ROLE OF ITK IN THE DEVELOPMENT OF INNATE T CELLS

A Dissertation in
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by
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ABSTRACT

The Tec family of tyrosine kinases transduces signals from antigen and other receptors in cells of the hematopoietic system. Tec kinases are composed of five domains- PH, TH, SH2, SH3 and kinase domain, which distinguish them from other nonreceptor tyrosine kinases. The unique domain composition of Tec kinases indicates a distinct structure and mode of regulation. In particular, Itk plays an important role in modulating T cell development and activation. Itk is activated by receptors via a PI3 kinase mediated pathway, which results in recruitment of Itk to the plasma membrane via its PH domain. We show here that membrane localization of Itk results in the formation of clusters of at least 2 molecules within 80 angstroms of each other, which is dependent on the integrity of its PH domain. More importantly, these clusters of Itk molecules form in distinct regions of the plasma membrane as only receptors that recruit PI3K reside in the same membrane vicinity as the recruited Itk. Our results indicate that Itk forms membrane dimers and that receptors that recruit Itk do so to specific membrane regions.

Although the structures of each domain of Itk have been determined, the full structure of Itk and other Tec kinases remain elusive. Models of Itk suggest either a head to tail dimer, with the SH2 domain interacting with the SH3 domain, or a folded monomer with the SH3 domain interacting with the proline rich region exists. We show here that in vivo Itk exists as a monomer, with the PH domain less than 80 Å from the C-terminus. Zn$^{2+}$ coordinating residues in the TH domain, not the proline rich region control this intramolecular interaction. These data have implications for our understanding of Tec family kinase structure.

Invariant natural killer T (iNKT) cells are a unique subset of innate T lymphocytes that are selected by CD1d. They have diverse immune regulatory functions via the rapid production of interferon-gamma (IFN-gamma) and interleukin-4 (IL-4). In the absence of ITK and Txk, Tec family non-receptor tyrosine kinases, mice exhibit a significant defect in iNKT cell development.
We now show here that although Itk is required for iNKT cell maturation, mice carrying an Itk mutant lacking its kinase domain, Tg(Lck-ItkΔKin)/Itk−/−, exhibit significant rescue of mature iNKT cells in the thymus compared with ITK null mice. Furthermore, this rescue correlates with the increased expression of CD122, and altered balance of two T-box transcription factor, T-bet and eomesodermin. These data indicate that ITK uses a scaffolding function in the signaling pathway leading to the maturation of iNKT cells, and indicate that the kinase activity of ITK is only partially required for maturation of iNKT cells.

Itk is critical for the development of αβ T cells as well as differentiation of CD4+ T cells into Th2 cells. Itk null mice have defect to produce Th2 cytokines, however they paradoxically have significant elevations in serum IgE. Here we show that Itk null mice have increased numbers of γδ T cells in the thymus and spleen. This includes elevated numbers of CD4+ γδ T cell, the majority of which carry the Vγ1.1 and Vδ6.2/3 γδ T cell receptor and exhibit distinct phenotype. The development of these CD4+ γδ T cells is T cell intrinsic, independent of either MHC class I or class II, and is favored during development in the absence of Itk. Itk null CD4+ γδ T cells secrete significant amounts of Th2 cytokines and can induce the secretion of IgE by WT B cells. Altogether, Itk plays important role in regulating γδ T cell development and function. The elevated IgE observed in Itk deficient mice is due to the enhanced development of CD4+ γδ T cells in the absence of Itk. CD4+ γδ T cells may therefore play critical roles in B cell IgE class switch
# TABLE OF CONTENTS

LIST OF FIGURES ................................................................................................................. viii

LIST OF COMMONLY USED ABBREVIATIONS ....................................................................... x

ACKNOWLEDGEMENTS ....................................................................................................... xi

Chapter 1 Introduction ............................................................................................................. 1

1.1 Tec family kinases in T cell signaling pathways ........................................................ 2
  1.1.1 T cell surface receptor induced signaling pathways ........................................ 2
  1.1.2 Domain structures of Tec family kinases ........................................................ 3
  1.1.3 Mechanism of Tec family kinase in T cell signaling pathways ...................... 5
  1.1.4 Tec family kinases in T cell development and function .................................. 7

1.2 iNKT lineage development and function ................................................................. 9
  1.2.1 iNKT cell development ................................................................................... 10
  1.2.2 Signaling proteins involved in iNKT cell development ................................ 13
  1.2.3 iNKT cell function .......................................................................................... 15

1.3 γδ T cell lineage development and function .............................................................. 16
  1.3.1 γδ T cell development .................................................................................... 17
  1.3.2 γδ T cell function ............................................................................................. 21

Chapter 2 Materials and methods .......................................................................................... 28

2.1 Plasmids ..................................................................................................................... 28
2.2 cell transfection ........................................................................................................... 29
2.3 Flow cytometric assay ............................................................................................... 29
2.4 Conical microscopy and FRET ................................................................................. 30
2.5 Mice ........................................................................................................................... 31
2.6 Cell sorting ............................................................................................................... 32
2.7 Rea-ltime PCR analysis .......................................................................................... 32
2.8 In-vivo BrdU incorporation assay .......................................................................... 33
2.9 Bone marrow chimeras ......................................................................................... 33
2.10 Cell transfers and serum analysis ......................................................................... 34
2.11 Data analysis .......................................................................................................... 34

Chapter 3 Itk forms membrane clusters specifically in the vicinity of recruiting receptors ... 35

3.1 Introduction ............................................................................................................... 35

3.2 Results ....................................................................................................................... 37
  3.2.1 Itk forms dimers specifically at plasma membrane of cells ............................ 37
  3.2.2 The PH domain is required for the formation of dimers of Itk at the plasma membrane .......................................................... 38
  3.2.3 Itk interacts specifically with receptors capable of recruiting PI3 kinase at the plasma membrane .............................................................................. 39
3.3 Discussion

Chapter 4 On the structure of Itk: Evidence for a folded monomer in vivo

4.1 Introduction

4.2 Results

4.2.1 Itk exits as a intramolecular folded monomer in the inactive state in-vivo

4.2.2 The SH2 and SH3 domains are not required to maintain the folded inactive monomer of Itk

4.2.3 The Zn\textsuperscript{2+} binding region in the TH domain is critical for maintenance of the folded inactive monomer of ITK

4.3 Discussion

Chapter 5 The role of Tec family kinases in iNKT cell development

5.1 Introduction

5.2 Results

5.2.1 Reduced frequency and number of iNKT cells in the absence of Itk and Tsk

5.2.2 iNKT cell development is partially dependent on the kinase domain of Itk

5.2.3 Itk kinase activity independent rescue of CD122 and ratio of T-bet to eomesodermin in developing iNKT cells

5.3 Discussion

Chapter 6 The role of Tec family kinases in γδ T cell development and function

6.1 Introduction

6.2 Results

6.2.1 Increased percentage and numbers of γδ T cells in the absence of Itk

6.2.2 Increased development of CD4\textsuperscript{+} γδ T cells in the absence of Itk

6.2.3 Altered phenotype of γδ T cells in the absence of Itk

6.2.4 Enhanced development of γδ T cells in the Itk null mice is cell intrinsic

6.2.5 Development of CD4\textsuperscript{+} γδ T cells in the absence of Itk is independent of MHC class I or MHC class II expression

6.2.6 Itk null CD4\textsuperscript{+} γδ T cells predominantly express V\textgreek{g}1.1/V\textgreek{d}2.3 γδ T cell receptor

6.2.7 Itk null CD4\textsuperscript{+} γδ T cells predominantly express Th2 cytokine IL-4 and carry increased IL-4 mRNA

6.2.8 Itk null mice have high levels of serum IgE of γδ T cells

6.2.9 Itk null CD4\textsuperscript{+} γδ T cells induce B cell class switch to produce IgE
6.3 Discussion ......................................................................................................................... 92

Chapter 7 Conclusion and future directions ............................................................................. 110

7.1 Discussion ......................................................................................................................... 110

  7.1.1 Comparison of BiFC and FRET ................................................................................. 110
  7.1.2 The conformation of Itk and other Tec family kinases ........................................ 111
  7.1.3 The role of Itk and Txk in the development of innate T cell populations .......... 112

7.2 Future directions ............................................................................................................. 115

  7.2.1 Dynamics of Itk during TcR stimulation ................................................................ 115
  7.2.2 The role of Txk in iNKT cell and γδ T cell development and function .......... 115
  7.2.3 Mechanism of the increased CD4+ γδ T cells in the absence of Itk ................. 117

References ............................................................................................................................. 122
LIST OF FIGURES

Figure 1-1: TcR-mediated signaling pathways and the role of Tec kinases in these pathways. 23

Figure 1-2: The domain organization of ITK and models of the conformation of ITK.............24

Figure 1-3: iNKT-cell development...........................................................................................25

Figure 1-4: iNKT-cell development....................................26

Figure 1-4: DN thymocyte development. ................................................................................27

Figure 3-1: Itk forms dimers specifically at plasma membrane of cells. ............................42

Figure 3-2: The PH domain is required for the formation of dimers of Itk at the plasma
membrane.............................................................................................................................44

Figure 3-3: Itk interacts specifically with receptors capable of recruiting PI3 kinase at the
plasma membrane...................................................................................................................45

Figure 3-4: Formation of dimers of Itk in T cells. ...............................................................47

Figure 4-1: Itk does not form dimers in the cytoplasm of cells. ........................................58

Figure 4-2: Quantification of fluorescence complementation ............................................60

Figure 4-3: Models of Itk as suggested by the experimental results. .................................62

Figure 4-4: Cytoplasmically localized Itk exists as a head to tail folded monomer in cells.....63

Figure 4-5: Interactions via cis/trans conformation, the PRR, SH3 or Cyclophilin A are
not required for the formation of the head to tail folded monomer of Itk..............65

Figure 4-6: The Zn²⁺ binding region in the TH domain of Itk is required to maintain the
head to tail folded monomer in cells..................................................................................67

Figure 5-1: The kinase activity of Itk is not required for generation of WT percentages of
iNKT cells, but is required for generating WT numbers of iNKT cells in the
thymus......................................................................................................................................77

Figure 5-2: CD1d, SLAM and Ly 108 were expressed at similar level in DP thymocytes of
WT, Itk<sup>−/−</sup> and Itk/Txk DKO mice ................................................................. 78

Figure 5-3: The kinase activity of ITK is not fully required for the maturation of iNKT cells ..................................................................................................................... 79

Figure 5-4: Kinase activity independent rescue of mature iNKT cell proliferation .......... 81

Figure 5-5: Kinase activity independent rescue of CD122 and ratio of T-bet to eomesodermin. ..................................................................................................... 82

Figure 6-1: Increased γδ T cells in mice lacking Itk ..................................................... 96

Figure 6-2: Surface phenotype of CD4<sup>+</sup> γδ T cells from WT and Itk<sup>−/−</sup> mice ...................... 98

Figure 6-3: Enhanced development of γδ T cells in the absence of Itk is bone marrow Intrinsic ...................................................................................................................... 100

Figure 6-4: Increased CD4<sup>+</sup> γδ T cells in the absence of Itk is independent of MHC class I or II expression ................................................................. 102

Figure 6-5: γδ TcR expression of γδ T cells from WT and Itk<sup>−/−</sup> mice. ...................... 103

Figure 6-6: Itk null CD4<sup>+</sup> γδ T cells develop independent of the γδ TcR they express. ....... 105

Figure 6-7: Itk null CD4<sup>+</sup> γδ T cells secrete IL-4 and induced B cell class switch to IgE..... 106

Figure 6-8: Itk<sup>−/−</sup> γδ T cells carry increased IL-4 mRNA ............................................... 108

Figure 7-1: Model of the conformation of Itk in the inactive state .................................. 119

Figure 7-2: Itk and Txk are important for the final maturation of iNKT cells .............. 120

Figure 7-3: Possible model of the role of Itk in γδ T cell development ....................... 121
## LIST OF COMMONLY USED ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>α-Galcer</td>
<td>α-galactosyl Ceramide</td>
</tr>
<tr>
<td>DN</td>
<td>Double Negative</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein;</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interlukine-4</td>
</tr>
<tr>
<td>Itk</td>
<td>Inducible T cell Kinase;</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant Natural Killer T cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural Killer T cell</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology domain</td>
</tr>
<tr>
<td>PRR</td>
<td>Proline Rich Region</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology domain 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src Homology domain 3</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell Receptor</td>
</tr>
<tr>
<td>TH</td>
<td>Tec Homology domain</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein;</td>
</tr>
<tr>
<td>Y1I</td>
<td>YFP1 fragment tagged to Itk;</td>
</tr>
<tr>
<td>Y2I</td>
<td>YFP2 fragment tagged to Itk.</td>
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Chapter 1

Introduction

Allergic asthma is the inflammatory disorder of the airways triggered by allergen induced abnormal reaction of the immune system. Patients with allergic asthma often need treatment, and asthmatic attacks can be life-threatening. Currently there is no drug that can cure asthma. Finding further targets that are pharmaceutically tractable will help treating this disease. Studies have shown that a number of T cell subtypes, such as Th2 cells (a subset of CD4^{+} \alpha \beta T cells), NKT cells, and \gamma \delta T cells, all play roles in the asthmatic response \cite{1}. IL-2 Inducible tyrosine kinase (Itk) is a non-receptor tyrosine kinase that plays a critical role in T cell signaling pathways, T cell development and T cell activation \cite{2}. Itk null mice showed dramatically reduced response to allergic asthma, which makes Itk a potentially good pharmacological target for inhibiting allergic diseases \cite{3-5}. However, the conformation of Itk and how Itk activity is regulated by conformational change are not clear, which makes it difficult to design specific Itk inhibitors. In addition, the role of Itk in unconventional innate T cell types, such as NKT cell and \gamma \delta T cells, is not clear, which makes it difficult to assess its suitability for targeting to control NKT and \gamma \delta T cell response. The rationale is that the studies on the conformation and conformational change of ITK, as well as the role of Itk in NKT cell and \gamma \delta T cell development and function, will provide a basis for designing selective ITK inhibitors of use in the treatment of allergic disease and help to rationally design methods to be able to control allergic diseases by NKT cells and \gamma \delta T cells.
Tec family kinases in T cell signaling pathways

1.1.1 T cell receptor induced signaling pathways

Signals from the T cell receptor (TcR) are required for T cell activation and the generation of an immune response. When TcR is activated, the immunoreceptor tyrosine-based activation motif (ITAMs) on the intracellular side of the TCR-CD3 complex is phosphorylated by Src family tyrosine kinase Lck and Fyn. The phosphorylation leads to the recruitment of Syk-family kinase member ζ chain-associated protein kinase of 70 kD (ZAP-70) to the TcR/CD3 complex, where it is phosphorylated and activated by Lck. Activated ZAP-70 further phosphorylates several signaling proteins, such as phospholipase C-γ1 (PLC-γ1) and adaptor protein LAT. Lck also phosphorylates and activates phosphoinositide 3-kinase (PI3K), which generates membrane phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) in cell membrane. Tec family protein tyrosine kinase IL-2-inducible T cell kinase (ITK) is then recruited to cell membrane through the interaction of its PH domain with PIP3, where it is phosphorylated and activated by Lck and interacts with other signaling proteins such as PLC-γ1, adaptor protein SH2-domain-containing leukocyte protein of 76 kD (SLP-76), LAT, Vav, and Gads, to form the signaling complex [6]. The formation of these signaling complexes is important for the full activation of PLC-γ1. Activated PLC-γ1 generates the second messenger inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) (Fig. 1-1). Whereas IP3 triggers increases in [Ca2+], which leads to the activation of nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB) signaling pathways, DAG activates Ras-dependent signals, such as the extracellular-signal-regulated kinase (ERK) pathway, which are important for the induction of cytokines such as IL-2 [7-9]. These signaling pathways further induce the transcription of multiple genes involved in T cell
proliferation and differentiation as well as the cytoskeleton reorganization that changes T cell shape and motility (Fig. 1-1).

### 1.1.2 domain structures of Tec family kinases

The Tec family of non-receptor protein tyrosine kinases are the second largest family of non-receptor tyrosine kinases which consist of five members: Tec, Btk, Itk, Txk and Bmx [10]. They share a similar domain organization. For example, Itk is structurally organized into five domains, an amino-terminal pleckstrin homology (PH) domain, followed by a Tec-homology (TH) domain which contains a Zn$^{2+}$-binding Btk homology (BH) motif and one proline-rich region (PRR), a Src homology 3 (SH3) and SH2 and a carboxyl-terminal kinase domain [11] (Fig. 1-2). Btk and Tec have a secondary PRR next to BH motif. Bmx does not have a PRR, while Txk has a cysteine-string motif, instead of a PH domain.

