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T-Cell Signaling Regulated by the Tec Family Kinase, Itk

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The Tec family tyrosine kinases regulate lymphocyte development, activation, and differentiation. In T cells, the predominant Tec kinase is Itk, which functions downstream of the T-cell receptor to regulate phospholipase C-γ. This review highlights recent advances in our understanding of Itk kinase structure and enzymatic regulation, focusing on Itk protein domain interactions and mechanisms of substrate recognition. We also discuss the role of Itk in the development of conventional versus innate T-cell lineages, including both αβ and γδ T-cell subsets. Finally, we describe the complex role of Itk signaling in effector T-cell differentiation and the regulation of cytokine gene expression. Together, these data implicate Itk as an important modulator of T-cell signaling and function.

The Tec family nonreceptor tyrosine kinases, Tec, Btk, Itk/Emt/Tsk, Rlk/Txk, and Bmx/Etk, are expressed primarily in hematopoietic cells and serve as important mediators of antigen receptor signaling in lymphocytes (Berg et al. 2005; Felices et al. 2007; Readinger et al. 2009). The demonstration that the human B-cell immunodeficiency, X-linked agammaglobulinemia (XLA), is caused by mutations in Btk first underscored the importance of this tyrosine kinase family in lymphocyte development and antigen receptor signaling (Rawlings et al. 1993; Thomas et al. 1993; Tsukada et al. 1993; Vetrue et al. 1993). T lymphocytes express three Tec kinases: Itk, Rlk and Tec. To date, only Itk has been found to have a clearly defined function in T cells, leading to the conclusion that Itk is the predominant Tec kinase in T cells. In this review, we will cover recent findings that highlight the critical role of Itk in T-cell signaling and function.

STRUCTURE AND REGULATION OF ITK ACTIVITY

Tec Kinase Domain Structure

Itk (inducible T-cell kinase; also known as Emt, expressed in mast cells and T lymphocytes, and Tsk, T-cell-specific kinase) was cloned in the early 1990s (Siliciano et al. 1992; Gibson et al. 1993; Heyeck and Berg 1993; Tanaka et al. 1993; Yamada et al. 1993). The domain organization of Itk and related Tec kinase family
members shares similarities with other tyrosine kinase families but also reveals unique features (Fig. 1A). Shared features include the common SH3-SH2-kinase cassette also found in the Src, Csk, and Abl kinases (Williams et al. 1998; Tsygankov 2003). Four of the five Tec tyrosine kinases (Itk, Btk, Tec, and Bmx) contain an amino-terminal region that includes a pleckstrin homology (PH) domain, followed by a Zn$^{++}$ binding region termed the Btk homology (BH) motif, and (except for Bmx) a proline rich region (PRR) that conforms to the consensus sequence of an SH3 ligand. Instead of the PH-BH region, Txk (also known as Rlk) contains a cysteine string motif at its amino terminus.

**Itk Activation Downstream of the TCR**

The Tec family kinases are involved in multiple receptor-mediated signaling pathways in many cell types; several recent reviews have covered these in depth (Berg et al. 2005; Felices et al. 2007; Gomez-Rodriguez et al. 2007; Mihara

![Figure 1. Itk domain organization and its role in T cell signaling. (A) The domain structure of Itk. (B) Schematic of T-cell signaling emphasizing the proximal signaling complex downstream of the TCR as described in the text. The trio of tyrosine kinases, Zap-70, Lck, and Itk that initiate signaling following TCR engagement are shown as gray, green, and brown circles, respectively. Arrows indicate early phosphorylation events on TCR/pMHC recognition. The amino-terminal region of Lck binds to the cytoplasmic tail of CD4 via coordination of Zn$^{++}$ (Huse et al. 1998). Active Lck phosphorylates the ITAMs, Zap-70, and Itk, which in turn phosphorylate their targets Slp-76, LAT (by Zap-70) and PLCγ1 (by Itk). Activated PLCγ1 then hydrolyzes PIP$_2$ (dashed arrow) to produce DAG and IP$_3$ leading to increased calcium flux. Multiple copies of these signaling molecules likely cooperate in the phosphorylation cascade and so stoichiometry as shown is overly simplified. Moreover, negative regulation of the signaling cascade in the form of phosphatases, kinases and other types of molecules also plays a significant role in T-cell function but are not represented here.](image-url)
and Suzuki 2007; Gilfillan and Rivera 2009; Koprulu and Ellmeier 2009; Prince et al. 2009; Readinger et al. 2009). For Itk, significant attention has been given to activation via T-cell receptor (TCR) stimulation (see Fig. 1B). Interaction of the TCR with peptide-MHC complexes on antigen presenting cells (APC) activates the Src kinase Lck, leading to phosphorylation of the CD3 immunoreceptor tyrosine-based activation motifs (ITAMs). Zap-70 then binds to the phosphorylated ITAMs and is phosphorylated by Lck, resulting in Zap-70 activation and subsequent phosphorylation of the adaptors LAT and SLP-76 (Pitcher and van Oers 2003; Houman et al. 2005; Au-Yeung et al. 2009; Smith-Garvin et al. 2009). Following activation of PI3K and accumulation of phosphatidylinositol (3,4,5) trisphosphate (PIP3) in the plasma membrane, Itk is recruited to the membrane via its PH domain (August et al. 1997; Ching et al. 1999; Yang et al. 2001). There, Itk interacts with the phosphorylated SLP-76/LAT adapter complex via Itk’s Src homology (SH3 and SH2) domains (Bubeck Wardenburg et al. 1996; Zhang et al. 1998; Shan and Wang 1999; Su et al. 1999; Bunnell et al. 2000; Ching et al. 2000; Sommers et al. 2004; Koretzky et al. 2006), permitting phosphorylation of Itk on its activation loop (Y511) by Lck (Heyeck et al. 1997).

