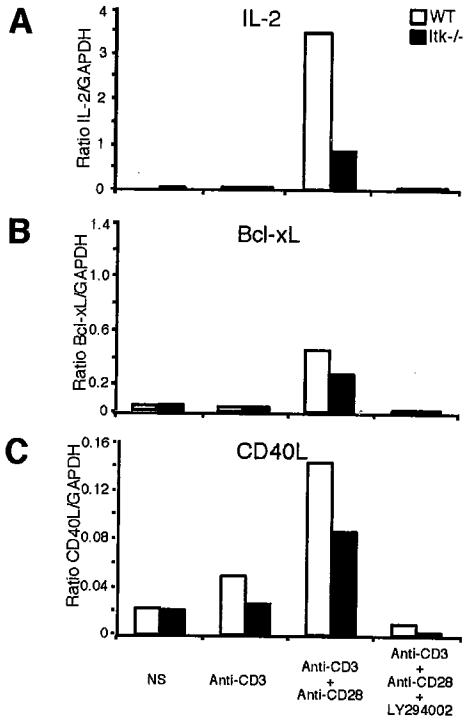

FIGURE 3. CD28-mediated enhancement of gene expression functions efficiently in the absence of Itk. Naive $CD4^+$ T cells from *WT* and $Itk^{-/-}$ mice were stimulated for 6 h with Ab-coated beads as indicated, with or without the PI3K inhibitor LY294002 at 10 μM. Levels of IL-2 (*A*), Bcl-xL (*B*), and CD40L (*C*) mRNA were determined by real-time quantitative PCR. Data were normalized to the expression of GAPDH mRNA in each sample and are representative of three experiments. The nonstimulated (NS) samples are the same data as shown in Fig. 1*E*.

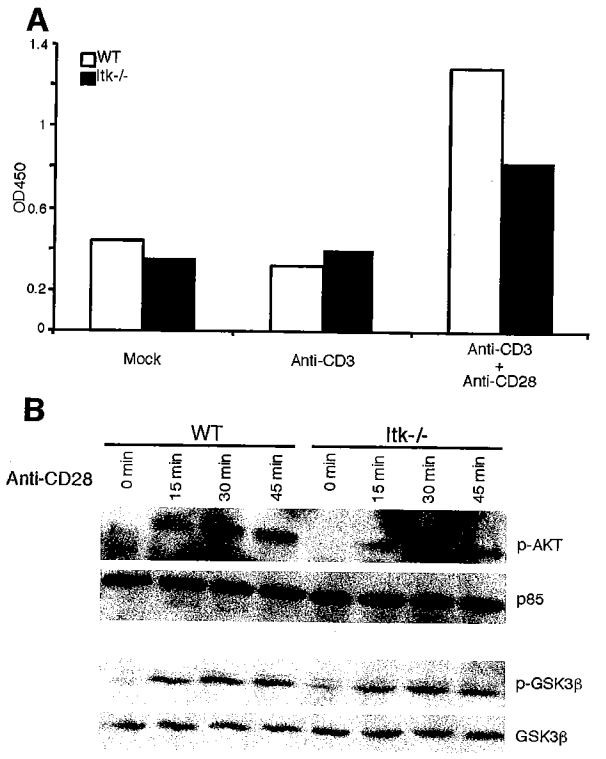

FIGURE 4. Downstream responses to CD28 signaling are functional in naive $Itk^{-/-}$ T cells. *A*, Naive $CD4^+$ T cells from WT and $Itk^{-/-}$ mice were stimulated for 1 h with Ab-coated beads as indicated. Activated NF-κB p65 in nuclear lysates was measured by ELISA. Data shown are representative of three experiments. Mock, cells stimulated with isotype-control Ab-coated beads. *B*, Naive $CD4^+$ T cells from WT and $Itk^{-/-}$ mice were stimulated with plate-bound anti-CD28 Ab for the indicated times. Akt phosphorylation (Ser^{473}) and GSK3β phosphorylation (Ser^9) were detected in total lysates by immunoblotting with phospho-specific Abs. Membranes were stripped and reprobed with Abs to the p85 subunit of PI3K and GSK3β as loading controls. Data shown are representative of three experiments.

of activated NF-κB in both WT and $Itk^{-/-}$ T cells. These data confirm the ability of CD28 costimulatory signaling to function in the absence of Itk.

Stimulation of CD28 alone induces phosphorylation of Akt and GSK3β in the absence of Itk

Although signaling through CD28 alone does not lead to functional changes in T cells, several biochemical events can be detected following CD28 stimulation. One such signaling pathway is the activation of PI3K, leading to the phosphorylation and activation of the serine/threonine kinase, Akt, and the subsequent phosphorylation of GSK3β (25). To examine whether these events occurred normally in the absence of Itk, naive $CD4^+$ T cells from WT and $Itk^{-/-}$ mice were stimulated with anti-CD28 Ab alone, and Akt and GSK3β phosphorylation were detected with phospho-Akt- and phospho-GSK3β-specific Abs. As shown in Fig. 4*B*, Akt and GSK3β were both phosphorylated comparably in WT and $Itk^{-/-}$ $CD4^+$ T cells. These data demonstrate that the CD28-PI3K-Akt-GSK3β signaling pathway is intact in the absence of Itk, indicating that Itk is not essential for CD28 signaling.

Using purified naive $CD4^+$ T cells and defined stimulation conditions, we have examined in detail the requirement for Itk in CD28 signaling and in CD28-mediated costimulation. Because Itk is required for optimal TCR signaling, it is difficult to ascertain whether CD28 costimulatory activity is equally effective in the presence vs the absence of Itk. Nonetheless, our data definitively demonstrate that CD28-mediated costimulation functions efficiently in the absence of Itk and, to a first approximation, is as effective in $Itk^{-/-}$ T cells as in WT T cells. This conclusion is supported by our biochemical data showing that two measurable outcomes triggered by CD28 stimulation alone, namely the phosphorylation of Akt and GSK3β, are completely independent of Itk. It is possible that efficient CD28 signaling in $Itk^{-/-}$ T cells is due to compensation by another Tec-kinase family member, Rlk or Tec, also expressed in T cells, although examination of Rlk and Tec expression in $Itk^{-/-}$ T cells has not indicated any compensatory up-regulation of these additional Tec kinases (Ref. 12 and data not shown). Overall, these findings demonstrate that Itk is not essential for CD28 signaling or function in naive $CD4^+$ T cells.

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Disclosures

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