A lot of studies have been done on the individual function of each domain. The kinase domain has catalytic activity to transfer phosphate to tyrosine residues in the substrate. The PH domain is responsible for the subcellular localization of Tec family kinases. When lymphocytes are not activated, Tec family kinases except Txk, which does not contain a PH domain, all localize predominantly in the cytoplasm in resting lymphocytes. When lymphocytes are activated, the PH domain can bind to the PI3-K kinase product PI(3,4,5)P3 in the plasma membrane, thus regulating membrane targeting of Tec family kinases from cytoplasm to the cell membrane where they can be activated [6]. In contrast, the cysteine-string motif is palmitoylated and recruits Txk to the lipid rafts in the cell membrane constitutively. The SH2, SH3 and TH domains all mediate protein-protein interactions that regulate the signaling pathways downstream of Tec family kinases and may help determine their varied functions in different signaling pathways [12].
Protein tyrosine kinases are maintained in an inactive conformation due to intramolecular interactions between domains, which block the ATP binding pocket of the kinase domain [13, 14]. When PTKs are activated by the upstream signaling, the intramolecular interactions of PTKs are broken and the kinase domain is exposed to the substrates, thus increasing their kinase activity [15, 16]. A number of studies have suggested that the conformation of protein tyrosine kinases is controlled by the self-interaction of domains, thus retaining them in the inactive state [17, 18]. Src family tyrosine kinases, which have similar overall structures as Tec kinases with the exception of the TH and PH domains, are folded via intramolecular interactions between carboxyl-terminal negative regulatory phosphotyrosine and the SH2 domain that keep the kinase in the inactive state prior to receptor stimulation [17]. The crystal structure of the full length of any Tec family kinase is still not clear, however, studies of the structures of the individual domains of Itk suggest that although Itk lacks the conserved carboxyl-terminal negative regulatory tyrosine phosphorylation site, Itk may also be regulated by intramolecular and/or intermolecular interactions among their domains.

When Itk is recruited to the cell membrane upon stimulation, Itk is phosphorylated on a conserved tyrosine residue Y512 in the activation loop in the kinase domain by Lck, followed by an autophosphorylation on a conserved tyrosine Y180 in the SH3 domain. This transphosphorylation has been shown to be important for the kinase activity of Itk, mutation of Y180 leads to severe defect in kinase activity [19]. The function of the autophosphorylation is suggested to be more important for the regulation of Itk-substrate protein interaction, rather than regulating Itk kinase activity. The research from Cheethman and colleagues showed the crystal structure of both the Y512 phosphorylated and unphosphorylated kinase domain of Itk [20]. Surprisingly, they found no significant conformational difference in the phosphorylated kinase domain compared to the unphosphorylated one [20]. These data suggest that the kinase activity of Itk may be regulated by its TH, SH2 and SH3 domains, instead of conformation changes in the
kinase domain after Y512 transphosphorylation. In addition, the structures of PRR, SH2 and SH3 domain have also been analyzed using NMR assays, and two types of inter- and intra-domain interactions in Itk have been suggested [21, 22].

An intramolecular interaction between Itk SH3 and PRR domains has been suggested, which may act to maintain Itk in a folded state and thus prevent the binding of each domain to its respective ligands [21]. A second type of intermolecular interaction has been suggested where the Itk SH2 domain interacts with the SH3 domain of a second Itk molecule, thus dimerizing Itk in a head-to-tail configuration [22]. This model also suggests that a proline-dependent conformational switch exists in the SH2 domain of Itk, which directs a cis or trans conformer of the SH2 domain. The cis-conformer favors the dimerization of Itk via reciprocal SH2-SH3 and may inhibit Itk kinase activity, while the trans-conformer favors the binding of phosphotyrosine containing ligands such as phosphorylated residues of SLP-76 [22, 23] (Fig. 1-2). These interpretations were from the results of NMR studies of isolated SH3 and SH2 domains, however since the crystal structure of full length Itk is not available, the exact conformation of Itk in the inactive state is still not clear.

1.1.3 Mechanism of Tec family kinase in T cell signaling pathways

There are three Tec family tyrosine kinases in T cells: Itk, Txk and Tec. Itk is the predominant Tec family kinase expressed in T cells, which is also expressed in mast cells, natural killer (NK) cells and NKT cells [12]. Txk expression is found in T cells, mast cells and NKT cells. Compared to Itk, the mRNA level of Txk is around 10-30% of that of Itk in resting mature T cells and NKT cells [23-25]. Tec is broadly expressed both in lymphocytes and other cell types. Its mRNA level is around 1% of Itk mRNA level in T cells. The role of Tec in T cell development and function has not yet been determined.
Tec family kinases regulate signals emanating from multiple receptors in T cells, including TcR, CD2, CD28 and CXCR4 [10, 26-30]. Itk has been shown to regulate TcR signals through phosphorylating and activating PLC-γ1, leading to increases in intracellular calcium, ERK/MAPK and activation of transcription factors NFAT and AP-1 [31, 32]. Itk is also activated and interact with T cell costimulatory receptor CD28 in the presence of Lck upon CD28 stimulation [33, 34]. The chemokine stromal cell–derived factor-1α (SDF-1α) modulates T cell development, migration, and costimulates the immune activation of T lymphocytes stimulated by the TCR [35-37]. CXCR4, which is the only receptor for SDF1-α, is coupled to the small G protein Gi, which can lead to the activation of PI-3K [38, 39]. SDF-1α can induce the translocation and activation of ITK in a PI3-K and Lck dependent manner, respectively [40]. Itk−/− T cells have defects in SDF1α-induced cell migration, with further deficiency in Itk−/−/Txk−/− T cells, suggesting that Txk is also involved in the chemokine responses in T cells [40, 41].

The role of Txk in T cell signaling is not clear since the Txk−/− T cells have no apparent defects, perhaps due to the functional redundancy among Tec family kinases. However, when compared to Itk−/− T cells, Itk−/−/Txk−/− T cells have a more severe defect in TcR induced PLC-γ1 activation and subsequent calcium influx, suggesting the role of Txk in TcR signaling [42].

As we mentioned before, the assembly of the Itk-containing signaling complex upon TcR stimulation is important for the phosphorylation and duration of signals, leading to full activation of downstream signaling pathways. The SH2 domain of Itk binds directly to Tyr145 within the N-terminus of SLP-76, and the SH3 domain of Itk binds the PRR of SLP-76 [15, 43]. The SH2 and SH3 domains of Itk and the P-I region in the central PRR of SLP-76 are important in mediating PLC-γ1 activation, indicating that multiple protein-protein interactions play a role in this process [44, 45]. The Itk, SLP-76, and PLC-γ1 complex also interacts with LAT through the growth factor receptor-bound protein 2 (Grb2)-related adaptor protein Gads, which bridges SLP-76 and LAT and interact with Vav (a guanine nucleotide exchange factor (GEF) for the Rho-
family small guanosine-triphosphatases). Itk phosphorylates Vav, and Vav positively regulates the tyrosine phosphorylation of Itk perhaps through the activation of PI-3 kinase[46-48]. All of these interactions suggest that Itk has an adaptor function besides its kinase activity. Indeed, studies have shown that the expression of a kinase-dead mutant of Itk rescues the defect of TcR induced membrane localization of Vav and actin polarization in Itk^{-/-} T cells, but either expression of either a PH mutant or a SH2 mutant of Itk fails to rescue it, suggesting Itk contributes to these process in a kinase independent manner [46, 49]. We have also shown that Itk activates the antigen receptor induced transcription factor Serum Response Factor (SRF) in the absence of its kinase activity [50]. All these data demonstrate that Itk also has kinase-independent scaffolding function.

1.1.4 Tec family kinases in T cell development and function

Studies on Itk null mice crossed to several TcR transgenic lines have shown that Itk regulates the development of αβ T cells such that positive selection and negative selection in the thymus are defective in the absence of Itk [51, 52]. These defects are exacerbated in mice lacking both Itk and Txk, indicating that Txk also plays a role in the positive selection and negative selection of αβ T cells [51]. These defects may reflect the role of Tec family kinases in T cell signaling pathways, some of which are important in thymic selection. Itk^{-/-} and Itk^{-/-}/Txk^{-/-} thymocytes have impaired calcium influx and reduced activation of ERK1/2 upon TcR stimulation [51]. Optimal calcium signaling is important for both positive selection and negative selection, and ERK signaling pathway is required for positive selection [53].

Itk is important for naïve T cell function. Itk^{-/-} T cells exhibit reduced IL-2 production and proliferation when stimulated via the TcR, which is exacerbated in Itk^{-/-}/Txk^{-/-} T cells [31, 32, 52]. It is now well established that Itk plays an important role in T helper 2 (Th2) cell function.
The mRNA and protein level of Itk are higher in Th2 cells compared to Th1 cells [23, 54]. CD4+ naïve T cells from Itk null mice have reduced secretion of Th2 cytokines IL-4 upon stimulation, which may be due to the failure to activate the calcium regulated transcription factor NFAT [32]. The defective Th2 response in Itk null mice has been confirmed in multiple murine models. Using an allergic asthma model, which is a Th2 cell induced immunological disease, our lab showed that Itk null mice have defects in developing allergen-induced allergic asthma [3]. Itk null mice also have defects in response to Th2-inducing parasitistic infection, such as *Nippostrongylus brasiliensis*, *Leishmania major* and *Shistosome mansoni*, all of which confirm that Itk is important for the Th2 cell function [32, 55]. Txk, in contrast, is preferentially expressed in Th1 cells but not Th2 cells. Overexpression of Txk in Jurkat T cells leads to increased expression of the Th1 cytokine IFNγ, suggesting a distinct role of Txk in Th1 cell function [42, 56]. However, analysis of Txk−/− mouse T cells does not exhibit reduction in IFNγ secretion or a defect in the response to Th1 pathogens [42, 55]. Furthermore, a recent study from our lab revealed that Tg(CD2-Txk)/Itk−/− transgenic mice, which overexpress Txk to the similar level of Itk in Th2 cells, rescue the defective Th2 response to allergic asthma and *S. mansoni* eggs, demonstrating that Txk have the same function as Itk in Th2 responses when it is expressed to the similar level of Itk. These data strongly suggesting that roles of Itk and Txk on Th cell function may result from their differential patterns of expression, but not due to intrinsic distinct function [57].

Several recent studies revealed the role of Itk and Txk in CD8+ and CD4+ cell development. Itk has been shown to be required for the development of conventional or naïve phenotype (CD44low) CD8+ and CD4+ T cells, but not for the development of innate or memory phenotype (CD44low) CD8+ and CD4+ T cells [58-62]. In the absence of Itk, around 85% of CD8+ T cells exhibit memory phenotype (CD44hi and increased expression of CD122, NK1.1), carry large amount of preformed message for IFNγ and rapidly secrete large amount of IFNγ upon activation [58, 60, 61, 63]. Compared to the severe developmental defect of naïve
phenotype CD8+ T cells in the absence of Itk, the development of naïve phenotype CD4+ T cells is less affected, although Itk is still required for their development [64].

1.2 iNKT lineage development and function

Natural killer T cells (NKT cells) are originally defined as a unique population of innate-like T lymphocytes that express the TcR and surface markers of NK lineage, such as NK1.1 and NKG2D[65]. However, this definition has recently been thought to be inaccurate as the expression of NK receptors is variable, depending on their developmental stages and activation states. In addition, innate phenotype T cells also express NK receptors. Currently NKT cells are defined as a distinct subset of T lymphocytes that recognize glycolipid antigen presented by the MHC class I-like molecule CD1d. The invariant NKT (iNKT) cells are the dominant subset of NKT cells that express an invariant TcRα chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) and certain Vβ chain (Vβ2, Vβ7 or Vβ8.2 in mice, Vβ11 in human) [66]. iNKT cells recognize the marine sponge-derived glycolipid antigen α-galactosyl ceramide (α-GalCer) and are identified using α-GalCer loaded CD1d tetramers[66]. iNKT cells represent about 0.5% of total thymocytes, about 2% of T cells in the spleen, and up to 30% of T cells in the liver. When activated, iNKT cells rapidly produce large amounts of IL-4 and IFNγ, along with a number of other cytokines and chemokines[67]. iNKT cells also regulate other immune cells, such as NK cells and B cells, thus playing multiple roles in immune responses. Another group of NKT cells express more diverse TcR. They also recognize CD1d, but the lipid ligands are not clear, which make them difficult to analyze.
1.2.1 iNKT cell development

iNKT cells develop in the thymus and first appear in the thymus a little later than conventional T cells. They develop from the DP (CD4⁺CD8⁺ double positive) thymocytes in the thymic cortex [68, 69].

In conventional T cell development, DP thymocytes first undergo positive selection, which requires the TcR recognition of peptides presented by MHC class I or class II molecules on thymic cortical epithelial cells. The DP thymocytes whose TcR fail to engage MHC:peptide mix presented by epithelial cells die. After positive selection, the immature thymocytes then undergo negative selection. The cells whose TcR is ligated to self peptide: MHC complex are eliminated from the population. During the positive and negative selection, T cells are matured to express either CD4 or CD8. In contrast, only positive selection is required for iNKT cell development. Studies have shown that the selection of iNKT cells requires their canonical Vα14 TcR recognition of glycolipid antigens presented by MHC class I-like molecule CD1d on other DP thymocytes. This is different from the positive selection of conventional T cells, which needs the peptide as ligand and the interaction with epithelial cells.

A lot of studies have been done in the search of the NKT ligands. The first NKT ligand found is marine sponge-derived glycolipid antigen α-galactosyl ceramide (α-GalCer). The finding of α-GalCer led to the identification of iNKT cells [70, 71]. The affinity between α-Galcer/CD1d and mouse iNKT TcR is very high and specific, which led to the generation of the first CD1d tetramer to identify mouse and human iNKT cells. Although α-GalCer is not considered to be a natural ligand, researchers have used it to search for glycosphingolipids in mammalian cells according to their structural similarity. Recent studies have identified a natural glycosphingolipid iGb3. Both plate bound CD1d/iGb3 and dendritic cell presented iGb3 leads to iNKT cell activation, demonstrating that iGb3 has physiological function [72-74]. In addition,
several studies suggested that iGb3 may work as a self antigen that is important for iNKT cell selection in the mice. However, recent studies challenge this point and showed that mice deficient in iGb3 synthase have normal iNKT cell development and function [75]. Thus the self glycolipid antigens that are responsible for iNKT cell selection are still not clear, although iGb3 is a candidate.

CD1d is indispensable in iNKT cell positive selection and CD1d null mice have a complete defect in iNKT cells. The importance of CD1d expression on DP thymocytes for iNKT cell development has been shown by several groups using different methods. The mixed chimera experiments with fetal liver cells that restrict CD1d expression and thymocyte development in certain stages and β2 microglobulin− fetal liver cells (β2M−, which do not express CD1d but capable of develop to major thymocyte population) with β2M− mice as host (which do not express CD1d and do not have any iNKT cells) showed that iNKT cells only developed from chimeric mice that have CD1d expression in the DP thymocytes (TcRα−), but not in the chimeric mice that lack CD1d expression in the DP thymocytes (TcRβ−) [76]. In addition, in pLck-CD1d/CD1d−/− transgenic mice with CD1d expression restricted mainly to DP thymocytes, iNKT cells developed normally and also maintained their effector function [77]. In the thymus, CD1d is not only expressed on thymocytes, but also on thymic CD11b+ macrophages, CD11c+ dendritic cells (DC), and epithelial cells, but the selection of iNKT cells are dependent mainly on DP thymocytes [77]. This raises the question why only CD1d expression on DP thymocytes but not on epithelial cells can help iNKT cell selection. The most probable explanation is that there are some other signals that could only be initiated by DP thymocytes that drive precursors to form the iNKT cell lineage.

Several studies demonstrated that iNKT cell development is completely blocked in either mice lacking the Src family tyrosine kinase Fyn or mice lacking the signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) [78-83]. Both of these two signaling
proteins are involved in SLAM family receptor initiated signaling pathway, raising the possibility that this signaling pathway and SLAM family receptors may be important for iNKT cell development. SLAM family of receptors are mainly homophilic self interaction proteins with several members (SLAM, Ly108, Ly9 and CD84) expressed on cortical thymocytes, which raises the hypothesis that the homotypic interaction between SLAM family members in DP thymocytes may activate SLAM-SAP-FYN signaling pathway, thus providing unique signals for positive selection of iNKT cells. Early studies showed that the development of iNKT cells was almost normal in mice lacking either SLAM, or Ly108, or Ly9, challenging the contribution of these signals in iNKT lineage selection [84-86]. However, a recent study provided important evidence that SLAM family receptors SLAM and Ly108 are important in iNKT cell selection [87]. By using mixed bone marrow chimeras designed to eliminate both SLAM and Ly108 signaling during CD1d ligand recognition in the thymus, the investigators found that in the absence of both SLAM and Ly108 signaling, iNKT development was strongly blocked, demonstrating that SLAM and Ly108 have essential but partly redundant roles through homophilic self interaction across the cell-cell synapse [87]. The finding that SLAM and Ly108 have functional redundancy is consistent with their shared recruitment of SAP and Fyn, and may explain the past failure to observe iNKT cell developmental defect in the absence of individual members of the SLAM family of receptors. Interestingly, SLAM and Ly108 are expressed only on thymocytes, but not on other thymic cell types, including epithelial cells and dendritic cells [87]. These data connect SLAM family receptor with SAP and Fyn and support the idea that the homotypic interactions between SLAM and Ly108 on the surface of DP-T cells during Vα14 TcR recognition of CD1d/ligand expressing cortical thymocytes lead to SAP-Fyn signaling and this second signal together with TcR signaling are required in iNKT lineage commitment.

After positive selection, the iNKT precursors(CD24^{high}CD44^{low}NK1.1^{-}) progress to the more mature CD24^{low} stage, followed by three developmental stages: CD44^{low}NK1.1^{-} iNKT
cells (stage 1) further develop through more mature CD44^{hi}NK1.1^- iNKT (stage 2) to finally mature CD44^{hi}NK1.1^+ iNKT cells (stage 3) in the thymus [88]. The NK1.1^- iNKT cells also appear to expand a lot, since they have a high turnover rate, which is very low in NK1.1^+ iNKT cells in the thymus [89]. A lot of CD44^{hi}NK1.1^- iNKT cells also migrate to periphery, where they upregulate the expression level of NK1.1 to final maturation, demonstrating that the maturation stage 3 could occur both in the thymus and in the periphery [89, 90]. During the development, around 30%-50% of iNKT cells downregulate their CD4 and become a double negative (DN, CD4^-CD8^-) population [91]. Since both immature and mature iNKT cells contain DN and CD4^+ population, their relationship in the development of iNKT cells is still not clear. When immature CD44^{hi}NK1.1^- iNKT cells progress to mature CD44^{hi}NK1.1^+ iNKT, it also associated with the upregulation of several cell surface receptors such as Ly49 family members, NKG2A, CD69 and CD122 (Fig. 1-3).