Once activated, Itk undergoes cis autophosphorylation on Y180 in its SH3 domain (Wilcox and Berg 2003; Joseph et al. 2007a). Itk also interacts with and directly phosphorylates its downstream target, PLCγ1 (Perez-Villar and Kanner 1999; Joseph et al. 2007c), resulting in phospholipase activation (Liu et al. 1998; Schaeffer et al. 1999; Wilcox and Berg 2003; Bogin et al. 2007). The kinetics characterizing many of these early phosphorylation events have been delineated, providing an understanding of the sequential nature of signaling following TCR engagement (Houtman et al. 2005). It is not known, however, whether Itk autophosphorylation occurs prior to, or following, phosphorylation of PLCγ1. Because autophosphorylation on Y180 does not affect Itk catalytic activity but instead modulates binding of the Itk SH3 domain to different targets (Wilcox and Berg 2003; Joseph et al. 2007a), Itk autophosphorylation may serve as a trigger to alter protein interaction partners or localization either before or after PLCγ1 activation. PLCγ1, once activated, hydrolyzes PIP2 to produce the second messengers IP3 and DAG (Rhee 2001). Downstream consequences include Ca2⁺ flux (Lewis and Cahalan 1995), Erk activation (Schaeffer et al. 1999; Schaeffer et al. 2000; Miller and Berg 2002), transcription (Crabtree and Olson 2002), cytokine release (Liao and Littman 1995; Liu et al. 1998), and actin reorganization (Labno et al. 2003), all of which are impaired in the absence of Itk. Importantly, these downstream consequences of TCR activation are not ablated by a deficiency in Itk, but instead, are substantially reduced, leading to altered development and differentiation of distinct T-cell lineages, as discussed later.

**The Itk Signaling Complex**

The Itk/SLP-76 interaction likely involves the SH3 and SH2 domains of Itk targeting a proline-rich stretch within SLP-76 (residues 184-195) and an adjacent phosphorylated tyrosine (Y145) in SLP-76, respectively (Su et al. 1999; Bunnell et al. 2000). In an elegant knockin mouse study, tyrosine 145 of SLP-76 was replaced with the nonphosphorylatable phenylalanine to probe the contribution of the ItkSH2/pY145 interaction to signaling (Jordan et al. 2008). Mutation of Y145 resulted in markedly diminished PLCγ1 phosphorylation, decreased Ca²⁺ flux, lowered phospho-ERK levels and gave rise to T cells that resembled those lacking Itk, as discussed later. This observation (Jordan et al. 2008) is consistent with findings that the Itk/SLP-76 interaction is required to activate Itk kinase activity in Jurkat cells (Bogin et al. 2007). Intriguingly, the majority of the catalytically active Itk in the cell consists of only the small fraction of Itk that is bound to SLP-76 (Bogin et al. 2007). Surprisingly, the SLP-76 Y145F mutation did not lead to loss of the Itk/SLP-76 association (Jordan et al. 2008) despite altered signaling. This finding suggests that cooperative multivalent interactions preserve Itk localization in this signaling complex even when a single contact site is disrupted.
The importance of the Itk PH domain/PIP<sub>3</sub> interaction (Fukuda et al. 1996; August et al. 1997) was also recently probed by inhibition of phosphatidylinositol-3 kinase (PI3K) activity in human peripheral blood T cells (Cruz-Orcutt and Houtman 2009). In a manner similar to SLP-76 Y145F, loss of the Itk PH domain/PIP<sub>3</sub> interaction resulted in impaired downstream signaling but did not affect the ability of Itk and other PH domain containing signaling proteins to assemble into the signaling complex (Cruz-Orcutt and Houtman 2009). In a separate study, soluble IP<sub>4</sub> was found to promote binding of the Itk PH domain to PIP<sub>3</sub> (Huang et al. 2007). Reminiscent of the Itk/SLP-76 interaction (Bogin et al. 2007), these studies also showed that only a limited pool of Itk in the cell is capable of binding PIP<sub>3</sub> even in the presence of excess PIP<sub>3</sub> (Huang et al. 2007). These separate findings suggest that multiple interaction sites exist to properly localize Itk following TCR stimulation (Graef et al. 1997; Garcon and Nunes 2006; Beach et al. 2007). Furthermore, part of the function of Itk and other proteins in the LAT/SLP-76 complex may be to function as adaptors and stabilize proteins in the complex. Kinase-independent functions for Itk have been observed in the regulation of actin polymerization (Dombroski et al. 2005) and activation of the transcription factor, SRF (Hao et al. 2006); these functions may be secondary to effects on recruitment of the guanine-nucleotide exchange factor Vav to this complex (Labno et al. 2003).

**Regulation of Itk Activity**

The regulatory mechanisms that control the activity of Lck and Zap-70 during TCR signaling have been the subject of intense investigation, leading to detailed insights into function (Palacios and Weiss 2004; Au-Yeung et al. 2009). In contrast, a mechanistic understanding of Itk regulation remains elusive, as Itk and other Tec kinases have, to date, resisted crystallization in their full-length form. Comparing the Tec and Src kinase families, it is evident that regulatory differences must exist. In spite of the shared SH3-SH2-Kinase cassette, the Tec kinases lack the carboxy-terminal regulatory tail that plays an important role in Src regulation (Brown and Cooper 1996; Xu et al. 1997; Xu et al. 1999; Cowan-Jacob et al. 2005). Moreover, removal of the noncatalytic domains from the Src kinase domain yields an active enzyme, whereas the isolated Itk and Btk kinase domains show poor catalytic activity (Fig. 2A) (LaFevre-Bernt et al. 1998; Hawkins and Marcy 2001; Guo et al. 2006; Joseph et al. 2007b). Mutation of a well-conserved tryptophan residue in the Itk and Btk SH2-kinase linkers (W355 in Itk, W395 in Btk) results in a significant decrease in kinase activity (Lin et al. 2005; Guo et al. 2006; Joseph et al. 2007b) whereas mutation of the corresponding tryptophan in the Src kinase Hck leads to a dramatic increase in the activity of that kinase (LaFevre-Bernt et al. 1998). In this regard, Itk is similar to Csk (carboxy-terminal Src kinase), another tyrosine kinase comprised of the common SH3-SH2-Kinase cassette and regulated in a manner opposite that of Src (Huang et al. 2009). The isolated Csk kinase domain shows negligible catalytic activity (Fig. 2A) and mutation of the conserved tryptophan (W188) decreases Csk activity (Lin et al. 2005). Based on similar biochemical profiles, a model for active Itk can be developed using the available structure of active Csk (Fig. 2B).

In agreement with the established interaction between active Itk and SLP-76 (Jordan et al. 2008; Bogin et al. 2007), the distance between the SH3 and SH2 binding pockets in the high-resolution structure of Csk closely matches the distance between the phosphotyrosine motif and proline-rich region in SLP-76 (residues 143–195). Moreover, the Itk PRR region adjacent to the Itk SH3 domain in the model of active Itk (Fig. 2B) could be available for binding to an SH3 domain from an exogenous signaling partner. The precise binding partner of the Itk PRR is not yet clear but candidates include the SH3 domains of Vav, Fyn, Src, PLCγ1 or Grb2 (Yang et al. 1995; Perez-Villar and Kanner 1999; Bunnell et al. 2000; Chamorro et al. 2001). Csk lacks the PH-BH region of the Tec kinases and so comparative model building for this region of Itk becomes
speculative. It is interesting to note however, that the PH-BH domain of Itk extends from the amino-terminus of the PRR region and, within the context of the Csk-based model, the PH-BH domain could pack between the SH3 and SH2 domains making contact with the small lobe of the kinase domain and/or the SH3-SH2/SH2-kinase linker regions potentially affecting kinase activity (Joseph et al. 2007b) (Fig. 2B).

**Mechanism of Itk Substrate Recognition**

Once activated and assembled within the SLP-76/Lck signaling complex, Itk meets its downstream substrate, PLCγ1 (Liu et al. 1998;...
Schaeffer et al. 1999; Wilcox and Berg 2003; Houtman et al. 2005; Bogin et al. 2007). Itk mediated phosphorylation of Y783 in PLCγ1 requires a remote specificity-determining element within the second SH2 domain of PLCγ1 (Joseph et al. 2007c). Mutation in the PLCγ1 SH2 domain that disrupts the Itk/PLCγ1 interaction results in decreased PLCγ1 activation as measured by calcium flux in Jurkat T cells (Braiman et al. 2006; Min et al. 2009). Earlier work also identified a TCR-regulated association between Itk and PLCγ1 involving the SH3 domain of PLCγ1 (Perez-Villar and Kanner 1999). Although PLCγ1 is the most well established substrate of Itk, additional candidate substrates have been described and include TFII-I (August 2009; Sacristan et al. 2009), Tim-3 (van de Weyker et al. 2006) and T-bet (Hwang et al. 2005); the molecular details of Itk recognition of these substrates has not been elucidated.

**Downregulation of Itk Kinase Activity**

Inactivation of kinase activity is an important regulatory feature that must appropriately balance activating signals. Although few negative regulators of Itk have been described, expression of PTEN, a lipid phosphatase, can decrease Itk activity by reducing PIP3 levels (Shan et al. 2000). Negative regulation of Itk by the peptidyl prolyl isomerase, cyclophilin A, has been described (Brazin et al. 2002; Colgan et al. 2004). Interestingly, another peptidyl prolyl isomerase, Pin1 (protein interacting with NIMA1), regulates phosphorylation and steady state levels of Btk (Yu et al. 2006). However, the mechanism of action remains poorly understood for both Pin1 and CypA (Joseph and Andreotti 2009; Mohamed et al. 2009).

As already described, the regulatory domains of the Tec kinases exert a positive influence on the kinase domain in a manner similar to Csk (Fig. 2B) and so conformational rearrangements that uncouple the regulatory domains (particularly the SH2-kinase linker region) from the catalytic domain would down-regulate Itk activity. In this context, the only structural data available for any full length Tec kinase comes from small-angle X-ray scattering (SAXS) analysis of Btk (Marquez et al. 2003). These data indicate that an inactive preparation of Btk (Marquez et al. 2003; Lin et al. 2009) adopts an extended conformation in solution with negligible contacts between domains (Fig. 2C). Whether the fully extended configuration derived from SAXS data represent a physiologically relevant state remains to be determined but the available data suggest that a conformational shift of the regulatory domains away from the kinase domain could provide one layer of negative regulation.

In addition to negatively regulating the catalytic domain, the configuration of the inactive kinase might also serve to sequester its ligand binding sites (Pawson and Kofler 2009). The SH3/PXXP interaction, in particular, is constitutive and not controlled by transient phosphorylation/dephosphorylation events. In down-regulated Itk, the SH3 domain-binding site might be masked in an intramolecular fashion by the adjacent PRR (Andreotti et al. 1997; Laederach et al. 2003) (Fig. 2C). However, it is experimentally difficult to probe the precise role of the PRR in kinase regulation because it is also likely involved in activating interactions with SH3 domain-containing signaling partners (Yang et al. 1995; Bunnell et al. 2000). Efforts that use deletion of the PRR region to probe its regulatory role (Hao and August 2002) are therefore complicated by the dual nature of the PRR.

**Regulation of Itk Activity by Multimerization**

Structural studies using different Tec kinase fragments indicate that the noncatalytic regulatory domains interact in an intermolecular fashion driving multimerization in solution (Andreotti et al. 1997; Brazin et al. 2000; Hansson et al. 2001; Laederach et al. 2002; Okoh and Vihinen 2002; Pursglove et al. 2002; Laederach et al. 2003; Severin et al. 2009). For Itk, multimerization is mediated by self-association of the PH domain (Huang et al. 2007) and intermolecular interactions between the SH3 and SH2 domains of different Itk molecules (Brazin et al. 2000; Severin et al. 2009). Native gel
electrophoresis indicates that the full-length Itk molecule, the SH3-SH2-kinase fragment, and the Itk SH3-SH2 fragment all form multimers (Severin et al. 2009) (Fig. 2D). The high resolution structure of the Itk SH3/SH2 complex has been solved and provides insight into how full length Itk might self-associate (Severin et al. 2009) (Fig. 2E). Functionally, disruption of the SH3/SH2 interface by mutation reduced oligomerization and increased Itk kinase activity both in vitro and in T cells (Severin et al. 2009; Min et al. 2010). Moreover, both binding experiments and structural data indicate that intermolecular association of Itk is mutually exclusive with binding of SLP-76 to the SH3 and SH2 domains (Brazin et al. 2000; Pletneva et al. 2006; Severin et al. 2009). Thus, down-regulation of Itk activity might involve intermolecular self-association whereas multimerization is disfavored for active, SLP-76 bound Itk. The interplay between specific clustering of signaling molecules (Houtman et al. 2006), the formation of TCR microclusters (Bunnell et al. 2006; Seminario and Bunnell 2008) and the specific role of Itk multimerization during T-cell signaling certainly deserve continued attention.