The different developmental stages and populations of iNKT cells also secrete different cytokines when activated through their TcR in-vitro. The CD44^{low}NK1.1^- iNKT only produce IL-4, while the CD44^{hi}NK1.1^- iNKT cells produce both IL-4 and IFN-γ and CD44^{hi}NK1.1^+ iNKT cells produce both IFNγ and IL-4 with a preference for IFNγ [89, 90].

1.2.2 Signaling proteins involved in iNKT cell development

A number of signaling molecules, transcription factors and cytokine receptors have been identified to be important for iNKT cell development through different stages. As we mentioned above, SLAM-SAP-FYN form signaling pathways that controls iNKT cell development at a very early stage [79-83, 87, 92, 93]. At least two signaling pathways initiate from this complex. The first one is to activate RAS GTPase-activating protein (RasGap) through SRC-homology-2-domain-containing inositol-5-phosphatase (SHIP1) and docking protein1/2 (Dok1/2), and finally
inhibit mitogen-activated protein kinase (MAPK) and the Ras signaling pathway, which may regulate signaling downstream of TcR and allow NKT cell positive selection [88]. The other one is to activate the NFκB pathway through protein kinase C0 (PKC0) [94]. The role of NFκB family members in thymic NKT cell development have been revealed by several studies. Mice lacking inhibitor of NF-κB kinase 2 (IKK2) and those expressing a dominant-negative inhibitor of NFκBα (IκBα) transgene had severe defects in NKT cell development, which was partially due to the lack of survival signal Bcl-2, suggesting a survival role for NFκB in NKT cell development [92, 95].

The transcription factor T-bet is a key factor in the final maturation of iNKT cells. T-bet null mice exhibited a very low iNKT cell number and a complete block of iNKT cell at CD44hiNK1.1- stage (stage 2) [96]. T-bet may play its role through activating IL-15 signaling pathway since T-bet induces the upregulation of IL-2/IL15 receptor β-chain (CD122). Mice lacking either IL-15 or CD122 have been shown to be deficient in NK1.1+ T cells, suggesting their role in the terminal maturation of iNKT cells [97, 98]. In addition, IL-15 is also important for the expansion and/or survival of iNKT cells [99] (Fig. 1-4).

Two recent studies have shown that Itk and Txk are also important for iNKT cell development and function. Itk null mice exhibited reduced iNKT cell number and the development of iNKT cells were blocked in CD44hiNK1.1- stage, which was exaggerated in Itk/Txk DKO mice [24, 62]. Analysis on the transcription factor level by Berg and colleagues suggest that the defect of iNKT development in mice lacking Itk and Txk may be due to downregulation of T-bet and CD122 in these cells [24]. In addition, these iNKT cells secrete dramatically decreased cytokine IL-4 and IFNγ, although the mRNA transcription of IL-4 and IFNγ is intact[62].
1.2.3 iNKT cell function

iNKT cells have diversified and distinct functions. Upon TcR activation, iNKT cells rapidly secrete large amount of both Th1 and Th2 cytokines, including IL-4 and IFNγ, as well as IL-2, IL-5, GMCSF, TNF, IL13 and IL-17 [100]. Injection of α-GalCer in-vivo could stimulate the production of both IL-4 and IFNγ by iNKT cells within 2 hours. This rapid production of cytokine may be partly due to the high levels of transcripts of IL-4 and IFNγ in the resting iNKT cells. Unlike conventional Th1 and Th2 cells, which only express one of the two transcription factors T-bet and GATA-3, respectively, iNKT cells express both, which contributes to their high mRNA level of both IL-4 and IFNγ. How this is regulated remains unclear.

The highly diversified cytokine secretion profiles by iNKT cells raise the possibility that iNKT cells may have functionally distinct subsets. Indeed, the CD4⁺ and CD4⁻ subset of iNKT cells in human have been reported to have different functions. CD4⁺ subset produce both Th1 and Th2 cytokines, whereas CD4⁻ subset prefers to only produce Th1 cytokine [67, 101]. An analysis of cytokine secretion by subsets of iNKT cells has been reported recently [102]. The authors purified three different subsets of mouse iNKT cells according to their surface expression of CD4 and NK1.1: CD4⁺NK1.1⁺, CD4⁺NK1.1⁻, CD4⁻NK1.1⁺ and CD4⁻NK1.1⁻; and analyzed their ability to secrete various cytokines upon CD3/CD28 stimulation during several time points. Mouse CD4⁺NK1.1⁺ and CD4⁻NK1.1⁺ iNKT cells secrete similar amounts of IL-4. Interestingly, the CD4⁻NK1.1⁻ subset is shown to be the main source for IL-17 production in iNKT cells [102].

Upon activation, iNKT cells have been implicated to influence a lot of other cell types in the immune system. When mice are injected with iNKT ligand such as α-GalCer, DCs present α-GalCer to iNKT cells, which activate iNKT cells to produce large amounts of cytokines. Activated iNKT cells and the cytokines they secreted rapidly activate NK cell, as well as enhance DC activation to prime adaptive CD4 and CD8 T cell immune responses [103-106]. Activated
iNKT cells can also provide help to B cells for antibody production directly and regulate the recruitment of neutrophils through their production of IFNγ [107, 108].

The rapid response of NKT cells may allow NKT cells to regulate adaptive immunity, such as providing protection against viral and bacterial infection, as well as regulate autoimmune diseases. iNKT cells have been found to be recognize and respond to the bacterial infections and participate in bacterial clearance [109-111]. They have also been suggested to be involved in immunity to a number of viruses including herpes simplex viruses (HSV) and hepatitis B[112, 113]. In addition, iNKT cells have been implicated in noninfectious diseases, such as tumor immunity, and certain autoimmune diseases, including diabetes, atherosclerosis, lupus and allergic asthma [100].

1.3 γδ T cell lineage development and function

T cells are divided into αβ T cells and γδ T cells according to their TCR expression. Compared to αβ T cells, γδ T cell is a minor population that is only around 5-10% of total T cells. Although the cell numbers are low, γδ T cells contain many distinct subsets, which reside in different tissues, including the secondary lymphoid organs and the epithelial layers of tissue such as the skin, intestinal epithelium and lung. The different subsets of γδ T cells express different specific γδTcRs. For example, skin γδ T cells uniquely express Vγ3/Vδ1 TcR which are called dendritic epidermal T cells (DETCs), whereas γδ T cells in the tongue, lung, and reproductive tract epithelium express Vγ4/Vδ1 TcR [114, 115]. γδ T cells in the secondary lymphoid organs predominantly express Vγ2, Vγ1.1 and Vγ1.2 with diverse Vδ chains [116-118].
1.3.1 \( \gamma\delta \) T cell development

\( \gamma\delta \) T cells develop in the thymus. The different subsets of \( \gamma\delta \) T cells generate in the thymus at different developmental stages. \( V\gamma 3^+ \gamma\delta \) T cells (DETCs) arise from fetal thymic precursor at around day 13, which are the first type of T cells detected in the fetal thymus. After development, these \( \gamma\delta \) T cells migrate to the skin from fetal thymus before the birth of mice [115]. \( V\gamma 4^+ \gamma\delta \) T cells are generated later than \( V\gamma 3^+ \gamma\delta \) T cells in the fetal thymus and migrate to epithelial layers of reproductive tract, lung, and tongue. The \( \gamma\delta \) T cells in the secondary lymphoid organs are only produced in the adult thymus [118-120]. Since the microenvironments in the fetal thymus and adult thymus are different, the production of distinct subsets of \( \gamma\delta \) T cells during different stages of ontogeny suggests that they have distinct developmental mechanisms. Indeed, the development of adult thymic \( \gamma\delta \) T cells requires DP T cells [121]. However, when DETCs arise and develop in the fetal thymus, DP T cells have not been detected yet, but DETC still develop normally, suggesting DP T cells are not required for the development of DETC. In this thesis, I will focus on the subset of \( \gamma\delta \) T cells that develop in adult thymus and localizes in secondary lymphoid organs after migration.

These \( \gamma\delta \) T cells arise from the most immature \( CD4^-CD8^- \) double negative (DN) thymocytes in the thymus [122]. DN thymocytes are at a very early stage of T cell development. They are further divided into four developmental stages according to the surface expression of CD25 (\( \alpha \) chain of the IL-2 receptor) and CD44, from most immature DN1 (CD44^-CD25^-) to DN2 (CD44^-CD25^-), then to DN3 (CD44^-CD25^-), and finally to more mature DN4 (CD44^-CD25^-) cells (Fig. 1-5). At DN1 stage, the genes encoding both chains of the TcR are in the germline configuration. At DN2 stage, TcR gene rearrangements are processing and have not finished yet. At DN3 stage, thymocytes finish gene rearrangements and express either TcR\( \beta \) chain paired with a surrogated \( \alpha \) chain called pre-T-cell-\( \alpha \) (pT\( \alpha \)) to assemble a pre-TCR or \( \gamma\delta \) TcR on the surface.
The cells that fail to express a functional TcR arrest in this stage and soon die. γδ T cells separate from αβ T cells at this stage, although the exact time point and the mechanisms involved in this process are still elusive (Fig. 1-5) [122, 123]. The pre-TCR then assembles with CD3 molecules in cell surface, leading to cell proliferation at DN4 stage and finally express both CD4 and CD8 to become DP thymocytes, followed by positive and negative selection to finally mature as SP T cells. Conventional αβ T cells proliferate extensively during development, which divide up to ten times after the formation of pre-TCR, whereas γδ T cells only divide one to three times in the thymus after diverging from αβ lineage. The difference of expansion rate between αβ T cells and γδ T cells at least partly contribute to big differences in cell numbers in thymus and secondary lymphoid organs.

The mechanism of γδ T lineage commitment is still elusive. The TcR gene rearrangements do not decide lineage commitment, since both mature αβ T cells and γδ T cells have complete rearrangement of TcR γ, δ, and β chain. However, whether γδ T lineage commitment is controlled by the expression of TcR is still not clear. Recently, a number of studies support the idea that the strength of the TcR signal is important for T cell lineage commitment. Stronger TcR signals favor the development of γδ T cells, while the weaker signals favor the development of αβ T cells [124-126]. The identification of γδ T precursors and signaling pathways that are required for γδ T lineage commitment in the future will solve this argument.

After T lineage commitment, the immature T cells need to go through several stages to mature. It is well known that DP αβ T cells undergo positive selection and negative selection to become mature SP T cells. Both MHC class I and MHC class II molecules expressed on epithelial cells are required for the positive selection. MHC I molecules are required for CD8 T cell development and MHC II molecules are required for CD4 T cell development.
About 90% of adult thymic γδ T cells do not express CD4 and CD8 receptors (CD4⁻CD8⁻, DN), the rest are CD4⁺CD8⁻. Studies on mice deficient in either MHC class I or MHC class II molecule showed no detected defect of the total γδ T cell development in the thymus [127, 128]. In addition, Mice lacking β2m, which lack the expression of both classical and non-classical MHC class I molecules, exhibited no obvious defect in γδ T cell development in the thymus [129]. All of these data demonstrate that MHC class I and MHC class II molecules may not be required for the positive selection of γδ T cells. Considering that the majority of γδ T cells are DN, this conclusion is reasonable, although we could not exclude the possibility that MHC class I and II molecules may be required for the selection of a subset of γδ T cells. In addition, since the natural ligands for γδ TcR are still unclear, it is still possible that γδ T cells undergo distinct positive selection.

The limited knowledge about natural ligands for γδ T cells makes it difficult to study the positive and negative selection of γδ T cells. In order to solve this problem, two γδ TcR transgenic mouse strains are established which are named as G8 and KN6. The transgenic γδ TcR in G8 recognize ligands T10 and T22, both of which are non-classical MHC I molecules expressed in H-2b. Since C57/BL6 mice are H-2b haplotype and BALB/c mice are H-2d haplotype, T10 and T22 are highly expressed in C57/BL6 mice but much lower in BALB/c mice, with no expression in β2M⁻/⁻ mice. The KN6 transgenic TcR only recognize T22 [132]. G8 and KN6 express different γδTcR, but they all contain a Vγ2 chain. The different expression levels of ligands in mice of different backgrounds lead to different signal strength of γδ TcR, thus allowing the study for the requirement of positive and negative selection in γδ T cells.

Studies on G8 and KN6 mice found that a high frequency and numbers of transgenic γδ T cells exist in the adult thymus of Balb/c background, which express very weak ligand for the transgenic γδ TcR [133, 134]. In contrast, transgenic mice in C57/BL6 background, which
express strong ligand and may lead to self-reactive γδ T cells, have much lower frequency and numbers of transgenic γδ T cells [133, 134]. These data demonstrate that the negative selection regulates the development of γδ T cells in adult thymus.

Whether positive selection is necessary for γδ T cells in the adult thymus is still controversial. Several studies found G8 mice in β2M−/− background, which do not express any T10 and T22, have much less transgenic γδ T cells in the adult thymus compared to those in BALB/c background, which express weak ligand, suggesting that interactions between transgenic γδ TcR and weak ligand in Balb/c mice is required for the positive selection [135, 136]. However, in another study using the same series of G8 mice, γδ T cells develop in a similar level in the β2M−/− background as in BALB/c background, thus challenging the finding of a requirement of positive selection [137]. In a recent study, the researchers used T22 tetramer to directly detect endogenous T10- and/or T22- specific γδ T cells in the non-transgenic mice, no defect in γδ T cell development in the adult thymus were found in β2M−/− mice, arguing that the positive selection is not required for γδ T cell development in the adult thymus. The finding of natural ligands for γδ T cells in the future will be greatly helpful to solve this discrepancy.

The proteins and signaling pathway that are important for γδ T cell development have been investigated. Some signaling pathways and corresponding genes that are required for the development of γδ T cell are also required for the development of αβ T cells, such as genes involved in Notch signaling pathway, which play decisive roles in mouse T cell vs. B cell commitment and TcR gene rearrangement [138-140].

Several signaling molecules that are important for TcR initiated signaling pathways have also been found to be required for the development of γδ T cells. As we mentioned earlier, LAT is an adaptor protein that is essential to form the signaling complex that are required in the proximal TcR signaling events. LAT null mice show severe defect in both αβ T cell development, which block the development of T cells at DN stage [141]. Interestingly, mice containing a LAT
mutation (Y136F), which selectively destroys the binding and activation of PLCγ1 in the signaling complex, exhibit an early partial block in αβ T cell development with later Th2 lymphoproliferative disorder but have no effect on γδ T cells [142, 143]. In contrast, mice containing another LAT mutation (Y175/195/235F), which mostly fail to recruit SLP-76 and more severely affect TcR signaling pathways than Y136F, show a complete block in αβ T cell development but affect the development of γδ T cells with an accumulation of γδ T cells in the periphery lymphoid organs [144]. In addition, Lck and SLP-76 are required for γδ T cell development in the adult thymus. Several other important signaling molecules, such as ZAP-70, Syk and Vav1, have been reported to be required for αβ but not γδ T cell development [145-148]. However, γδ T cell development is defective in mice lacking both Syk and ZAP-70, suggesting the functional redundancy of these two kinases in γδ T cell signaling. These data suggest that αβ and γδ T cells have different requirements in their development, may be through different signaling pathways and/or different gene expression patterns. Future studies on the role of signaling molecules in the development of γδ T cells are needed.

1.3.2 γδ T cell function

Like αβ T cells, γδ T cells can be differentiated into Th1-like or Th2-like cells and secrete either IFNγ or IL-4 in-vitro, respectively, and the IL-4 secreting γδ T cells are associated with the expression of CD4 [149]. In addition, γδ T cells have been reported to produce either IL-4 or IFN-γ in response to the Th1-or Th2- stimulating pathogens in-vivo [150]. γδ T cells are also able to secrete IL-17 [151]. A recent study demonstrated that γδ T cells secrete either IL-17 or IFNγ dependent on whether they interact with antigen in the thymus. The investigators used the T10 and T-22 specific γδ T cells as a model and defined γδ T cells as antigen naïve (CD122low) or
antigen-experienced cells (CD122\textsuperscript{hi}). The antigen naïve lymphoid γδ T cells preferentially to secrete IL-17, whereas the antigen experienced γδ T cells preferentially to secrete IFNγ [152].

A lot of data suggest that γδ T cells play a role in the humoral immune responses. Mice lacking αβ T cells showed normal B cell phenotypes, germinal center formation and production of antibodies, particularly IgG1 and IgE, which are suggested to be due to the IL-4 production by γδ T cells [153, 154]. Human γδ T cells could also induce class switching in B cells to produce IgE [155]. In an allergic asthma model, mice lacking γδ T cells had decreased production of IgE and, which were rescued by adding IL-4, suggesting that γδ T cells are important for IL-4 production and help the production of IgE and IgG1 [156]. The LAT mutant (Y175/195/235F) mice, which have no αβ T cells but accumulate high numbers of γδ T cells in peripheral lymphoid organs, exhibit high serum IgE and IgG1 level, suggesting that γδ T cells induces B cell responses [144].