A split YFP/fluorescence complementation approach has been used to probe the different conformational and oligomeric states of Itk in cells (Qi et al. 2006; Qi and August 2009). Itk that is doubly tagged at the amino- and carboxy-termini results in fluorescence complementation in the cytosol and at the membrane. The authors suggest that the observed fluorescence complementation within a single Itk molecule is indicative of a folded compact structure. Taking the linker lengths between Itk and each of the split YFPs (two linkers each ~40 Å) and the diameter of YFP itself (~30 Å) into account, fluorescence complementation in this system would occur for distances of approximately 110 Å between the Itk termini. It should be noted that the distance between the amino- and carboxy-termini of Btk in the fully extended model derived from SAXS data is 125 Å and given the flexibility inherent in the extended model (Marquez et al. 2003), it seems likely that fluorescence complementation would be observed for almost any domain arrangement of Itk; compact or extended. Thus, the fluorescence “ruler” used in these experiments is unlikely to discriminate between different domain arrangements.

Intermolecular fluorescence complementation between differentially tagged Itk molecules is consistently observed only at the cell membrane and not in the cytosol (Qi et al. 2006; Qi and August 2009). Although this data alone does not rule out multimerization in the cytosol, the spatial segregation of the stronger fluorescence signal to the membrane is certainly interesting and suggests that Itk clusters are at least stabilized upon or after membrane localization. Notably, Itk autophosphorylation enhances the affinity between the SH3 and SH2 domains (Joseph et al. 2007a), possibly triggering sequestration of Itk into clusters and down-regulation of kinase activity. Release of Itk from the membrane, promoted by dephosphorylation of PIP3 and the SLP-76/LAT complex (Shan et al. 2000; Baker et al. 2001; Yang et al. 2001; Tomlinson et al. 2004), would then result in decreased local Itk concentration, which could cause Itk oligomers to disassociate into inactive, monomeric species in the cytosol. Regardless of aggregation state, the inactive species would adopt a conformation that decouples the regulatory region (in particular the SH2-kinase linker) from the Itk kinase domain.

ITK REGULATION OF T-CELL FUNCTION

Naïve T-Cell Activation is Impaired in the Absence of Itk

The central role of Itk in the LAT-SLP-76 complex suggests that Itk is an important component of TCR signaling. Early studies using primary murine Itk−/− T cells showed that Itk is required for robust T-cell activation in response to TCR plus costimulatory receptor signaling. However, these studies also revealed that Itk is not absolutely required for all responses downstream of the TCR (Liao and Littman 1995; Liu et al. 1998; Schaeffer et al. 1999). One aspect of this modulatory role for Itk is the partial redundancy between Itk and a
second Tec kinase family member, Rlk. Thus, a combined deficiency in both Itk and Rlk results in TCR signaling defects that are significantly more profound than is seen in T cells lacking Itk alone (Schaeffer et al. 1999). Overall, the message from these experiments was that Itk functions to amplify TCR signaling, rather than as an all-or-nothing component of the TCR signaling pathway.

T-Cell Differentiation and Effector Functions are Regulated by Itk

In spite of the relatively modest impact of the Itk deficiency on naïve T-cell activation, numerous studies have shown that Itk signaling plays a critical role in regulating T-cell differentiation and T-cell effector functions (Fig. 3). The first of these studies showed that Itk-/- Balb/c mice were unable to generate the TH2 response to Leishmania major infection typically seen in wild-type Balb/c mice and instead, mounted a protective TH1 response that cleared the infection (Fowell et al. 1999). These results highlighted the importance of Itk signaling in the generation of TH2 responses, a function of Itk that has been studied in more detail both in vitro and in vivo.

Itk Promotes TH2 Differentiation and Cytokine Production

The initial findings that Itk-deficient mice were unable to mount a protective TH2 response to infection were verified in a number of distinct infectious disease systems including Leishmania major in Itk-/- Balb/c mice, as well as Nippostrongylus brasiliensis and Schistosoma mansoni, two parasites that are eliminated by TH2-dependent recruitment and activation of granulocytes.
Tec Kinase Itk in T-Cell Signaling

(Fowell et al. 1999; Schaeffer et al. 2001). Analyses of cytokine responses of the parasite-specific T cells from these infected mice indicated that Itk−/− T cells produced decreased IL-4, IL-5, and IL-10 (Schaeffer et al. 2001). Similarly, in vitro experiments showed that Itk−/− Balb/c CD4+ T cells stimulated under Th2-polarizing conditions produced much less IL-4 than their wild-type counterparts (Fowell et al. 1999). These cytokine responses correlated with defective TCR-induced activation of the calcium-dependent transcription factor NFAT; by Itk−/− T cells, providing a potential explanation for the reduced Il4 transcription (Fig. 3).

However, it is difficult to infer from these experiments the precise nature of the T-cell defect resulting in impaired protection to parasitic infections. Reductions in T-cell activation, T-cell differentiation, T-cell migration, or elicitation of recall responses at the site of infection could each contribute to the poor protection observed in Itk−/− mice. An additional complication is the presence of a large population of previously activated/memory CD4+ T cells in Itk−/− mice; global gene expression analysis has confirmed that Itk−/− CD4+ T cells are not comparable to wild-type CD4+ T cells prior to their activation (Blomberg et al. 2009), and thus, might respond differently than naïve T cells to parasite infections.

Nonetheless, useful information has been gleaned from direct infection of Itk−/− mice with pathogenic microorganisms. One elegant approach to evaluating T-cell defects in the absence of Itk was taken by Au-Yeung and colleagues, who crossed Balb/c Itk−/− mice to reporter mice that express GFP under the control of the IL-4 promoter (Au-Yeung et al. 2006). Following infection of these mice with Leishmania major, equal numbers of CD4+GFP+ T cells were found at the sites of infection in wild-type versus Itk−/− mice, indicating that Itk was not required for the initiation of the Th2 response or for the migration of IL-4-competent T cells to these sites. Instead, the effector T-cell recall response at the sites of infection were impaired in the absence of Itk, leading to reduced IL-4 production by Itk−/− T cells following restimulation. However, these studies are not in complete agreement with an independent set of experiments examining T-cell-mediated airway hyperresponsiveness in Itk−/− mice (Mueller and August 2003). In this system, in addition to impaired Th2 cytokine production, T-cell recruitment to the lungs was also impaired following inhaled antigen challenge in Itk−/− mice compared to controls. Interestingly, a partial inhibition of Itk kinase activity was able to block T-cell migration to the lung in this model system, but had no effect on the priming of Th2 effector responses in secondary lymphoid organs (Sahu et al. 2008a). These findings indicate roles for Itk in both T-cell priming and T-cell migration, findings that are supported by studies showing a role for Itk in signaling downstream of chemokine receptors (Fischer et al. 2004; Takeda et al. 2004).