γδ T cells have also been indicated to have functions in host defense and tumor immunology. Mice and Human γδ T cells have been reported to be involved in the defense to parasite infection through early secretion of IFN-γ [157, 158]. γδ T cells are also implicated in protection against virus through the production of IFNγ [159]. Using different bacteria models, γδ T cells are considered to be an important source of IL-17 against bacterial infection [151, 160, 161].

In conclusion, Itk is expressed in T cells and NKT cells, and has been shown to be important in T cell signaling, development and function. The regulatory mechanism of Itk and the role of Itk in NKT and γδ T cell development are unclear.
Figure 1-1. TcR-mediated signaling pathways and the role of Tec kinases in these pathways.

TCR leads to rapid activation of Lck and Zap-70, which phosphorylate multiple downstream signaling molecules and form signaling complex, which phosphorylate PLC-γ and activate subsequent multiple signaling pathways, as well as help actin reorganization and cell adhesion.

Adapted from Science STKE 396: 39-42 (2007)
Figure 1-2. The domain of ITK and models of the conformation of ITK.  

a) Domain organization of Itk. b) The folded conformation model of ITK regulated by intramolecular SH3-PRR interaction. c) The homodimer head-to-tail conformation model of ITK regulated by intermolecular SH2-SH3 interaction

*Adapted from Science STKE 396; 39-42 (2007)*
Figure 1-3. iNKT-cell development. iNKT cells arise in the thymus. iNKT-cell precursors undergo selection and development through a series of developmental stages that ultimately become mature.

Adapted from Nature Review Immunology 7(7); 505-18 (2007)
Figure 1-4. Signaling pathways that regulate iNKT-cell development. The three main signaling pathways that affect iNKT cell development, which are SLAM–SAP–FYN pathway, the TCR-signaling pathway and IL-15 pathway.

*Adapted from Nature Review Immunology 7(7); 505-18 (2007)*
**Figure 1-5. DN thymocyte development.** DN thymocytes develop from the most immature DN1 cells to more mature DN4 cell. γδ T cells separate from αβ T cells during DN thymocytes, but the exact time point is not clear.
Chapter 2

Material and Methods

2.1 Plasmids

Antibodies against Itk were a gift of Dr. Gordon Mills (MD Anderson Cancer Center) [162], or from Cell Signaling. Anti-GFP antibody was from Roche Biosciences. The pCDNA3.1-YFP1-zipper, pCDNA3.1-YFP2-zipper were a kind gift from Dr. Stephen Michnick (University of Montreal, Montreal, Canada)[163]. The lentivirus vector FUGW and packaging plasmids pΔ8-9, pVSVG were a kind gift from Dr. David Baltimore (California Institute of Technology, Pasadena, CA)[164]. The pCDNA3.1-YFP1-Itk was generated by deleting the zipper sequence of pCDNA3.1-YFP1-zipper and replacing it with the human Itk cDNA generated by PCR and sequenced to confirm the integrity. pCDNA3.1-YFP2-Itk plasmid was generated by deleting the YFP1 sequence of pCDNA3.1-YFP1-Itk and replacing with YFP2 fragment. pCDNA3.1-YFP1-Itk-YFP2 plasmid was generated by deleting the zipper sequence of pCDNA3.1-zipper-YFP2 and replacing with YFP1-Itk fragment. The pG-ICOS-YFP1 and pMex-CD8α-YFP1 were generated by subcloning linker-YFP1 to the C-terminus of pG-ICOS or pMex-CD8 fragment lacking the cytoplasmic tail [165], respectively.

Point mutation R29C was introduced to pCDNA3.1-YFP1-Itk, Itk-YFP2, YFP2-Itk, YFP1-Itk-YFP2 plasmids by using QuikChange II site-directed mutagenesis kit (Stratagene). Point mutations P287G, W208K, P158AP159A, and C132GC133G were introduced to pCDNA3.1-YFP1-ItkR29C-YFP2 plasmid using the same method. The FUGW-YFP1-Itk, FUGW-YFP2-Itk and FUGW-YFP1-ItkR29C-YFP2 plasmid were generated by substituting the GFP fragment for YFP1-Itk, YFP2-Itk or YFP1-ItkR29C-YFP2 in the FUGW vector, respectively. The pGFP-Itk was generated by inserting GFP fragment to the N-terminus of HA-Itk [166]. The
caspase reporter SCAT used as a positive control for the FRET experiments was a kind gift of Dr. Masayuki Miura (University of Tokyo, Japan) [167]. The ECFP and Venus variant of EYFP fluorescent proteins used in the SCAT construct were ligated to the N-terminus of R29C Itk in the plasmid pG-R29C Itk.

2.2 Cell Transfection

HEK 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum. The plasmids were transfected into HEK-293T cells by using TransIT-293T (Mirus). Jurkat E6-1 T cells were cultured in RPMI supplemented with 5% fetal bovine serum and were transfected by electroporation or transduced using lentiviral infection. Human PBL T cells were expanded as previously described, then infected with lentiviral vectors carrying the indicated Itk cDNAs [164]. The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (CypA-/- Jurkat T cells) from (Jeremy Luban).

2.3 Flow cytometric assay

For bifluorescence complementation (BiFC) assay, 24 hours after transfection, the cells were incubated at 30 °C overnight to promote fluorophore maturation. The cells were cultured, washed twice with PBS, resuspended in PBS containing 2% FBS (staining buffer) followed by flow cytometric analysis. YFP Fluorescence was corrected for expression by analysis of Itk expression either by western blot, or in most experiments, by analysis of YFP expression using an antibody against GFP and intracellular staining.

For intracellular staining to analyze YFP expression level, the same transfected cells were resuspended in 500 µL of 4% paraformaldehyde in PBS and incubate at 4°C for 30min. The fixed
cells were then washed once by staining buffer, resuspended in 500 µl of 0.1% saponin in staining buffer (permeabilization buffer) and incubated for 15 min at room temperature (RT). After permeabilization, cells were spun down and resuspended in anti-GFP antibody (Roche) in 100 µl permeabilization buffer (1:250 dilution) and incubated at RT for 30 mins. After staining, cells were washed twice using permeabilization buffer and resuspended in PE anti-mouse IgG in 100 µl permeabilization buffer (1:500 dilution). After 30 min of incubation at RT, cells were washed twice using permeabilization buffer and resuspended in 500 µl of staining buffer to flow analysis. The values of PE fluorescence intensity were obtained for each sample and were compared to untransfected control. YFP fluorescence was then corrected for expression using the corresponding PE fluorescence. These values were then compared to control cells transfected with YFP1-Itk alone, which was set at 1.

For animal experiments, lymphocytes were incubated with Fc block (BD bioscience) containing staining buffer (1:200 dilution) for 10 minutes on ice, and then were incubated with fluorescent antibodies for 30 min on ice. PE-PBS57/CD1d tetramer and unloaded/CD1d tetramer were provided by the National Institute of Health tetramer core facility. anti-PECy5-TCRβ, PECy5-CD44, FITC-γδTCR, PECy5-CD4, FITC-TCR Vγ2, and PECy7-CD4 were from BD Bioscience; anti-PECy7-NK1.1, PE-γδTCR, APC-PECy7-CD62L, PE-CD24, PE-CD3, PECy7-CD8, biotin-CD122, PECy5-strepavidin, APC-alexa750-TCRβ were purchased from eBiosciences.; Anti-CD4-ECD and CD8-ECD were purchased from CalTag Lab; Anti-PECy7-CD3 was purchased from BioLegend.

2.4 Confocal microscopy and FRET

HEK 293T cells were grown on glass slides in 6-well plate. 24 hours after transfection, the cells were incubated at 30°C overnight and the fluorescence image analyzed by confocal
microscopy. For immunofluorescence microscopy, cells were fixed using 4% paraformaldehyde for 20-30 min at room temperature. The fixed cells were then incubated with anti-Itk antibody in blocking buffer (2% FBS and 0.1% Triton in PBS) for 1 hour. After washing three times with PBS, cells were then stained with Alexa 663-conjugated secondary antibody for 30 min in dark. The unbound antibody was removed by washing three times with PBS. The cells were then incubated in PBS and analyzed by confocal microscopy. Since each cell is of a different size, and may express varying levels of protein, an algorithm was developed to compare the localization of Itk molecules in cells of different sizes and varying levels of expression using the program CCDi Image (Stellar Image Software) as follows: 1) a line was drawn across the cells excluding the nucleus and the fluorescence intensity collected for each cell and map data points were collected for each cell; 2) points along the line were set as a percentage of the cell from one end of the line to the other (0-100%); 3) the fluorescence at each point was calculated as a percentage of the maximum fluorescence observed for that cell; 4) these values were then plotted for each cell as % maximum fluorescence (y-axis) vs. % cell (x-axis) for each cell. In general, the cell membrane represented that 15% of the cell at the edges (0-15% and 85-100%).

For FRET analysis, HEK 293T cells were grown on glass slides in 6-well plate. 24 hr after transfection, cells were analyzed in confocal microscope (Olympus, Flowview 1000) using YFP, CFP and FRET channel. The data were analyzed using PFRET software (Olympus).

2.5 Mice

Wild-type (WT), Itk null, Itk/Txk DKO, KN6Tg, β2m−/− and MHCII−/−and Tg(Lck-ItkΔKin)/Itk−/− mice were on C57BL/6 background and kept in pathogen-free conditions. Tg(Lck-ItkΔKin)/Itk−/− mice were generated as described [58]. β2m/Itk DKO, MHCII/Itk DKO, TcRδ/Itk DKO and KN6Tg/Itk−/− mice were generated by breeding Itk−/− mice with β2m−/−, MHCII−/−, TcRδ−/−
and KN6TgItk−/− mice, respectively. All the mice used were 6-10 wk of age and kept in pathogen-free conditions. Experiments were approved by the IACUC at the Pennsylvania State University.

2.6 Cell sorting

To purify CD4+ and CD4− γδ T cells, splenocytes were first incubated with biotin-B220 and biotin-TCRβ followed by negatively purification using streptavidin-magnetic beads (Miltenyi Biotech). Remaining cells were further stained with anti-FITC-γδTCR, PE-CD3 and PECy5-CD4 and sorted using Cytopeia Influx Cell Sorter.

For B cell purification, splenocytes from each WT mice were resuspended in 100 µl staining buffer containing a master mix of biotinylated antibodies. The mix biotinylated antibodies include: anti-CD11b, DX5, Gr1, CD8, CD4, CD3, Ter119, CD25. After incubation on ice for 20 mins, the cells were washed twice by staining buffer and resuspended in staining buffer containing strepavidin magnetic beads, followed by negative purification using magnetic columns. The purity of B cells is above 95%.

For other cell purification, cells were stained with corresponding fluorescent antibodies and sorted using Cytopeia Influx Cell Sorter.

2.7 Real-time PCR analysis.

RNA samples from purified cells were extracted using RNase Mini kit (Qiagen). cDNA was generated with You Prime First-Strand beads kit (GE Health) and quantitative real-time PCR was performed using primer/probe sets for target genes with GAPDH as a housekeeping gene (Applied Biosystems). All of the primer/probe sets are Taqman genes from Applied Biosystem, which are T-bet, GATA-3, IL-4, eomesodermin and IFNγ. Data were analyzed using the
comparative threshold cycle ΔΔCT method, normalized to the expression of GAPDH and the values were expressed as fold increase compared to WT cells, which was set as 1.

**2.8 In vivo BrdU incorporation assay**

WT, Itk null and Tg(Lck-ItkΔKin)/Itk−/− mice were injected intraperitoneally with 1 μg BrdU and placed on drinking water containing 0.8 mg/ml BrdU for the next 6 days. BrdU-containing drinking water was changed every two days. 6-days after injection, mice were sacrificed and thymocytes were isolated and stained with PE-PBS57/CD1d tetramer, followed by fixation and permeabilization, BrdU was detected using APC-BrdU kit purchased from BD biosciences.

**2.9 Bone marrow chimeras**

Bone marrow cells were isolated from femurs and tibia of the donor mice. 1x10⁷ cells were resuspended in 100 μl PBS and intravenously transferred to lethally irradiated recipient mice. For mixed chimera transfer, 5x10⁶ cells from each donor mice (1:1 mixture) were mixed in 100 μl PBS and intravenously injected to irradiated recipient mice. Mice were analyzed 8 wk after transfer.

**Cytokine secretion assays**

Purified CD4⁺ and CD4⁻ γδ splenocytes cells were stimulated in-vitro at 2x10⁴ cells/well in duplicate with 10 μg/ml plate-coated anti-γδ TcR antibody (BD bioscience) for 72 h.
Supernatants were then harvested and assayed for the presence of IL-4, IL-17 and IFNγ by Bioplex (Bio-Rad).

### 2.10 Cell transfers and serum analysis

1x10^6 purified T cells and B cells were mixed in 200µl PBS and injected intraperitoneally to Rag^-/- mice and blood was collected 4 wk after transfer. Blood samples from indicated mice were collected and supernatants were obtained after spinning for 10 min at 4000 rpm. IgE level was analyzed by ELISA using IgE ELISA kit (BD bioscience).

### 2.11 Data Analysis

Data was analyzed using Microsoft Excel and Prizm, with evaluation using the Student’s \( t \) test (for two groups) or one way ANOVA (for three or more groups) and a probability value of \( p<0.05 \) considered statistically significant.
Chapter 3

Itk forms membrane clusters specifically in the vicinity of recruiting receptors

Chapter adapted from the manuscript entitled: “Tec kinase Itk forms membrane clusters specifically in the vicinity of recruiting receptors”

Authors: Qian Qi, Nisebita Sahu and Avery August


3.1 Introduction

The Tec family of kinases, including Itk, is involved in transducing signals from a number of receptors, including the TcR, BcR, FcεR, c-kit, CD28, CD2, CD32 and the EPOR [10, 26-30]. A common feature of these kinases is that they require that activation of PI3 kinase, as well as Src kinase activation for their activity [168-171]. These kinases, including Itk, have a PH domain that allows them to be recruited to the plasma membrane by an activated PI3 kinase [10]. Thus membrane recruitment is a critical component of their activation, and Itk can be found at the plasma membrane of cells, although other events are required for its full activity [162, 171, 172]. It is however, not clear if this is general membrane localization of the protein in anticipation of activation, or a specific localization in certain regions of the plasma membrane.

The structure of Itk in cells is not known, although it has been suggested to form dimers in its inactive state, with a resultant monomerization upon activation [22, 23]. We have examined these issues using a split YFP system [163], and find that Itk forms dimers or higher order
clusters, however, it does so only at the plasma membrane, and only in the vicinity of a receptor that can recruit PI3 kinase. Our data shed new light on the regulation, structure and localization of this family of kinases in cells.
3.2 Results

3.2.1 Itk forms dimers specifically at plasma membrane of cells

Itk is recruited to cell membrane after receptor stimulation [27, 28, 173-175]. However, whether the localization of Itk results in specific clustering or a more dispersed distribution is not clear. In order to answer this question, we utilized the split YFP system to probe the interaction between molecules of Itk [163]. Either YFP1 or YFP2 was fused to the N-terminus of Itk using a linker peptide of 10 amino acids, thus constructing the protein designated as YFP1-Itk or YFP2-Itk respectively (Fig. 3-1a). Since the average distance of each amino acid is approximately 4 angstroms, the fluorescence can be detected when Itk molecules fused to the two fragments of YFP are within an average distance of 80 angstroms. These constructs were expressed in 293T cells and fluorescence analyzed by flow cytometry and confocal microscopy.

The expression of either YFP1-Itk or YFP2-Itk alone in cells did not show any detectable fluorescence compared to non-transfected cells (data not shown), suggesting that expression of individual YFP fragment does not exhibit fluorescence, similar to what has been previously determined for other fusions (Fig. 3-1b) [163]. However, when YFP1-Itk and YFP2-Itk were co-expressed, strong fluorescence was detected (Fig 1b). Since the YFP fluorescence can be detected when Itk molecules are in close proximity (<80 angstroms), this data indicates that Itk proteins formed specific dimers or higher order clusters in living cells that are within this range. In order to determine the subcellular sites of Itk clustering, we analyzed the localization of YFP fluorescence in these cells using confocal microscopy. The YFP fluorescence was found predominantly at the cell membrane. In comparison, analysis of cells with an anti-Itk antibody revealed that Itk was distributed in both cytoplasm and cell membrane (Fig. 3-1c, control non-transfected 293T cells are negative for Itk staining by western and immunofluorescence, data not
shown, see also Fig. 3-2b). In addition, GFP tagged Itk also showed the same pattern of subcellular localization as that seen with the anti-Itk antibody (Fig. 3-1c). Quantification of the localization of these proteins dramatically illustrated this point, that the YFP1 and YFP2 Itk only interacts at the membrane and thus fluorescence is only detected at that location, while GFP Itk is found in both the cytoplasm and the membrane (Fig. 3-1d). Together, these results suggested that although localized both in the cytoplasm and at the cell membrane, Itk only forms dimers or higher order clusters as detected by complementation of YFP1 and YFP2 and subsequent fluorescence emission at the cell membrane.