These in vivo experiments have been complemented by a series of in vitro experiments performed with naïve Itk−/− CD4+ T cells. All of these studies agreed that Itk−/− CD4+ T cells produced substantially less IL-4, IL-5, and IL-13 than wild-type T cells, even after differentiation in a powerful Th2-polarizing environment (Fowell et al. 1999; Schaeffer et al. 2001; Miller et al. 2004; Au-Yeung et al. 2006). In these studies, the defects in effector cytokine production were not because of impaired Th2 differentiation, as molecular analyses indicated that Itk−/− T cells polarized into the Th2 lineage produced all of the hallmarks of bona fide Th22 effector cells (Schaeffer et al. 2001; Miller et al. 2004; Au-Yeung et al. 2006). Interestingly, the requirement for Itk in effector cytokine production is shared by αβTcR+ NKT cells specific for α-galactosylceramide bound to CD1d; in the absence of Itk, NKT cells stimulated in vivo or in vitro produce substantially less IL-4 or IFN-γ than wild-type NKT cells (Au-Yeung and Fowell 2007; Felices and Berg 2008).

Th11 Differentiation and Effector Functions are Modestly Impaired in the Absence of Itk

Unlike the essential role for Itk in protective immunity to helminthic parasites, Itk signaling in T cells is largely dispensable for protective
CD4+ TH1 responses to intracellular pathogens. Thus, Itk−/− mice clear infections of *Leishmania major*, and are only modestly more susceptible to sublethal doses of *Toxoplasma gondii* than wild-type mice (Fowell et al. 1999; Schaeffer et al. 1999). These observations may reflect the fact that IFNγ production by Itk−/− CD4+ T cells is not reduced to the same extent as production of IFNγ by naive TH2 cytokines (Fowell et al. 1999; Schaeffer et al. 2001; Miller et al. 2004; Au-Yeung et al. 2006). Indeed, under certain conditions that normally lead to TH2 cytokine production, such as stimulation with altered peptide ligands, Itk−/− CD4+ T cells produce IFN-γ (Miller et al. 2004). A second aspect of this differential requirement for Itk in TH1 versus TH2 effectors is the interesting expression pattern of the two predominant Tec kinases in T cells, Itk and Rlk (Fig. 3). Unlike naive T cells which express both Itk and Rlk, TH2 effector cells lose Rlk and express only Itk; in fact, the levels of Itk are several-fold increased in TH2 cells compared to naive T cells (Miller et al. 2004). In contrast, TH1 effector cells continue to express both of these Tec kinases. As a consequence, whereas Itk−/− TH1 cells continue to express Rlk, TH2 cells lack both Itk and Rlk, thus accounting for the more severe functional defect observed in Itk−/− TH1 responses. Corroborating this notion, ectopic expression of Rlk in effector TH2 cells restores wild-type T cell responses in Itk−/− mice injected with *Schistosoma mansoni* eggs or immunized to induce allergic airway hypersensitivity (Sahu et al. 2008b).

Itk Is Required for IL-17A Production in TH17 Effector Cells

In addition to the global defects in effector T-cell responses described above for TH12, and to a lesser extent, TH1 responses, T cells lacking Itk display more selective alterations in cytokine production that highlight the complex nature of the signaling pathways that regulate T-cell effector responses. One recent example is the role of Itk in the regulation of cytokine production by TH17 cells, a recently recognized CD4 effector T-cell lineage that differentiates in response to IL-6 and TGF-β and expresses the pro-inflammatory cytokines IL-17A, IL-17F, IL-21 and IL-22 (Korn et al. 2009). TH17 cells are important for antimicrobial activity, particularly in the gastrointestinal system, but are also hallmarks of inflammation involved in multiple autoimmune disorders.

Although the requirements for cytokine signals for TH17 differentiation have been extensively examined, the contribution of TCR signaling to the regulation of TH17 cytokine production was unknown. To evaluate this question, naive CD4+ T cells from Itk−/− and Rlk−/−Itk−/− mice were differentiated under TH1 and TH17 polarizing conditions, and cytokine production evaluated by intracellular staining (Gomez-Rodriguez et al. 2009). Although both cell types could generate large percentages of IFNγ producing TH1 cells, Itk−/− and even more dramatically Rlk−/−Itk−/− T cells showed reduced IL-17A production. These defects did not appear to be because of altered thymic development, because they could be reversed by re-introduction of Itk by retroviral transduction before TH17 differentiation. Further analyses showed decreased *Il17a* message in Itk−/− T cells (Fig. 3). However, surprisingly, expression of other TH17 cytokines including the closely linked *Il17f* gene, was relatively intact, as was the expression of genes encoding the master regulators RORγt and RORα. Although phosphorylation of STAT3 in response to IL-6 was slightly attenuated in Itk-deficient T cells, re-expression of a constitutively activated STAT3 construct did not restore normal IL-17A production.

The selective role for Itk in IL-17A production resulted from a requirement for robust calcium signaling, leading to NFAT activation, in the transcription of the *Il17a* gene (Fig. 3). Thus, differentiation of Itk−/− T cells in the presence of anti-CD3 plus ionomycin, which can rescue Ca2+ mobilization in Itk-deficient T cells (Liu et al. 1998), did restore IL-17A production, directly implicating defective TCR-driven Ca2+ mobilization in this phenotype (Gomez-Rodriguez et al. 2009). Supporting this idea, stimulation of wild-type T cells with decreasing amounts of anti-CD3 during differentiation preferentially reduced expression of IL-17A, whereas leaving IL-17F relatively spared.
Similarly, differentiation of wild-type T cells in the presence of low-dose CyclosporinA or FK506, two inhibitors of Calcineurin that prevent NFAT activation (Winslow et al. 2003), also preferentially decreased IL-17A expression. Conversely, expression of a constitutively active mutant of NFATc1 (Neal and Clipstone 2003) restored IL-17A production by Itk−/− T117 cells, suggesting that Itk-mediated activation of NFAT contributes to the regulation of IL-17A. Sequence analyses showed a conserved NFAT binding site located 3.5 kb upstream of the start site of the Il17a gene and ChIP analyses showed that this site was occupied in wild-type but not Itk-deficient cells differentiated under T117 conditions. Although potential NFAT binding sites were found upstream of the Il17f gene, none were conserved cross-species. Nonetheless, conserved noncoding sequences in the region around both the linked Il17a and Il17f genes showed epigenetic modifications suggestive of open chromatin conformations (Wilson et al. 2009).