3.2.2 The PH domain is required for the formation of dimers of Itk at the plasma membrane

To further test the possibility that interaction between Itk molecules in the cytoplasm could occur, we generated an Itk mutant, R29C, which disrupts the ability of the PH domain to interact with lipids, rendering this mutant unable to be recruited to the membrane [176, 177]. Thus this mutant is totally constrained to the cytoplasm. The expression level of this mutant fused to YFP1 and YFP2 was determined by western blot (data not shown), and although expressed at similar level as wild-type Itk, the R29C PH mutants did not show any detectable fluorescence compared with untransfected 293T cells (Fig 3-2a, b). Specific anti-Itk antibody analysis of Itk, as well as YFP fusion to this Itk mutant confirmed that it is localized only in the cytoplasm (Fig 3-1c, d; Fig. 3-2b). More importantly, even when wild-type YFP1-Itk was coexpressed with the mutant YFP2-R29CItk, no interaction was detected as measured by YFP fluorescence and confocal microscopy (Fig 3-2b). Thus, we conclude that the dimers and perhaps higher order clusters of Itk form only at the cell membrane, and thus YFP fluorescence of YFP1-
Itk plus YFP2-Itk can only be detected at the cell membrane, but not in the cytoplasm. This experiment also indicates that the interaction between Itk molecules at the membrane is specific.

3.2.3 Itk interacts specifically with receptors capable of recruiting PI3 kinase at the plasma membrane

In order to investigate the specificity of Itk dimerization/clustering at the cell membrane, we determined whether Itk could interact with a transmembrane protein that does not normally interact with Itk. We deleted the cytoplasmic tail of the human CD8α receptor and fused it to YFP1 at the C-terminus. CD8α is randomly distributed in the cell membrane and if Itk was also randomly distributed, then the two proteins should be able to interact. Flow cytometric analysis demonstrated that co-expression of CD8α-YFP1 and YFP2-Itk did not result in the emission of any YFP fluorescence compared with non-transfected 293T cells (Fig 3-3a), suggesting that there was no interaction between these two proteins at the plasma membrane. The expression of the CD8α-YFP1 was confirmed by flow cytometry (Fig 3-3a, Itk expression was confirmed by western blot as well as flow cytometry, data not shown). By contrast, when we analyzed whether Itk could interact with ICOS, a receptor which has a PI3 kinase binding motif and recruits PI3 kinase to the plasma membrane [86, 178] using a similar approach, we found that Itk could interact with this receptor (Fig. 3-3b). Confocal analysis indicates that this interaction occurs at the cell membrane (Fig. 3-3b). This interaction was dependent on recruitment of Itk to the membrane by PI3 kinase as a R29C PH mutant of Itk could not interact with ICOS (Fig. 3-3c). Together, these data indicate that Itk forms specific dimers/clusters at specific regions of the cell membrane, around the vicinity of receptors capable of activating PI3 kinase.

Since Itk is predominantly expressed in T cells, we analyzed the ability to form dimers/clusters at the plasma membrane in Jurkat T cells. Expression of YFP1-Itk and YFP2-
ITK in Jurkat T cells demonstrated that Itk forms similar dimers/clusters in these cells at the cell membrane (Fig. 3-4a). In addition, similar to what we found in 293T cells with CD8α and ICOS, Itk only interacted with ICOS, but not with CD8α in Jurkat T cells (Fig. 3-4b, c). All together, our data suggest that Itk is recruited to specific regions of the plasma membrane, where it can form dimers/clusters, and this occurs in the vicinity of receptors that can recruit and activate PI3-kinase.
3.3 Discussion

We have shown that Itk is recruited to the plasma membrane in a PI3-kinase and PH domain dependent manner, however, whether this recruitment results in clustering/dimerization of Itk was not known.

Our data demonstrates that at least dimers of Itk form specifically at the plasma membrane of cells. In addition, receptors capable of recruiting this kinase specifically interact with Itk, although we note, less efficiently than the Itk dimers. This could be that all the receptors are not capable of interacting with Itk at the exactly same time, thus their binding efficiency to Itk is reduced. While we cannot distinguish between the formation of dimers or higher order clusters, the data suggest that at least dimers are formed. In addition, our data also indicate that these dimers are at least 80 angstroms apart, and that the interaction between Itk and ICOS is also at least the same distance.

Our data also suggest Itk localize at specific region at cell membrane close to receptors that can recruit and activate PI-3K. Another study on the enzyme ItpKB found that ItpKB contributes to the activation of Itk by generating soluble IP4 that interact with the PH domain of Itk. This data is complementary to our conclusion and further confirmed that Itk localize in the cell membrane specifically when activated [179].
Figure 3-1: Itk forms dimers specifically at plasma membrane of cells. a) Model of split YFP fusions to Itk. b) 293T cells were transfected with Itk fused to YFP1 along with Itk fused to YFP2, both at the N-terminus. Cells were then analyzed by flow cytometry for YFP fluorescence (right). Fluorescence in several such experiments was quantified, with control cells transfected with YFP1-Itk alone set at 1 (left). c) 293T cells were transfected with Itk fused to YFP1 along with Itk fused to YFP2, both at the N-terminus (top left panel). Cells transfected with Itk fused to GFP (top middle panel) or R29C Itk fused to GFP (top right panel). Cells were analyzed by
confocal microscopy. Bottom panel: Cells were transfected with Itk fused to YFP1 along with Itk fused to YFP2, both at the N-terminus. Analyzed for YFP fluorescence (left), Fixed and stained with anti-Itk antibody and analyzed by confocal microscopy (right). d) Cells transfected as in (b & c) were analyzed as described in materials and methods. Note localization of dimerized Itk only at the cell membrane, while Itk can be found throughout the cell.
Figure 3-2. The PH domain is required for the formation of dimers of Ick at the plasma membrane. a) 293T cells were transfected with R29CItk fused to YFP1 along with R29CItk fused to YFP2. Cells were then analyzed by flow cytometry for YFK fluorescence (right) or quantified as in fig. 1 (left). Bottom, fluorescence of cells transfected with YFP1 fused to Itk along with R29CItk fused to YFP2. b) (i) Cells transfected with R29CItk fused to YFP1 along with R29CItk fused to YFP2. Top panel: YFP fluorescence. Bottom panel: Cells stained with anti-Itk antibody. (ii) Cells transfected with Itk fused to YFP1 along with R29CItk fused to YFP2. Top panel: YFP fluorescence. Bottom panel: Cells stained with anti-Itk antibody. Cells were analyzed by confocal microscopy.
Figure 3-3. Itk interacts specifically with receptors capable of recruiting PI3 kinase at the plasma membrane. a) 293T cells were transfected with cytoplasmic tail deleted CD8 fused to YFP1 at its C-terminus, along with Itk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of CD8 using a specific antibody (right panel). b) Cells were transfected with full length ICOS fused to YFP1 at its C-terminus, along with Itk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of ICOS using a specific antibody (right panel). C) Cells were transfected with full length ICOS
fused to YFP1 at its C-terminus, along with R29CItk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of ICOS using a specific antibody (right panel).
Figure 3-4. Formation of dimers of Itk in T cells. a) Jurkat T cells were infected with lentiviruses carrying YFP1-Itk and YFP2-Itk. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel) or by confocal microscopy (right). b) Cells were transfected with full length ICOS fused to YFP1 at its C-terminus, along with Itk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of ICOS using a specific antibody (right panel). c) Cells were transfected with cytoplasmic tail deleted CD8 fused to YFP1 at its C-terminus, along with Itk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of CD8 using a specific antibody (right panel).
Chapter 4

On the structure of Itk: Evidence for a folded monomer in vivo

4.1 Introduction

Itk is structurally organized into five domains, an amino-terminal pleckstrin homology (PH) domain, followed by a Tec-homology (TH) domain which contains a Zn$^{2+}$-binding Btk homology (BH) motif and one proline-rich region (PRR), Src homology 3 (SH3) and SH2 domains, and a carboxyl-terminal kinase domain. During stimulation of the TcR, phosphatidylinositol-3-kinase (PI3K) is activated, resulting in the formation of cell membrane phosphoinositides, to which the PH domain of Itk binds. Itk also forms dimers specifically at the plasma membrane in the vicinity of receptors that activated PI3K [180]. Once Itk is recruited to the membrane, it is phosphorylated by Src family kinases [162, 168]. Upon activation, Itk is enriched in membrane rafts and interact with other signaling proteins through its SH2, SH3 and TH domain. Subsequently, Itk activates several downstream signaling components, including phospholipase C$\gamma$1 and regulated the Ca$^{2+}$ signaling pathway [181].

Although the structure of each of the individual domains of Itk is known, that of the full length protein is unknown. A number of studies have suggested that the conformation of protein tyrosine kinases is controlled by the self-interaction of domains, thus retaining them in the inactive state [17, 18]. Src family tyrosine kinases, which have similar overall structures as Tec kinases with the exception of the TH and PH domains, are folded via intramolecular interactions between carboxyl-terminal negative regulatory phosphotyrosine and the SH2 domain that keep the kinase in the inactive state prior to receptor stimulation [17]. Although Itk lacks the conserved
carboxyl-terminal negative regulatory tyrosine phosphorylation site, Itk may also be regulated by intramolecular and/or intermolecular interactions among their domains. Indeed, two types of inter- and intra-domain interactions in Itk have been suggested. An intramolecular interaction between Itk SH3 and PRR domains has been suggested, which may act to maintain Itk in a folded state and thus prevent the binding of each domain to its respective ligands [21]. A second type of intermolecular interaction has been suggested where the Itk SH2 domain interacts with the SH3 domain of a second Itk molecule, thus dimerizing Itk in a head-to-tail configuration [22]. This model also suggests that a proline-dependent conformational switch exists in the SH2 domain of Itk, which directs a cis or trans conformer of the SH2 domain. The cis-conformer favors the dimerization of Itk via reciprocal SH2-SH3 and may inhibit Itk kinase activity, while the trans-conformer favors the binding of phosphotyrosine containing ligands such as phosphorylated residues of SLP-76 [22, 23]. These interpretations were arrived from the results of NMR studies of isolated SH3 and SH2 domains, however since the crystal structure of full length Itk is not available, the exact conformation of Itk in the inactive state is still not clear.

We have examined the existence of Itk superstructures using fluorescence complementation assays [163, 182]. Our data do not support an intermolecular dimerized head to tail conformation in the inactivated state. Instead, our data suggest a intramolecular folded conformation for Itk in the inactive state, where the PH domain is within 80 Å from the C-terminus. Furthermore, our data indicate that the TH domain of Itk is critical for maintaining the intramolecular fold. We therefore propose that in cells, Itk exists as a monomer, folded intramolecularly in a similar fashion to the Src and Abl non-receptor tyrosine kinases [183, 184].
4.2 Results

4.2.1 Itk exits as a intramolecular folded monomer in the inactive state in-vivo

We utilized fluorescence complementation assays using the split YFP system to examine the conformation of Itk in-vivo [163]. The N-terminal YFP1 fragment was fused to the N-terminus of Itk and the C-terminal YFP2 fragment was fused to the C-terminus of the same Itk molecule, thus constructing YFP1-Itk-YFP2 (Fig. 4-1A). Itk tagged with full length GFP at the N- or C- termini have been demonstrated to maintain function and behavior [174, 177]. However, we verified that this modified YFP1-Itk-YFP2 was able to rescue antigen receptor mediated activation of the SRF transcription factor in DT40 cells lacking Tec kinases (BTK−/− DT40 cells) as we have previously demonstrated for Itk [50, 185] (Fig. 4-1B).

Both structural models proposed for Itk (intramolecularly folded monomer and head to tail dimer) support the view that the N-terminus and C-terminus of Itk should be in close proximity (see Fig. 4-1A). Using the YFP fusions described in Figure 4-1A, this should result in complementation of the YFP fragments and the generation of fluorescence. As expected, when YFP1-Itk-YFP2 was expressed in cells, strong YFP fluorescence was detected by flow cytometry (see examples in Fig 4-2, quantified in Fig. 4-1C). The fluorescence of YFP1-Itk-YFP2 was located at both the cell membrane and cytoplasm, which was similar to the distribution pattern of Itk when analyzed by anti-Itk antibodies (Fig. 4-1C). Furthermore, expression of this protein in the Jurkat T cell line, as well as human peripheral blood T cells, also resulted in fluorescence complementation (Fig. 4-1D). These data suggest that the N-terminus and C-terminus are in close proximity (within 80 Å apart based on the length of the linkers used [180]) in the cytoplasm of these cells, suggesting that one or both models of Itk exist in-vivo (see Fig. 4-3A for model).
Since the YFP molecule will be complemented (and thus be fluorescent) regardless of whether Itk molecules fold intramolecularly as a monomer or intermolecularly as a dimer (Fig. 4-3A), we next determined which of these two models were more likely by analyzing Itk fused only to YFP1 at the N-terminus (YFP1-Itk) expressed along with YFP2 fused to the C-terminus (Itk-YFP2). If the intermolecular dimer model is more likely, then co-expression of YFP1-Itk and Itk-YFP2 should result in fluorescence complementation (see Fig. 4-3A). However, if the intramolecular monomer is more likely, then this combination may result in little fluorescence complementation (Fig. 4-3A). Analysis of cells expressing Itk tagged at either end by flow cytometry also revealed similar complementation of the YFP (Fig. 4-1E). However, the reconstituted YFP fluorescence was only found at the cell membrane although Itk was localized at the cell membrane and in the cytoplasm, suggesting that Itk only forms dimers or higher order clusters at the cell membrane (Fig. 4-1F, Fig. 4-3A, [180]). Similar results were found in Jurkat T cells (Fig. 4-1F). Since Itk is only activated after it has been recruited to the cell membrane and the clusters of Itk also localize in the cell membrane, these data suggest that these clusters of Itk are active [162, 168]. In contrast, our data do not support the homodimer model and suggest that when Itk is in the inactive state in the cytoplasm, it is more likely to exist in an intramolecularly folded conformation.

To test this interpretation, we generated an Itk mutant, R29C, which disrupts the binding of the PH domain to lipids at the cell membrane, thus keeping Itk in the cytoplasm and in the inactive state (see Fig. 4-3B [180]). Analysis of cells expressing N-terminally tagged YFP1-Itk\textsuperscript{R29C} and C-terminally tagged Itk\textsuperscript{R29C}-YFP2 indicated that Itk may not form head-to-tail homodimers when it cannot be recruited to the plasma membrane and is entirely cytoplasmic (Fig. 4-4A, Fig. 4-3C). Similar experiments performed where WT Itk fused to YFP1 was co-expressed with the Itk\textsuperscript{R29C}-YFP2 mutant revealed that co-expression of the WT Itk could not
induce the formation of dimer at any location in the cell (membrane or cytoplasm) suggesting that Itk is unlikely to form dimers prior to being recruited to the plasma membrane (Fig. 4-4D).

In contrast, R29C Itk tagged at both ends with the split YFP showed high fluorescence in both HEK-293T cells and Jurkat T cells, with complete cytoplasmic localization (Fig. 4-4B-C, Fig. 4-3C). Together, these data further confirm that Itk most likely exists as an intramolecularly folded monomer in the inactive state in cells (Fig. 4-3D). However, since the YFP fluorescence was only complemented when the distance of N-terminus and C-terminus of Itk was within ~80 Å, we could not exclude the possibility that Itk could also form homodimer with N-terminus and C-terminus farther apart or it represents a small percentage of the total species of molecules in these cells.

4.2.2 The SH2 and SH3 domains are not required to maintain the folded inactive monomer of Itk

Since Itk forms clusters in the cell membrane which complicates the analysis, we used the R29C mutation as a base to analyze specific domain mutants of Itk (Figs. 4-1 & 4-4, [180]). The dimer model proposes that the SH2 domain of Itk exists in both a cis- and trans- conformation, around P287, the conformers of which are regulated by Cyclophilin A (CypA). In experiments using isolated SH2 and SH3 domains of Itk, the cis conformer of SH2 domain has been shown to interact with the SH3 domain of Itk in trans via P287. These experiments also suggest that the isolated SH2 domain mutant, P287G, primarily exists in the trans-conformation and does not participate in SH2-SH3 domain interactions, favoring instead a monomer form of Itk [22, 23]. We introduced the P287G mutation into the Itk and the fluorescence intensity of this mutant was determined by flow cytometry. The results show that this mutant had similar fluorescence
complementation to the “WT” R29C mutant, suggesting that if a head-to-tail homodimer of Itk exists, it does not contribute to the YFP fluorescence in WT R29C, which further confirm that there is intramolecular folded monomeric conformation of Itk in the inactive state. These data also suggest that R29C/P287G mutant has a similar structure as the “WT” R29C “cytoplasmically trapped” inactive Itk (Fig. 4-5A, B, C, Fig. 4-3E(i)). To further prove this demonstration, we also utilized Jurkat T cells lacking CypA [186], where Itk should exist in a predominantly trans conformation around the P287 in the SH2 domain. If the folded monomer model of correct, then expression of the R29C Itk tagged at both ends with the split YFP in these cells should result in fluorescence complementation. Indeed, expression of the R29C PH mutant of Itk tagged at both ends with the split YFP in these cells resulted in similar fluorescent complementation to that seen in WT Jurkat cells, arguing that CypA does not alter the overall structure of Itk in cells, and that cis-trans isomerization of Itk around P287 may not affect the structure of Itk (Fig. 4-5C, Fig. 4-3E(i)). However, we cannot rule out that in the absence of CypA, other prolyl isomerases can act on Itk, although treating YFP1-R29CItk-YFP2 expressing cells with Cyclosporine A results in no change in fluorescence complementation (data not shown). Together, these data further confirm that intramolecular folded conformer of Itk exists in vivo and indicate that the cis/trans conformation of Itk SH2 domain is not important for its intramolecular folded conformation. W208 is centrally located and solvent exposed in the ligand-binding pocket of Itk SH3 domain. Mutation of this conserved W to K prevents the SH3 domain from mediating protein-protein interactions [22]. A W208K mutant should therefore eliminate any SH3 interactions with either the cis-conformer of the SH2 domain, or any SH3-PRR interactions if they exist. However introducing this mutant into YFP1-R29C-YFP2 (i.e. YFP1-Itk$^{R29C/W208K}$-YFP2) did not change fluorescence complementation, indicating that SH3 domain interactions, at least via the classical binding site, may not alter the intramolecular folded conformation of Itk (Fig. 4-5D, Fig. 4-3E(ii)). PRR mutants P158A/P159A in the TH domain which disrupt SH3
domain binding to the PRR region should also destroy any SH3-TH interaction [187]. However, this mutation did not change YFP fluorescence complementation of the R29CItk, further confirming that the SH3-PRR interaction is not critical for maintaining this conformation of Itk (Fig. 4-5D, Fig. 4-3E(iii)). The folded monomer model proposes that specific domains of Itk interact with a N-terminal PRR in the TH domain of Itk to maintain the monomer [22, 23]. However, the fact that we can alter both the SH3 binding pocket as well as proline residues in the PRR suggest that although this mode of interaction may exist in-vivo, it may not be strong enough to maintain a folded monomer.

4.2.3 The Zn\textsuperscript{2+} binding region in the TH domain is critical for maintenance of the folded inactive monomer of ITK

Since disrupting interactions between the two predicted domains (\textit{cis}-SH2 interaction with SH3 or SH3 interaction with PRR) proposed by the existing models do not alter the intramolecular folded monomer conformer of Itk, we deleted the PRR region and part of Zn\textsuperscript{2+}-binding Btk Homology motif (BH) motif in the R29C mutant of Itk (resulting in a mutant referred to as \(\Delta\text{TH}\)) and determined its ability to induce complementation of YFP fluorescence [166]. Strikingly, we found that this mutant had dramatically decreased YFP fluorescence complementation (Fig. 4-6A, Fig. 4-3F(i)). When the PRR was restored, including key amino acids required for the proposed interaction between PRR region and SH3 domain giving the mutant \(\Delta\text{TH15C}\), YFP fluorescence was not recovered (Fig. 4-6B, Fig. 4-3F(iii)). However, when amino acids N-terminal to the PRR were restored (giving mutant \(\Delta\text{TH10N}\)), fluorescence was largely recovered (Fig. 4-6B, C, Fig. 4-3E(ii)). Since the deletion variant \(\Delta\text{TH15C}\) missing only a portion of the BH motif had reduced complementation, while the mutant \(\Delta\text{TH10N}\) that
included the BH domain maintained fluorescence, this data suggest that the BH motif is important for maintaining the intramolecular folded conformation of Itk. The BH motif of Tec family kinases is conserved, with amino acids involved in coordinating a molecule of Zn\textsuperscript{2+} that are in identical positions [188, 189]. C155 is one of the conserved zinc ligands and when mutated in BTK, C155G has been reported to disrupt the Btk motif [188]. To further test the role of the BH motif in the conformation of ITK, C132 and C133, two corresponding reserved amino acids in Itk, were substituted to G in YFP1-Itk\textsuperscript{R29C}-YFP2 (to generate YFP1-Itk\textsuperscript{R29C/C132G/C133G}-YFP2), and this mutant tested. The data show that this Zn\textsuperscript{2+} binding mutant of Itk had significantly decreased fluorescence complementation, suggesting that the folding was disrupted (Fig. 4-6C, D, Fig. 4-3E(iv)).

To confirm that the fluorescence complementation assay was able to faithfully report the structure of Itk in cells, we also used FRET between ECFP and Venus in Itk appropriately tagged. The data (Fig. 4-6E, F), show that FRET only occurred when Itk was tagged at the N and C-terminus with ECFP and Venus, but not when individually Itk molecules tagged with ECFP or Venus were expressed together in cells. By contrast, expression of the SCAT molecule, consisting of ECFP and Venus separated by a 18 amino acid peptide (~72 Å apart) served as a positive control and gave similar robust FRET (Fig. 4-6E, F, [167]). Thus the data support the conclusion that the N- and C-terminus of Itk are in close proximity to each other, and that Itk form an intramolecular monomer in the cytoplasm of cells. All taken together, our data provide support for a model of Itk where the inactive protein in the cytoplasm exists as a folded monomer, with the N-terminus within ∼80 Å from the C-terminus. Our data also suggest that the Zn\textsuperscript{2+} binding region of the BH motif of Itk is important for its ability to maintain a monomeric intramolecularly folded conformation.
4.3 Discussion

We show here that the intramolecular folded monomeric conformation of Itk exists when Itk is in the inactive state in-vivo. Luban and colleagues have demonstrated that Itk can form dimers using co-immunoprecipitation assay. We also detected dimer of Itk in the cell membrane. Although our data do not support the head to tail homodimer of Itk, it may still exist. One possibility is that it only represents a small percentage of the total species of molecules in the cells, the other possibility is that the distance of N-terminus and C-terminus of Itk was much more than 80 Å when it forms head-to-tail dimer such that our system could not detect it.

While the crystal structure of a full length Tec family tyrosine kinase has not yet been reported, Márquez et al. have predicted that Btk likely exists as a monomer with little interaction between its domains [190]. This prediction was based on X-ray synchrotron radiation scattering and rigid body modeling, using purified protein from bacteria. Our experiments were performed in mammalian cells, including T cells, and it is possible that the cellular environment provides the proper requirements for the folding patterns that we detect. It is also possible that Btk has a different conformation from Itk. We should note that the BH motif within the TH domain has been previously suggested to regulate the activity of Btk. Huang and colleagues have reported that the Gqα protein interacts with Btk via the TH motif, and that this interaction is involved in the ability of this G-protein to regulate the activity of Btk [191-193]. Schreiber and colleagues have also suggested that the TH domain may be involved in stabilizing the intramolecular folded conformation of Itk.

Crystallographic studies of other non-receptor tyrosine kinases of the Src, Abl and Syk family have all shown that in the inactive state, these kinases fold as monomers with the N-terminus near the C-terminus of the protein [183, 184]. However, the mechanisms involved in maintenance of these structures are varied. In the case of Src family kinases, the SH2 domains
interact with a C-terminal phosphorylated tyrosine, while the SH3 domain forms contacts with pseudo-proline regions to maintain the inactive structure. Similar analysis of c-Abl reveals an analogous structure, except that the SH2 and SH3 domains make other contributions to the maintenance of the inactive protein. More recently the structure of the Syk kinase was solved and revealed a similar folded inactive conformer [194].

Our work here supports the view that non-receptor tyrosine kinases Src, Abl and Tec have similar modes of regulation from the perspective of folding. The data also suggest that active Itk may be in an open configuration, allowing enhanced kinase activity. We were only able to determine the “folded” conformation of Itk using a PH mutant that is cytoplasmically localized, avoiding membrane associated dimers or higher order clusters. Thus we were unable to determine if the Zn\textsuperscript{2+} binding mutant (C132 and C133) of Itk with the proposed “open” conformation has elevated kinase activity since Itk requires phosphorylation by Src family kinases at the membrane for activity [162, 168]. While we cannot rule out that small pools of Itk may exist in a head to tail dimer as previously suggested, our data support a model where the majority of Itk molecules existing in a folded monomer, with the N- and C-terminus in close proximity, a conformation that requires the Zn\textsuperscript{2+} binding region of the BH motif in the TH domain. Our data also suggest that the reported CypA/Itk interaction may not significantly affect the structure of Itk in cells.
Figure 4-1. Itk does not form dimers in the cytoplasm of cells. A) Model of split YFP fusions to Itk. B) BTK^+/− DT40 B cells were transfected with SRF-Luciferase along with YFP1-Itk-YFP2.
or GFP. Cells were then stimulated with left unstimulated or anti-IgM antibodies and harvested 8 hours later. Cells were lysed and luciferase determined. *p<0.05 compared to cell transfected with GFP. C) (i) 293T cells were transfected with Itk fused to YFP1 at the N-terminus and YFP2 at the C-terminus. Cells were then analyzed by flow cytometry for YFP fluorescence and quantified as described. (ii) Confocal analysis of cells expressing YFP1-Itk-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk, Red). D) (i) Flow cytometry plots of Jurkat T cells transduced with lentiviruses expressing YFP1-Itk-YFP2, YFP fluorescence (left panel), Itk expression as detected by anti-GFP antibodies (right panel). (ii) Confocal analysis of Jurkat T cells expressing YFP1-Itk-YFP2 (YFP fluorescence, Green). (iii) Confocal analysis of peripheral blood T cells expressing YFP1-Itk-YFP2 (YFP fluorescence, Green). E) 293T cells were transfected with Itk fused to YFP1 at the N-terminus along with Itk fused to YFP2 at the C-terminus and YFP fluorescence quantified. F) (i) Confocal analysis of cells expressing YFP1-Itk along with Itk-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk antibodies, Red). (ii) Confocal analysis of Jurkat T cells transfected with YFP1-Itk along with Itk-YFP2 (YFP fluorescence, Green). *p<0.05.
Figure 4-2. Quantification of fluorescence complementation. A) 293T cells were transfected with Itk fused to YFP1 at the N-terminus and YFP2 at the C-terminus. Cells were then fixed, permeabilized and Itk expression determined by staining with an anti-Itk antibody (which recognizes these YFP tagged proteins), followed by a PE tagged anti-mouse IgG. The cells were then analyzed by flow cytometry for PE fluorescence (left 2-color plot). Since fixation reduces the YFP fluorescence (unpublished), we also determined in parallel the YFP fluorescence in live cells to quantify the YFP fluorescence complementation (right histogram). In the histogram, the control cells were transfected with Itk fused to YFP1 at the N-terminus. B) 293T cells were transfected with the R29C mutant of Itk fused to YFP1 at the N-terminus and YFP2 at the C-terminus. Cells were then fixed, permeabilized and Itk expression and YFP fluorescence complementation determined as in (A). In the histogram, the control cells were transfected with Itk fused to YFP1 at the N-terminus. C) 293T cells were transfected with Itk fused to YFP1 at the N-terminus along with Itk fused to YFP2 at the C-terminus. Cells were then fixed, permeabilized and Itk expression and YFP fluorescence complementation determined as in (A). In the
histogram, the control cells were transfected with R29CItk fused to YFP1 at the N-terminus along with Zip fused to YFP2 at the C-terminus. D) 293T cells were transfected with the R29C mutant of Itk fused to YFP1 at the N-terminus along with R29C Itk fused to YFP2 at the C-terminus. Cells were then fixed, permeabilized and Itk expression and YFP fluorescence complementation determined as in (A). In the histogram, the control cells were transfected with R29CItk fused to YFP1 at the N-terminus along with Zip fused to YFP2 at the C-terminus.
Figure 4-3. Models of Itk as suggested by the experimental results. A) Models that account for the results of experiments shown in figure 1. B) WT and R29C PH mutant of Itk. C) Model that account for the results of experiments shown in figure 2A. D) Models that account for the results of experiments shown in figure 2B & C. E)(i) Model that account for the results of experiments shown in figure 3A & B. (ii)& (iii) Models that account for the results of experiments shown in figure 3C. F) Models that account for the results of experiments shown in figure 4.
Figure 4-4. Cytoplasmically localized Itk exists as a head to tail folded monomer in cells. A) (i) 293T cells were transfected with R29CItk fused to YFP1 at the N-terminus along with R29CItk fused to YFP2 at the C-terminus, or R29C/P287GItk fused to YFP1 at the N-terminus along with R29C/P287GItk fused to YFP2 at the C-terminus, and YFP fluorescence quantified. (ii) Confocal analysis of cells expressing YFP1-R29CItk along with R29C Itk-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk, Red). B) (i) 293T cells were transfected with R29CItk fused to YFP1 at the N-terminus and YFP2 at the C-terminus and YFP fluorescence quantified.
fluorescence quantified. (ii) Confocal analysis of cells expressing YFP1-R29CItk-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk, Red). C) (i) Flow cytometry plots of Jurkat T cells transfected with YFP1-R29CItk-YFP2. YFP fluorescence (left panel), Itk expression as detected by anti-GFP antibodies (right panel). (ii) Confocal analysis of Jurkat T cells expressing YFP1-R29CItk-YFP2 (YFP fluorescence, Green). D) 293T cells were transfected with WT Itk fused to YFP1 at the N-terminus along with R29CItk fused to YFP2 at the C-terminus. Cells were then analyzed by flow cytometry for YFP fluorescence and quantified. *p<0.05.
Figure 4-5. Interactions via *cis/trans* conformation, the PRR, SH3 or Cyclophilin A are not required for the formation of the head to tail folded monomer of Itk. A) 293T cells were transfected with YFP1-R29C/P287GItk-YFP2 and YFP fluorescence quantified. B) (i) Flow cytometry plot of Jurkat T cells transfected with YFP1-R29C/P287GItk-YFP2 (YFP fluorescence). (ii) Confocal analysis of Jurkat T cells expressing YFP1-R29CItk-YFP2 (YFP fluorescence, Green). C) WT or CypA−/− Jurkat T cells were transduced with lentivirus expressing YFP1-R29CItk-YFP2 and YFP fluorescence quantified. Similarly, WT Jurkat T cells were
transduced with lentivirus expressing YFP1-R29C/P287GItk-YFP2 followed by quantification of YFP fluorescence. D) (i) 293T cells were transfected with YFP1-R29C/W208KItk-YFP2 or YFP1-R29C/P158AP159AItk-YFP2, followed by quantification of YFP fluorescence. (ii) Confocal analysis of cells expressing YFP1-R29C/W208KItk-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk, Red). (iii) Confocal analysis of cells expressing YFP1-R29C/PPAAItk-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk, Red).
Figure 4-6. The Zn$^{2+}$ binding region in the TH domain of Itk is required to maintain the head to tail folded monomer in cells. A) (i) 293T cells were transfected with YFP1-R29C/ΔTHItk-YFP2 followed by quantification of YFP fluorescence. (ii) Confocal analysis of cells expressing YFP1-R29C/ΔTHItk-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk, Red). B) (i) 293T cells were transfected with YFP1-R29C/ΔTHItk-YFP2, YFP1-R29C/ΔTHItk+10N-YFP2, YFP1-R29C/ΔTHItk+15C-YFP2 or YFP1-R29C/C132GC133GItk-YFP2, followed by quantification of YFP fluorescence. (ii) Confocal analysis of cells expressing YFP1-R29C/ΔTHItk+10N-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk, Red). (iii) Confocal analysis of cells expressing YFP1-R29C/ΔTHItk+15C-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk, Red). (iv) Confocal analysis of cells expressing YFP1-R29C/C132GC133GItk-YFP2 (YFP fluorescence, Green, Itk expression as detected using anti-Itk, Red). C) Jurkat T cells were transduced with lentiviruses expressing the indicated Itk mutants tagged at the N-terminus with YFP1 and the C-terminus with YFP2, followed by quantification of YFP fluorescence. *p<0.05. D) 293T cells were transfected with either ECFP along with Venus alone (not shown), ECFP-R29CItk along with R29CItk-Venus, ECFP-R29CItk-Venus or ECFP-SCAT-Venus (as a positive control), followed by analysis by confocal microscopy for ECFP fluorescence (left panels), Venus fluorescence (middle panels) or normalized FRET (right panels). E) Quantification of FRET efficiency (max=1) for the above experiments. *p<0.05.
Chapter 5

The role of Tec family kinases in iNKT cell development

5.1 Introduction

Natural killer T cells (NKT cells) are a unique population of innate-like T lymphocytes that play important functions in diverse immune responses. Invariant NKT (iNKT) cells are the dominant subset of NKT cells that express an invariant TCRα chain (Vα14-Jα18). When activated, iNKT cells rapidly produce large amounts of IL-4 and IFNγ, along with a number of other cytokines and chemokines. iNKT cells also regulate other immune cells, such as NK cells and B cells, thus playing multiple roles in immune responses.

iNKT cells develop from the DP (CD4+CD8+ double positive) thymocytes and are selected by the MHC class I-like molecule CD1d itself on DP cells. Recent studies have shown that homotypic interactions between signaling lymphocytic-activation molecule (SLAM) family members SLAM and Ly108 on DP-T cells are also important for iNKT cell lineage selection [87]. After selection, the iNKT precursors (CD44lowNK1.1-) further develop through more mature CD44bNK1.1- iNKT to finally mature CD44bNK1.1+ iNKT cells. A number of signaling molecules, transcription factors and cytokine receptors have been identified to be important for iNKT cell development through different stages. For example, SLAM, SLAM-associated protein (SAP), and Src-family kinase FYN and NFκB have been suggested to form a signaling pathway that controls iNKT cell development at a very early stage [79-83, 87, 92, 93]. The cytokine IL-15, the vitamin D receptor, PTEN, SLP-76 and transcription factors T-bet and AP-1 are all involved in the final maturation of iNKT cells [96-99, 195-197].

Itk and Txk are two Tec family non-receptor tyrosine kinases expressed in T cells. Itk and Txk are required for the TcR induced phosphorylation and full activation of phospholipase-γ1 and
subsequent calcium influx, as well as activation of multiple signaling pathways, such as NFκB, NFAT and Ras [12]. Itk null mice show impaired Th2 cell development, as well as cytokine secretion. In addition, both Itk and Txk regulate the development of naïve phenotype CD4<sup>+</sup> and CD8<sup>+</sup> T cells [57, 60, 61]. Altogether, signals through Itk and Txk are important for T cell development and function.