These results suggest that a TCR-driven pathway involving Itk-mediated regulation of Ca2+ and NFAT is required for full expression of IL-17A and that this pathway distinguishes expression of different T117 cytokines. These data support the growing view that there are distinct subclasses of T117 cells that produce overlapping sets of cytokines based upon distinct inputs. Thus, TCR signaling may be particularly important for expression of IL-17A, which is more strongly proinflammatory than IL-17F (Yang et al. 2008; Ishigame et al. 2009). This regulation operates during the differentiation of T117 cells, because the defect in IL-17A production is seen even upon restimulation of cells with PMA and ionomycin (Gomez-Rodriguez et al. 2009). NFATc1 autoregulates its own expression and previous data showed defective NFATc1 induction in Itk-deficient cells (Nurieva et al. 2007), perhaps accounting for the requirement for Itk during T117 differentiation. Alternatively, other chromatin remodeling factors that are recruited by transcription factors, including the BRG proteins, may not assemble properly on the IL-17A locus in the absence of efficient NFAT activation (Berg 2009). Although the exact mechanism of this defect awaits further study, these detailed investigations of Itk-deficient T lymphocytes have revealed another feature of how TCR signaling can affect the differentiation of distinct cytokine producing cells.

TCR Signaling in CD8+ T Cells Requires Itk

Although fewer studies have addressed the role of Itk in CD8+ T-cell responses, the conclusions thus far indicate that primary responses to viral infection are largely intact in the absence of Itk, or Itk and Rlk (Bachmann et al. 1997; Atherly et al. 2006). Biochemical studies show that Itk−/− and Rlk−/−/Itk−/− CD8+ T cells share a similar deficiency in TCR-induced PLC-γ1 activation, calcium mobilization, ERK activation, and cytokine production as CD4+ T cells lacking the Tec kinases (Atherly et al. 2006). Nonetheless, Itk−/− mice do mount protective immune responses to LCMV, vaccinia virus, and VSV, and Rlk−/−/Itk−/− mice to LCMV, although kinetics of viral clearance were often slightly delayed. Tracking of virus-specific CD8+ T cells indicated that Itk−/− and Rlk−/−/Itk−/− T cells were unable to expand to the extent seen with wild-type T cells, resulting in an overall deficit in the magnitude of the CD8+ T-cell response. This reduction in T-cell expansion could not be accounted for by impaired CD4+ T cell help in Itk−/− mice, as adoptive transfer of wild-type LCMV-immune T cells (CD4+ and CD8+) into Itk−/− mice prior to LCMV challenge was unable to rescue the CD8+ T-cell defects. In addition, the impaired response of Itk−/− CD8+ T-cells was not because of the predominant population of “innate-like” CD8+ T-cells present in these mice (see the following), as OT-1 TCR transgenic Itk−/− CD8+ T-cells, which develop as conventional naïve T-cells, also shared the defect seen with polyclonal Itk−/− CD8+ T-cells in non-TCR transgenic mice. It still remains to be determined whether Itk−/− CD8+ T-cells can generate functional memory cells. Although virus-specific T-cells persist in Itk−/− mice well after virus has been cleared from the animals, the ability of these cells to provide protection, and to mount an effective recall response, has not yet been tested.
This issue is complex, as defects in Itk−/− CD4+ T-cells could impact the generation of robust memory CD8+ T-cells; thus, appropriate studies will be required to distinguish CD4+ T-cell-intrinsic from CD8+ T-cell-intrinsic contributions to antiviral memory responses.

**TEC KINASES IN T-CELL DEVELOPMENT**

**Role of Itk and Rlk in Conventional T-cell Development**

TCR signaling plays a critical role in the development of T cells within the thymus, where the strength or duration of TCR signaling helps determine the survival, maturation, and differentiation of thymocytes into mature T cells. Double negative CD4−CD8− (DN) cells that have rearranged their TCRβ chains must receive competent signals through the pre-TCR to progress through the DN3 stage and expand in the DN4 to double positive (DP) transition (Ciofani and Zuniga-Pflucker 2006). At the double positive stage, TCR signaling regulates cell survival and differentiation of conventional T cells (Jameson et al. 1995). Thymocytes that receive very poor or no TCR signals undergo “death by neglect.” Thymocytes that receive strong TCR signals, as from agonist peptides, particularly in the context of costimulation, undergo negative selection and also die within the thymus. Only thymocytes that receive weak but adequate signals will progress onto CD4/CD8 selection, where the duration of TCR signaling, in large part dictated by the expression patterns of the co-receptors CD4 and CD8 upon TCR engagement, will determine their ultimate lineage as CD4 or CD8 single positive (SP) cells (Bosselut 2004).

It is therefore not surprising that Itk−/− and Rlk−/−Itk−/− T cells show altered thymocyte development (Fig. 4). Itk−/− mice have smaller thymi and show phenotypes consistent with impaired pre-TCR signaling. Although these defects are relatively subtle, competitive bone marrow transfers using mixtures of WT and Itk−/− bone marrow indicated that both Itk−/− and Rlk−/−Itk−/− cells had a selective disadvantage at the DN-DP transition (Lucas et al. 2007). However, Rlk−/−Itk−/− T cells also showed a competitive advantage compared to WT cells at earlier stages (prior to DN3), perhaps reflecting a negative signaling role for Rlk in cell survival/cytokine signaling pathways.

Defects in positive selection of thymocytes lacking Itk have been shown using a variety of MHC Class I and Class II-restricted TCR transgenes (Liao and Littman 1995; Schaeffer et al. 2000; Lucas et al. 2002; Lucas et al. 2003). The extent of these defects depended on the selecting TCR, with the weakest selecting TCRs showing the largest effects (Lucas et al. 2002). Defects in positive selection were exacerbated in Rlk−/−Itk−/− mice, providing evidence that these genes are partially redundant, and supporting the idea that decreased TCR signaling reduces positive selection of conventional T cells (Schaeffer et al. 2000). Supporting partial redundancy of these genes, overexpression of Txk/Rlk partially rescued positive selection defects in Itk−/− mice (Sommers et al. 1999).