Itk null mice also show reduced iNKT cell number, impaired maturation and cytokine secretion, which is exaggerated in Itk/Txk DKO mice [24, 62]. Besides its kinase activity, Itk also functions as a scaffolding protein in signaling pathways. Kinase-inactive Itk have been shown to activate the transcription factor Serum Response Factor and induce antigen induced actin polarization in T cell lines [46, 49, 50]. However, the kinase activity independent function of Itk has not been examined in iNKT cell development in-vivo. We show here that a kinase-domain deleted Itk partially rescues the maturation defect of ITK null iNKT cells. This rescue correlates with increased expression of CD122 and T-bet, as well as reduction in the elevated levels of eomesodermin observed in ITK null iNKT cells. These data suggest that ITK has both kinase dependent and kinase independent function in iNKT cell development.
5.2 Result

5.2.1 Reduced frequency and number of iNKT cells in the absence of Itk and Txk

Itk<sup>−/−</sup> mice have been shown to either have similar percentage and number of iNKT cells in the thymus, or reduced percentage and number of thymic iNKT cells, with a further decrease in percentage and number of iNKT cells observed in mice lacking both Txk and Itk [24, 198]. We examined iNKT cells in the thymi of Itk<sup>−/−</sup> and Itk/Txk double knockout mice (DKO) and found that these cells were present in decreased numbers and percentage in Itk<sup>−/−</sup> mice compared with WT mice, with a further decrease in the Itk/Txk DKO mice (Fig. 5-1). These data are consistent with the work of Berg and colleagues and indicate that both Itk and Txk play role in the development of iNKT cells. CD1d was expressed at similar levels on DP thymocytes from WT, Itk<sup>−/−</sup> and Itk/Txk DKO mice, indicating that it is not responsible for the reduced iNKT cell numbers in these mice (Fig. 5-2). Similarly, expression of the SLAM family receptors, SLAM and Ly108, on DP thymocytes was not different in the different mice, also ruling out the possibility SLAM family receptors deficiency as a cause of iNKT cell development defect (Fig. 5-2).

5.2.2 iNKT cell development is partially dependent on the kinase domain of Itk

Itk is involved in the phosphorylation of substrates downstream of the T cell receptor during T cell activation. However, we and others have shown that Itk also has adaptor function and can activate specific pathways in a kinase independent manner [46, 50]. We have also shown that conventional T cells require the kinase domain of Itk for development [58]. In order to determine if the kinase activity of Itk is required for the development of iNKT cells, we compared the iNKT population in WT, Itk<sup>−/−</sup> and mice carrying a mutant ITK lacking it’s kinase domain
instead of WT ITK (Tg(Lck-Itk∆Kin)/Itk−/−) [58]. Compared with Itk−/− mice, the percentage of thymic iNKT cells in the Tg(Lck-Itk∆Kin)/Itk−/− mice was significantly increased, although it was still lower than WT mice (Fig. 5-1). In contrast, the numbers of iNKT cell were comparable between Tg(Lck-Itk∆Kin)/Itk−/− and Itk−/− mice. Together, these results suggest that the generation of iNKT cells may be partially independent of the kinase activity of ITK.

Three well characterized stages of development have been recognized during the development of iNKT cells based on the expression of NK1.1 and CD44 following expression of the invariant NKT cell receptor (which binds CD1d/αGalCer tetramers): the most immature iNKT cells are NK1.1−CD44− (stage 1), which differentiate into NK1.1−CD44+ (stage 2), followed by further development into NK1.1+CD44+ iNKT cells (stage 3). Analyzing these stages, we found that the absence of Itk results in significant block of iNKT cell development in stage 2 (Fig. 5-3a). Analysis of Itk/Txk DKO mice revealed that the percentage of stage 2 NK1.1+CD44+ iNKT cells was further increased over that seen in the absence of Itk, suggesting that Txk is also involved in the iNKT cell development in the stage 2 (Fig. 5-3a). By contrast, the percentage of thymic NK1.1+CD44+ iNKT cells in the Tg(Lck-Itk∆Kin)/Itk−/− transgenic mice was significantly increased (60-70% vs. 40-50%) compared with Itk−/− mice. This data suggests that the kinase activity of Itk is not required for allowing the development of iNKT cells through the immature stage 2 to the more mature stage 3. However, since the percentage of thymic NK1.1+CD44+ iNKT cells in the Tg(Lck-Itk∆Kin)/Itk−/− mice is still lower than that in the WT mice, the kinase activity of Itk is still required for the full maturation of iNKT cells. These data suggest that Itk may regulate the development of iNKT cells not only through its kinase activity, but also via its kinase independent functions.

There are two defined subsets of murine iNKT cells, CD4+ or CD4−CD8− (DN). Analyzing these two populations, we found that about 50% of thymic iNKT cells in WT mice
were DN, but it was decreased to only around 20% in Itk\textsuperscript{+/} mice, demonstrating that Itk is important in \textit{i}NKT subset development. This DN subset was further decreased to 5% in Itk/Txk DKO mice, suggesting that Txk also plays role in DN population formation (Fig. 5-3b). Previous studies show that the DN population appear later than CD4\textsuperscript{+} population in \textit{i}NKT cell development, although the exact time for the division is still not clear [90]. Thus the reduced DN population at least partly reflects the higher percentage of immature \textit{i}NKT cells in these mice. However, Analysis of \textit{i}NKT cell subsets in \textit{Tg(Lck-Itk}\textsuperscript{ΔKin})/Itk\textsuperscript{+/-} mice revealed that the percentage of thymic DN \textit{i}NKT cells in these mice was similar to that in Itk\textsuperscript{+/} mice, indicating that the kinase activity of Itk is important for the \textit{i}NKT subset division (Fig. 5-3b). Since kinase mutant of Itk partly rescues the maturation of \textit{i}NKT cells, this data also suggests that the role of Itk in subset distribution of \textit{i}NKT cells is not only due to the more immature \textit{i}NKT cells in these mice.

We further examined the proliferation of thymic \textit{i}NKT cells by BrdU incorporation. Compared with WT \textit{i}NKT cells, much higher percentage of Itk\textsuperscript{+/} \textit{i}NKT cells labeled with BrdU (Fig. 5-4). Since thymic NK1.1\textsuperscript{-} \textit{i}NKT cells incorporate BrdU much faster than NK1.1\textsuperscript{+} \textit{i}NKT cells, this difference may reflect the higher proportion of immature \textit{i}NKT cells in Itk\textsuperscript{+/-} mice [89]. Indeed, BrdU feeding studies revealed that most BrdU\textsuperscript{+} \textit{i}NKT cells in Itk\textsuperscript{+/-} mice are NK1.1\textsuperscript{-}, and the percentage of BrdU\textsuperscript{+} \textit{Tg(Lck-Itk}\textsuperscript{ΔKin})/Itk\textsuperscript{+/-} \textit{i}NKT cells was lower those from Itk\textsuperscript{+/-} mice, consistent with the higher percentage of mature NK1.1\textsuperscript{+} \textit{i}NKT cells in the \textit{Tg(Lck-Itk}\textsuperscript{ΔKin})/Itk\textsuperscript{+/-} mice (Fig. 5-4). These data further confirm that the role of Itk in \textit{i}NKT development is partly independent of its kinase activity.
5.2.3 Itk kinase activity independent rescue of CD122 and ratio of T-bet to eomesodermin in developing iNKT cells

Several studies have shown that IL-15 is required for the final maturation of iNKT cells and that the IL-2/IL-15 receptor β-chain is important for NKT cell development [98, 99]. Thus the defect of final maturation in Itk\textsuperscript{-/-} iNKT cells may be due to the lower CD122 expression in these cells. In support of this, Berg and colleagues have reported that Itk deficient iNKT cells express lower levels of CD122 than WT iNKT cells. Our analysis of Itk deficient iNKT cells indeed reveals that these cells have reduced levels of CD122 (Fig. 5-5a). More importantly, we found that the CD122\textsuperscript{+} iNKT population in the Tg(Lck-Itk\textsuperscript{ΔKin})/Itk\textsuperscript{-/-} mice was significantly higher than that in Itk\textsuperscript{-/-} mice, indicating that the increased expression of CD122 may contribute to the increased maturation of iNKT cell in the mice carrying the mutant Itk. These data also suggest that Itk may regulate CD122 expression in a kinase independent fashion.

The transcription factor T-bet is also a key regulator in iNKT cell development, and iNKT cells are completely blocked in immature stage 2 in the absence of T-bet [96]. T-bet can also regulate the expression of CD122, and Berg and colleagues have shown that T-bet expression level is lower in the Itk\textsuperscript{-/-} iNKT cells in the thymus, along with the reduced expression of CD122 [24]. As we observed rescue of CD122 expression on developing iNKT cells in the thymus of Tg(Lck-Itk\textsuperscript{ΔKin})/Itk\textsuperscript{-/-} mice, we examined sorted populations of these cells for levels of T-bet message. We found that Tg(Lck-Itk\textsuperscript{ΔKin})/Itk\textsuperscript{-/-} iNKT cells had significantly increased levels of T-Bet mRNA compared to Itk\textsuperscript{-/-} iNKT cells. Examination of the expression level of eomesodermin, another transcription factor of T-box family that also regulates CD122, revealed that while eomesodermin was not detected in WT iNKT cells, it was highly expressed in the Itk\textsuperscript{-/-} iNKT cells (Fig. 5-5b). Interestingly, the expression of the Itk mutant strongly reduced eomesodermin expression in Itk\textsuperscript{-/-} iNKT cells (i.e. iNKT cells that develop in the Tg(Lck-Itk\textsuperscript{ΔKin})/Itk\textsuperscript{-/-} mice), suggesting that kinase independent signals from Itk may block signaling
pathways leading to eomesodermin expression in iNKT cells. The ratio of T-bet:eomesodermin may be critical for proper development of iNKT cells, and our data suggest that this ratio may be partially regulated by Itk in a kinase independent manner.

Our data suggest that the IL-15 signaling pathway and the ratio of T-bet to eomesodermin may contribute to the defect of Itk in iNKT cell development. Indeed, GATA-3 transcription is not affected in Itk null iNKT cells [24, 62]. In addition, while two recent studies have shown that the transcription factor PLZF is important for iNKT cell development at early stage [199, 200], PLZF mRNA levels are similar between Itk null and WT iNKT cells (Fig. 5-5b), suggesting that PLZF does not contribute to the defect in Itk−/− iNKT cells. However, we cannot exclude the possibility that some other transcription factors and signaling pathways may also be involved in Itk signals to iNKT cell development.
5.3 Discussion

Upon TcR stimulation, Itk is recruited to the cell membrane through its PH domain binding to PIP3 in the cell membrane, where Itk is phosphorylated and activated, as well as interact with other signaling proteins, including SLP-76, LAT, GADS, PLCγ and Vav, to assemble the productive signaling complex and subsequently initiate the downstream signaling pathways. Itk interacts with other proteins mainly through its SH2 and SH3 domains and this adaptor function is very important for the downstream signaling pathways. Using T cell lines, two studies showed that Itk regulation of Vav localization and TCR-induced actin polarization is independent of its kinase activity, but requires its PH and SH2 domains [46, 49]. Our previous study also showed that the SH3 domain but not kinase activity of Itk is required for the antigen receptor induced transcription factor SRF activation [50]. In addition, the kinase domain deleted Itk can partially rescue the antigen receptor induced activation of Erk in Tec kinase null DT40 cells, indicating that antigen receptor induced ERK/MAPK signaling pathway may be partially Itk kinase activity independent [50]. These signaling pathways may contribute to the kinase independent role of Itk in iNKT cell development in-vivo.

Altogether, our data demonstrates that Itk and Txk are important for the development of iNKT cells. More importantly, our data demonstrates that the kinase activity of Itk is required for generating WT levels of iNKT cell numbers, but not fully required for the maturation of iNKT cells. Itk may therefore utilize its scaffolding function in regulating signaling pathways that contribute to the maturation of iNKT cells. These pathways may regulate the expression level of CD122, which in turn may be regulated by the balance of T-bet and eomesodermin expressed in these cells.
Figure 5-1. The kinase activity of Itk is not required for generation of WT percentages of iNKT cells, but is required for generating WT numbers of iNKT cells in the thymus. A) Flow cytometric analysis of thymocytes from WT, Itk null, Itk/Txk DKO and $Tg(Lck-\hspace{1em}Itk\Delta Kin)/Itk^-\hspace{1em}iNKT$ cells. Numbers indicate the percentage of iNKT cells. B) Percentages and absolute numbers of iNKT cells in the thymus. Data were from four independent experiments. *p<0.05.
Fig. 5-2 CD1d, SLAM and Ly 108 were expressed at similar level in DP thymocytes of WT, Itk$^{-/-}$ and Itk/Txk DKO mice. The staining patterns of double positive thymocytes from WT, Itk$^{-/-}$ and Itk/Txk DKO mice for CD1d, and SLAM family members SLAM and Ly108.
Figure 5-3. The kinase activity of ITK is not fully required for the maturation of iNKT cells. A) Thymocytes from WT, Itk null, Itk/Txk DKO and Tg(Lck-ItkΔKin)/Itk−/− mice were gated on αGalcer/CD1d tetramer positive cells and analyzed for the maturation markers CD44 and NK1.1. The percentage of CD44⁺NK1.1⁺, CD44⁺NK1.1⁻ and CD44⁻NK1.1⁺ iNKT cells are also shown (bottom graph). B) Thymocytes were gated on iNKT cells and analyzed for CD4 and CD8. The percentage of thymic CD4⁺ iNKT cells are also shown (bottom graph). Data were from four independent experiments.
Figure 5-4. Kinase activity independent rescue of mature iNKT cell proliferation. Thymocytes were gated on iNKT cells and were analyzed for BrdU vs. α-Galcer/CD1d tetramer (top) or BrdU vs. NK1.1. Data were from two independent experiments.
Figure 5-5. Kinase activity independent rescue of CD122 and ratio of T-bet to eomesodermin. A) Thymocytes from WT, Itk null and $Tg(Lck$-$Itk\Delta Kin)/Itk^{-/-}$ mice were gated on $\iota$NKT cells and analyzed for the expression of CD122. B) $\iota$NKT cells were sorted from the thymus of WT, Itk null and $Tg(Lck$-$Itk\Delta Kin)/Itk^{-/-}$ mice and mRNA levels of T-bet, PLZF and eomesodermin were determined by real-time quantitative PCR. Data were representative of two independent experiments.
Chapter 6

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

6.1 Introduction

Itk signals regulate the development of Th2 cells such that in its absence, T cells from Itk null mice have defects in the production of Th2 cytokines, and these mice have defects in generating Th2 response in several infection and allergic asthma models [201-204]. Despite this defect in the generation of effective Th2 responses and secretion of Th2 cytokines, Itk null mice paradoxically exhibit increased class switch in B cells to IgE, and elevated levels of serum IgE [3, 32]. It is not clear what the source of cytokines that could drive the increase in class switch to IgE.

T cells are divided into αβ T cells and γδ T cells according to their TCR expression. Both αβ and γδ T cells arise from the most immature CD4⁻CD8⁻ double negative (DN) thymocytes in the thymus. DN thymocytes are divided into four developmental stages according to the surface expression of CD25 and CD44, from most immature DN1 to more mature DN4 cells. γδ T cells separate from αβ T cells at DN stages, although the exact time point and the mechanisms involved in this process are still elusive [122, 123]. Studies on γδ TCR transgenic mice demonstrated that negative selection is required for the development of γδ T cells in adult thymus, but whether positive selection is necessary is still controversial.

γδ T cells can produce Th1, Th2 and Th17 cytokines and thus having multiple functions in the modulating immune responses such as host defense and tumor immunity [149, 150, 152, 205-208]. Both murine and human γδ T cells have been suggested to provide help to B cells,
which is correlated with their production of the Th2 cytokine IL-4 [154, 155, 209]. More interestingly, several studies showed that only the CD4+ γδ T cells are able to produce IL-4 [149, 150].

We now show here that mice lacking Itk have altered γδ T cell development such that they have more of these cells. We also show that the CD4+ population of γδ T cells is expanded in the absence of Itk, and that this population can induce B cells to class switch to generate increased levels of serum IgE. Our data suggest that the elevated levels of serum IgE observed in the absence of Itk is due to altered γδ T cells, and that these cells may be able to regulate the development of allergies by enhancing B cell class switch to IgE.
6.2 Results

6.2.1 Increased percentage and numbers of $\gamma\delta$ T cells in the absence of Itk

During our analysis of T cell populations in mice lacking Itk, we noticed that these mice have a higher percentage of $\gamma\delta$ TcR bearing T cells. Careful analysis of these populations revealed that the Itk$^{-/-}$ mice have a higher percentage of $\gamma\delta$ T cells in the thymus, spleen and lymph node (Fig. 6-1A, B). This increased percentage represented increased numbers of these T cells (Fig. 6-1B).

6.2.2 Increased development of CD4$^+$ $\gamma\delta$ T cells in the absence of Itk

Further analysis of thymic $\gamma\delta$ T cells revealed that about 40% of these cells carried the CD4 receptor, while WT thymus had a much lower percentage of CD4$^+$ $\gamma\delta$ T cells (Fig. 6-1C, D). This skewing of the $\gamma\delta$ T cell population to CD4 expressing cells carried over into the periphery, where about 30% of $\gamma\delta$ T cells in the spleen were CD4 bearing (Fig. 6-1C). Again, this increased percentage reflected increased numbers.