Itk-deficient animals also show reduced or delayed negative selection in multiple TCR transgenic systems (Liao and Littman 1995; Schaeffer et al. 2000; Lucas et al. 2002). In Rlk−/−Itk−/− mice, negative selection is more clearly impaired, leading to positive selection of normally deleted HY TCR transgenic cells in male Rlk−/−Itk−/− mice (Schaeffer et al. 2000). These data support a model where negative selection is converted to positive selection in the context of impaired TCR signaling and may account for the increased SP cells in Rlk−/−Itk−/− mice.

**Development of Innate T-Lymphocyte Populations**

Recent data have revealed surprising new findings on thymic selection in mice deficient in Itk and Rlk (Atherly et al. 2006; Broussard et al. 2006; Dubois et al. 2006; Berg 2007; Hu et al. 2007). Although Itk−/− and Rlk−/−Itk−/− mice show decreased positive selection of conventional T cells using both MHC Class I and Class II-restricted TCR transgenes, surprisingly, Itk−/− and Rlk−/−Itk−/− mice have increased numbers of CD8 SP cells (Liao and Littman 1995;
Further examination of these CD8 SP populations suggested that these cells do not resemble conventional naive CD8 SP thymocytes. Instead, virtually all of the CD8 SP thymocytes in these mice showed markers seen on memory or activated T cells, including CD44 and CD122 (the IL-2Rβ chain), and rapidly expressed cytokines upon activation (Atherly et al. 2006; Broussard et al. 2006; Dubois et al. 2006; Hu et al. 2007). These phenotypes are characteristic of memory cells in the periphery—but, developmental studies and fetal thymic organ cultures provided evidence that Itk-deficient CD8 cells developed these characteristics within the thymus (Atherly et al. 2006; Broussard et al. 2006; Dubois et al. 2006; Schaeffer et al. 2000; Lucas et al. 2002). Currently, the cell–cell interactions required for γδ T-cell development are unknown.

Figure 4. Regulation of T-cell lineages by Itk. In the thymus, progenitor T cells (CD4⁻CD8⁻CD3⁻; DN) develop into γδ T cells and αβ T cells. Cells developing in the αβ T-cell lineage progress through an intermediate CD4⁺CD8⁺ (DP) stage prior to their maturation into mature conventional αβ T cells, CD1d-restricted αβ NKT cells and other subsets of innate-like αβ T cells. Conventional αβ T cells, which are selected on classical MHC molecules expressed on thymic stromal cells, and αβ NKT cells, which are selected on the MHC class Iβ molecule, CD1d, expressed on hematopoietic cells (HC) and dependent on SLAM receptor signaling, are greatly reduced in the absence of Itk. In contrast, innate-like αβ T cells, which are also selected by recognition of MHC molecules on HC and require SLAM receptor signaling, and “innate-like” γδ T cells (γδ NKT) are substantially increased in number in Itk⁻/⁻ mice. A third subset of cells, illustrated by conventional γδ T cells, appear unaffected by the presence versus the absence of Itk.
Although genetic crosses showed a requirement for MHC Class I for their development, transfers of bone marrow from Itk\(^{-/-}\) or Rlk\(^{-/-}\) mice into irradiated B2m-deficient mice, which lack MHC-Class I on their radio-resistant thymic stroma, showed that Itk\(^{-/-}\) and Rlk\(^{-/-}\) CD8\(^+\) thymocytes could be selected on hematopoietically derived cells (Broussard et al. 2006). Although these observations are atypical for conventional thymocytes, these features are all reminiscent of NKT cells and other related innate T-cell lineages; thus, many innate T-cell lineages have been shown to be selected on double-positive thymocytes, to express activation or memory cell markers, and to rapidly produce cytokines even in the thymus (Berg 2007). Like NKT cells, Itk\(^{-/-}\) and Rlk\(^{-/-}\) Itk\(^{-/-}\) CD8\(^+\) cells also expressed NK cell markers and high levels of the transcription factor Eosmosederm, and were dependent on IL-15 (Atherly et al. 2006; Dubois et al. 2006). Moreover, like NKT cells, development of these CD8 cells was dependent on SAP, a small adaptor molecule that is required for signaling from the SLAM family of receptors that are expressed on hematopoietic cells, but not on the thymic epithelium (Horai et al. 2007). Together these characteristics suggested that Itk-deficiency led to the preferential selection of an innate CD8 cell population on hematopoietic cells within the thymus (Fig. 4).

Although this phenotype is primarily seen in CD8 cells, there is also a small population of CD4 cells that show these properties in Itk\(^{-/-}\) mice. These CD4\(^+\)CD4\(^+\) cells are also dependent on SAP and express PLZF, a transcription factor responsible for the innate characteristics of NKT cells (PLS and LJB, unpublished data and Broussard et al. 2006; Hu and August 2008; Raberger et al. 2008). Although the selecting cell population for the innate type T cells in Itk\(^{-/-}\) mice is yet undetermined, the large number of CD8 cells with this characteristic (and relatively low numbers of CD4 cells) suggests that it is likely to be DP thymocytes, which express MHC Class I but not Class II, and are the selecting cells for NKT cells (Bendelac 1995).

Why loss of Itk leads to the preferential development of CD8 cells with this phenotype is not clear. Reciprocal transfers of Itk\(^{-/-}\)/B2m\(^{-/-}\) bone marrow provided evidence that the development of these innate phenotypes required selection on hematopoietic cells (Horai et al. 2007). One possibility is that poor selection of conventional thymocytes could permit selection or expansion of cells selected on hematopoietic cells. However, transfer of WT bone marrow into irradiated B2m-deficient hosts prevents selection of conventional CD8\(^+\) T cells and yet does not give rise to a large number of innate CD8 cells, suggesting that development of these cells is normally suppressed. Given the rapid production of cytokines showed by these cells, it is likely that the generation of these cells may be under tight control.

Several theories could account for the development of this population in Itk\(^{-/-}\) mice. Itk could play a negative role in signaling pathways required for the development of these lineages, such as those downstream of SLAM family receptors. Alternatively, these cells might normally be deleted in wild-type animals—negative selection can occur on hematopoietic cells, which express costimulatory molecules, such as CD28, thought to be important in this process (Punt et al. 1994). Alternatively, defective TCR signaling may make these cells more susceptible to signals from costimulatory molecules, such as SLAM family members, or cytokines that may be required either for selection or development of these phenotypes (Atherly et al. 2006; Dubois et al. 2006; Horai et al. 2007). Further analyses will provide clues to the regulation of these interesting lineages that appear to be at the boundary of innate and adaptive immune responses (Berg 2007).

**Itk Signaling Regulates αβ and γδ NKT-Cell Maturation**

In addition to defects in the development and selection of conventional naïve CD4\(^+\) and CD8\(^+\) T-cells, αβTCR\(^+\) NKT-cell maturation is also impaired in the absence of Itk and Rlk (Gadue and Stein 2002; Au-Yeung and Fowell 2007; Felices and Berg 2008; and Fig. 5). In these Tec kinase-deficient mice, αβ NKT cell numbers are substantially reduced; furthermore, of
the cells that remain, few represent the most mature αβ NKT subset characterized by high expression of NK1.1 and CD122, and down-regulation of CD4. *Itk*−/− and *Itk*+/−/Rlk−/− NKT cells also show poor responses to TCR stimulation, producing little to no IL-4 and IFNγ following in vivo activation. Thus, similar to conventional αβ T cells, αβ NKT cells require Tec kinases for their development and function.

In stark contrast to the requirements for αβ TCR+ T cells, the development of γδ T cells does not require Itk signaling (Fig. 4). The major γδ T-cell populations that arise in the fetus and adult are largely unchanged in *Itk*−/− mice (Felices et al. 2009; C. Yin and LJB, unpub. observ.). Indeed, overall γδ T-cell numbers in *Itk*−/− mice are significantly increased compared to wild-type mice, because of dramatic increases in one particular γδ T-cell subset, the γδ NKT cells (Felices et al. 2009; Qi et al. 2009). This subset expresses the Vγ1.1/Vδ6.3 TCR; however, in all other regards, these γδ T cells share characteristics of CD1d-specific αβ NKT cells rather than traits associated with most adult-derived γδ T cells (Vicari et al. 1996; Azuara et al. 1997; Lees et al. 2001). For instance, the majority of γδ NKT cells express CD4, NK1.1, and the signature NKT cell transcription factor, PLZF (Felices et al. 2009; Kreslavsky et al. 2009). As a result, γδ NKT cells, like their αβ NKT cell counterparts, produce both IFNγ and Tnfα cytokines. In addition, these cells preferentially home to the liver, rather than to lymphoid organs such as the spleen (Lees et al. 2001; Felices et al. 2009).

Surprisingly, this expanded population of γδ NKT cells is responsible for a spontaneous hyper-IgE syndrome observed in *Itk*−/− mice (Felices et al. 2009; Qi et al. 2009). When *Itk*−/− mice are crossed to mice lacking γδ T cells, the elevated serum IgE and germinal center hyperplasia seen in *Itk*−/− mice disappear. *Itk*−/− splenic γδ NKT cells constitutively express ICOS, and readily upregulate CD40-ligand and OX40 following γδ TCR stimulation in vitro (Felices et al. 2009). These findings suggest that *Itk*−/− γδ NKT cells are being activated in vivo, allowing them to provide T cell help to B cells and secrete cytokines that promote switching to IgE. As with the majority of γδ T cells, the ligand recognized by the Vγ1.1/Vδ6.3+ TCR is unknown, nor is it known whether activation of these cells requires signaling through the γδ TCR.

These findings raise several interesting issues regarding the development and signaling properties of γδ T cells. In contrast to αβ T cells, γδ T cells are not reduced in the absence of Itk; further, the γδ NKT cell population is, in fact, greatly expanded. These data indicate that the signals regulating γδ T-cell development do not require Itk, a situation comparable to that seen for innate CD8+ αβ T cells. The expansion of both the γδ NKT and the innate CD8+ population in *Itk*−/− mice may reflect the fact that the majority of these cells normally undergo negative selection, but that their deletion is impaired in the absence of Itk.

A second interesting issue is the apparently robust TCR signaling in *Itk*−/− γδ T cells. αβ T cells lacking Itk have substantially reduced responses to TCR signaling, particularly in their ability to synthesize effector cytokines. *Itk*−/− γδ T cells, however, produce copious amounts of cytokines in response to γδ TCR stimulation. These data suggest that the biochemical pathways downstream of the γδ TCR are likely distinct from those in αβ T cells. Consistent with this idea, mice carrying a mutant version of the adapter protein LAT also have few αβ T cells but greatly increased numbers of γδ T cells that overproduce Tnfα cytokines; further, these mice share the hyper-IgE syndrome seen in *Itk*−/− mice (Nunez-Cruz et al. 2003). This LAT mutant is lacking a critical tyrosine in the cytoplasmic tail that is essential for efficient TCR signaling in αβ T cells, reinforcing the notion that αβ and γδ TCR signaling have distinct biochemical requirements.

**ITK AND HUMAN DISEASE**

Although mutations affecting Btk were identified as the cause of X-linked Agammaglobulinemia 17 years ago (Rawlings et al. 1993; Thomas et al. 1993; Tsukada et al. 1993; Vetrie et al. 1993), alterations in Itk have only recently been associated with human disorders. These
include Itk promoter polymorphisms associated with atopy (Graves et al. 2005) and the expression of an Itk-Syk fusion protein generated by a chromosomal translocation in T-cell lymphomas (Streubel et al. 2006); in this latter case, the Itk PH domain becomes fused to the kinase domain of Syk, leading to constitutive activation of the kinase. Most recently, a mutation affecting Itk was found in 2 siblings who died from fatal Epstein-Barr Virus infection (Huck et al. 2009). This mutation changes a single amino acid in the Itk SH2 domain and destabilizes the Itk protein. Although the basis for this particular immunodeficiency is unknown, it may be related to Itk function in cytotoxic CD8+ T cells (Bachmann et al. 1997; Atherly et al. 2006). Importantly, the implication of Itk in a human primary immunodeficiency has validated its role as a critical regulator of T lymphocyte function. Given the depth of information on lymphocyte biology provided through the study of Tec kinases, it is clear that their study will continue to provide important insights into the signaling pathways involved in immune cell development, homeostasis and function.

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Tec Kinase Itk in T-Cell Signaling


T-Cell Signaling Regulated by the Tec Family Kinase, Itk

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