6.2.3 Altered phenotype of $\gamma\delta$ T cells in the absence of Itk

Analysis of the CD4$^+$ and CD4$^-$ populations of thymic $\gamma\delta$ T cells in WT and Itk null mice for expression of CD44 and CD24, markers that can identify developmental stages, revealed that most of the WT CD4$^+$ $\gamma\delta$ T cells are CD44$^\text{lo}$/CD24$^\text{hi}$, similar to that seen in CD4$^+$ $\gamma\delta$ T cells and the $\gamma\delta$ T cell population as a whole (Fig. 6-2A). However, Itk null $\gamma\delta$ T cells had a higher percentage of cells that were CD44$^\text{hi}$/CD24$^\text{lo}$, suggesting that they may be more mature than WT
γδ T cells. More importantly, while the Itk<sup>−/−</sup> CD4<sup>−</sup> γδ T cell population were more like the WT γδ T cell population with regards to CD44 and CD24 expression, the Itk<sup>−/−</sup> CD4<sup>+</sup> γδ T cells were largely CD44<sup>hi</sup>/CD24<sup>lo</sup>, suggesting that they were more mature.

Analyzing γδ splenocytes for the expression of CD44 and CD62L, useful markers in determining effector T cells, showed that the CD4<sup>+</sup> and CD4<sup>−</sup> γδ T cell populations differed with regards to the expression of these markers. The WT CD4<sup>+</sup> γδ T cells including a higher percentage of the CD62L<sup>lo</sup>/CD44<sup>hi</sup> effector phenotype γδ T cells than the CD4<sup>+</sup> γδ T cell population. By contrast, Itk null γδ T cells contained a larger population of CD62L<sup>lo</sup>/CD44<sup>hi</sup> cells, and more strikingly, the Itk null CD4<sup>+</sup> γδ T cell population was largely CD62L<sup>lo</sup>/CD44<sup>hi</sup>, while the CD4<sup>−</sup> population was more like the WT γδ T cell population (Fig. 6-2B).

Further analysis of the surface properties of the γδ T cells in Itk null mice revealed that there was also an increase in NK1.1 expressing γδ T cells (Fig. 6-2C). Indeed, a large percentage of the CD4<sup>+</sup> γδ T cells were NK1.1<sup>+</sup>, although there was a significant percentage of CD4<sup>+</sup> γδ T cells that were NK1.1<sup>+</sup> (Fig. 6-2D). Itk null mice have reduced development of classical NKT cells [24, 62, 198]. To determine if this NK1.1 expression contributes to the CD4 expression on Itk<sup>−/−</sup> γδ T cells, we analyzed mice lacking both T-bet and Itk. T-bet null mice have defective NKT cell development [96], and they lack NK1.1<sup>+</sup> γδ T cells (Fig. 6-2D). More importantly, mice lacking both Itk and T-bet also lack NK1.1<sup>+</sup> γδ T cells, and now carry increased percentage of CD4<sup>+</sup> γδ T cells, suggesting that NK1.1 expression is not required for the development of these CD4<sup>+</sup> γδ T cells in the absence of Itk.
6.2.4 Enhanced development of γδ T cells in the Itk null mice is cell intrinsic

To test whether the enhanced development of CD4⁺ γδ T cells in the Itk null mice was due to cell-intrinsic mechanism or microenvironment, we performed bone marrow transfer experiments. Transfer of Itk⁻/⁻ bone marrow into sub-lethally irradiated WT or Itk⁻/⁻ mice resulted in the same enhanced development of total γδ T cells as well as CD4⁺ γδ T cells (Fig. 6-3A,B). By contrast, transfer of WT bone marrow into sub-lethally irradiated Itk⁻/⁻ mice resulted in the same level of γδ T cell development as when WT bone marrow was transferred into sub-lethally irradiated WT mice (Fig. 6-3A,B).

Thus, our data supported that this altered γδ T cell development was due to the intrinsic enhanced development of these cells in the absence of Itk. To further prove this, we performed competitive mixed bone marrow chimera experiments. Bone marrow from WT (carrying the CD45.1/Thy1.2 alleles allowing for ease of monitoring) and Itk null mice (carrying CD45.2/Thy1.2 alleles) were mixed in a 1:1 ratio and transferred into sub-lethally irradiated WT mice carrying the Thy1.1 allele to distinguish host derived cells. Following recovery of the hematopoietic system (8 weeks), the thymus and spleen were analyzed for donor derived γδ and αβ T cells (Fig. 6-3C). Our results show that Itk null donors exhibited enhanced development of γδ T cells in thymus and spleen, while in the same animals, Itk null donors exhibited reduced αβ T cells as we have recently reported [64]. Thus, the altered γδ T cell development in Itk⁻/⁻ mice is cell intrinsic.
6.2.5 Development of CD4+ γδ T cells in the absence of Itk is independent of MHC class I or MHC class II expression

Previous studies suggested that MHC class I and MHC class II molecules may not be required for the selection of γδ T cells in the thymus. To determine if the expression of MHC class I or class II molecules were required for the development of the CD4+ γδ T cells in the absence of Itk, we analyzed mice lacking both β2m and Itk (thus lacking the expression of MHC class I) as well as mice lacking both MHC class II and Itk. These mice maintain the enhanced development of the CD4+ γδ T cell population in the absence of Itk, indicating that unlike NKT and conventional T cells, the development of these CD4+ γδ T cells is MHC independent (Fig. 6-4).

6.2.6 Itk null CD4+ γδ T cells predominantly express Vγ1.1/Vδ6.2/3 γδ T cell receptor

γδ T cells usually carry oligoclonal TcRs, with a few receptors predominating. We therefore determined if the Itk null CD4+ γδ T cells differed in the TcR that they carried. Thymic CD4+ γδ T cells in the Itk null mice did not differ with regards to expression of Vγ2, however, a higher percentage of Itk null CD4+ γδ T cells expressed Vδ6.2/3 (Fig. 6-5). In addition, the large population of the γδ T cells in both WT and Itk null mice carry the Vγ1.1 TcR (Fig. 6-5). Indeed, while the CD4+ and CD4- γδ T cells in WT had equal percentage of cells that express the Vγ1.1/Vδ6.2/3 TcR, a majority of the CD4+ γδ T cells in the Itk null mice expressed this TcR (Fig. 6-6A). Thus in the absence of Itk, a large percentage of the CD4+ γδ T cells that develop carry the Vγ1.1/Vδ6.2/3 TcR.

To determine if this the expression of this Vγ1.1/Vδ6.2/3 TcR was driving the enhanced development of CD4+ γδ T cells in the absence of Itk, we analyzed mice carrying the KN6
transgenic mice which carry the Vγ2 TcR [210], for the presence of these cells. While KN6 transgenic mice on a WT background had few CD4⁺ γδ T cells, KN6 transgenic mice on an Itk null background had a higher percentage of CD4⁺ γδ T cells (Fig. 6-6B). These data argue against an antigen driven response or preferential expression of a particular TcR as the explanation for the enhanced development of CD4⁺ γδ T cell in the absence of Itk.

6.2.7 Itk null CD4⁺ γδ T cells predominantly express Th2 cytokine IL-4 and carry increased IL-4 mRNA

γδ T cells have been reported to secrete IL-4, IFNγ and IL-17 both ex-vivo and in-vivo [149, 150, 152]. We therefore analyzed cytokine secretion response by sorting WT and Itk null CD4⁺ and CD4⁺ γδ T cells and stimulated them with anti- γδ TcR for 72 hours, followed by analysis of the supernatants for the presence of IL-4, IL-17 and IFNγ. We found that both WT and Itk⁻/⁻ CD4⁺ γδ T cells secrete significant levels of IL-4, but no IL-17 or IFNγ (Fig. 6-7A). Itk null CD4⁺ γδ T cells also had higher levels of preformed transcripts for IL-4, suggesting that they are poised to secrete this cytokine (Fig. 6-8). In addition, they had slightly elevated levels of T-bet, GATA3 and significantly reduced levels of eomesodermin transcripts (Fig. 6-8). In contrast, only CD4⁺ γδ T cells from WT and Itk null mice secreted IL-17, while CD4⁺ γδ T cells from WT but not Itk null mice secreted appreciable levels of IFNγ, suggesting that Itk is required for signaling to IFNγ but not IL-17 secretion (Fig. 6-7A). However, WT and Itk⁻/⁻ CD4⁺ γδ T cells have comparable levels of IFNγ mRNA level, indicating that the expression of IFNγ transcript is Itk-independent (Fig. 6-8). Thus CD4⁺ γδ T cells are selective in their cytokine secretion pattern, secreting IL-4, but not IL-17 or IFNγ, while CD4⁺ γδ T cells can secrete IL-17 and IFNγ with different requirements for Itk.
6.2.8 Itk null mice have high levels of serum IgE of γδ T cells

We and others have previously reported that mice lacking Itk have elevated levels of serum IgE and higher IgE class switch of B cells, although their CD4⁺ αβ T cells have defects in secreting IL-4 (Fig. 6-7B) ([3, 55]). As we have now identified increased numbers of CD4⁺ γδ T cells in Itk null mice that can secrete significant levels of IL-4, we hypothesized that they may be responsible for the enhanced class switch of B cells to IgE, and the elevated levels of serum IgE observed in these mice. We therefore analyzed the serum of mice lacking Itk and γδ T cells to determine if removing these T cells would reduce the elevated levels of IgE seen in the absence of Itk. We found that mice lacking both Itk and γδ T cells had much reduced serum IgE compared to mice lacking Itk alone, although not as low as that seen in mice lacking γδ T cells or WT mice, suggesting that the γδ T cells in Itk null mice are important for the elevated serum IgE level in these mice (Fig. 6-7B).

6.2.9 Itk null CD4⁺ γδ T cells induce B cell class switch to produce IgE

To determine if Itk null CD4⁺ γδ T cells can induce B cell class switch for secretion of IgE, we set up a cell transfer model to analyze class switch in vivo. In these experiments, we used cells from WT or Itk null mice to transfer into Rag null mice, followed by analysis of serum for IgE 4-weeks later. Our experiments show that transfer of Itk null splenocytes results in increased levels of serum IgE compared to equivalent transfer of WT splenocytes (Fig. 6-7C). Furthermore, transfer of Itk null CD4⁺ T cells along with WT B cells resulted in enhanced serum IgE levels, while transfer of Itk null B cells along with WT CD4⁺ T cells, did not, suggesting that Itk⁻/⁻ CD4⁺ T cells is responsible for the production of IgE by B cells in Itk null mice. To test which subset of
Itk<sup>−/−</sup> T cells is important for increased IgE level, we transferred same numbers of WT B cells together with distinct subset of Itk<sup>−/−</sup> T cells to Rag null mice. Only mice received Itk<sup>−/−</sup> CD4<sup>+</sup> γδ T cells and WT B cells displayed elevated serum IgE after 4 weeks, whereas mice received Itk<sup>−/−</sup> CD4<sup>+</sup> αβ T cells did not. Since WT B cells were used for all the experiments, we exclude the possibility that the difference in IgE production is caused by an increase in IgE production from IgE-expressing B cells. These data indicate that CD4<sup>+</sup> γδ T cells can induce class switch in B cells, resulting in elevated serum IgE. Altogether, our data indicate that Itk null CD4<sup>+</sup> γδ T cells secrete IL-4 and induce class switch to IgE, leading to elevated serum IgE in these mice.
6.3 Discussion

We show here that Itk regulates the differentiation and function of γδ T cells. In the absence of Itk, there is enhanced development of CD4⁺ γδ T cells. These cells carry a predominant γδ TcR, although this is not a requirement for their development. These cells develop in the absence of the expression of MHC class I or class II, suggesting an independent developmental pathway from classical T cells as well as non-conventional T cells such as NKT cells and innate memory phenotype T cells [60, 61, 63, 64, 211]. Finally, our data support the role of these cells as IL-4 secreting cells in vivo, which can enhance B cell class switch to IgE, and explain the paradox of elevated serum IgE in Itk null mice, despite the defect in Th2 development exhibited by the αβ CD4⁺ T cells in these mice.

Recent studies have shown that T cell populations have different developmental requirements of Itk. For example, the “naïve phenotype” CD44loCD62Lhi CD4⁺ and CD8⁺ αβ T cells require Itk for their development and function, while the “innate memory phenotype” CD44hiCD62Llo CD4⁺ and CD8⁺ αβ T cells do not. Here we show that development and function of CD4⁺ and CD4⁺ γδ T cells also have different requirements for Itk. Itk deficiency does not change the development of CD4⁺ γδ T cells in the thymus and spleen, but their ability to secrete IFNγ through TCR signaling pathway is defective. Itk deficiency instead leads to accumulation of mature CD4⁺ γδ T cells with effector phenotypes, indicating that it plays an important role in modulating the development of these cells. However, Itk does not seem to be required for the secretion of IL-4 by CD4⁺ γδ T cells. Negative selection has been reported to be important in the development of γδ T cells in adult thymus. Itk is downstream of TcR and it works as an amplifier of TcR signals. The lack of Itk may therefore weakens the TcR signals necessary for γδ T cell negative selection and led to the survival of CD4⁺ γδ T cells in Itk null mice. It would be
interesting to investigate this question in the future studies. Since the natural ligands for γδ TCR is still unknown, Itk null mice in γδ TCR transgenic background will be needed to answer this question.

A number of studies have suggested that γδ T cells are able to rapidly secrete IL-4 both in-vivo and ex-vivo [149, 150]. It has also been suggested, but not directly shown, that these IL-4 producing γδ T cells participate in helping B-cells produce IgE [144, 156]. In particular, the presence of the CD4⁺ population of γδ T cells has been correlated with elevated levels of serum IgE. For example, mice with a LAT mutation have dramatically increased percentage of CD4⁺ γδ T cells, which produced large amounts of IL-4 when stimulated ex-vivo and are correlated with elevated IgE levels in these mice [144]. Similar findings have been reported for mice deficient in Itch, an E3 ubiquitin ligase, which have a population of IL-4 producing γδ T cells and are correlated to the elevated level of IgE in these mice, although these investigators did not determine if these cells expressed CD4 [212]. Our results now suggest that the CD4⁺ γδ T cell population is most likely responsible for the elevated serum IgE observed in these mice.

Both Itk and LAT are part of the same αβ TCR signaling complexes, with LAT lying upstream of Itk [213]. The increased population of CD4⁺ γδ T cells in both types of mice suggests that the γδ TcR signaling pathway modulates this pathway, and also suggests that Itk and LAT are also in the same γδ TcR signaling complexes. Besides the similarity, LAT mutant and Itk⁻/⁻ CD4⁺ γδ T also have some differences. The LAT mutant mice do not have higher numbers of γδ T cells until about 20-weeks, and the population only exists in the periphery [144]. In comparison, increased numbers of γδ T cells were found both in the thymus and periphery in young Itk⁻/⁻ mice and 20-week-old Itk⁻/⁻ mice did not show any further increases in γδ T cell number (data not shown). In addition, both WT and Itk⁻/⁻ CD4⁺ γδ T cells secrete large amounts of IL-4 when stimulated with anti-γδ TcR, suggesting that the lack of Itk does not affect the γδ TcR signaling
leading to IL-4 secretion. However, LAT mutant γδ T cells can only secrete IL-4 following stimulation with PMA and ionomycin, but not TcR cross-linking, indicating that the signaling pathway from the γδ TcR is defective in the absence of LAT for induction of IL-4 secretion [144]. LAT is upstream of Itk and this LAT mutation may affect Itk function or incorporation into or affect the signaling complex [144, 214]. Thus, these differences in γδ T cells between Itk and LAT mice may be due to differences in TcR signal strength.

One recent study has demonstrated that antigen naïve γδ T cells preferentially secrete IFNγ, while antigen experienced γδ T cells preferentially secrete IL-17 [152]. CD4+ γδ T cells in the spleen are CD44hi/CD62low, an effector phenotype, and thus may be predicted to preferentially secrete IL-17. However, neither WT nor Itk−/− CD4+ γδ T cells secreted IL-17 or IFNγ. In contrast, large amounts of IL-17 and IFNγ were secreted by CD4− γδ T cells, suggesting that CD4+ and CD4− γδ T cells may be two independent subsets of γδ T cells, and that CD4+ γδ T cells may secrete IL-4 by default. Consistent with this data, the CD4+ population of γδ T cells had dramatically increased IL-4 mRNA compared with CD4+ γδ T cells both in the thymus and spleen (Supplemental Fig. 2). The key transcription factors for differentiation of αβ T cells to Th1 or Th2 cells and subsequent cytokine secretion are T-bet and GATA-3. However, although splenic CD4+ γδ T cells had significantly higher levels of GATA-3 mRNA, there is little difference in the thymus. In addition, Itk−/− γδ T cells also expressed higher amounts of T-bet mRNA than WT cells. Since T-bet is largely only expressed in effector but not naïve γδ T cells, this increased T-bet in Itk−/− γδ T cells may reflect their effector like phenotype [215]. Eomesodermin is another T-box family transcription factor that has been shown to correlate with the development of innate CD8+ T cells in Itk−/− mice [61]. However, it is interesting that the expression of eomesodermin is dramatically decreased in Itk−/− γδ T cells. These data suggest that perhaps the ratio of transcription factors may be important for the development and function of CD4+/CD4− γδ T cells.
and the molecular mechanism involved in this process may be different from that seen in αβ T cells.

Several studies on allergic asthma have shown that γδ T cells, including Vγ1.1 bearing γδ T cells, contribute to allergic airway inflammation by producing IL-4 and thus inducing IL-4-dependent IgE and IgG1 responses [156, 216, 217]. We have shown that mice lacking Itk are resistant to developing allergic asthma, suggesting that despite the presence of the CD4⁺ γδ T cell population in these mice, this is insufficient to derive the development of allergic asthma if the conventional αβ T cell cannot secrete Th2 cytokines [3, 25, 32, 55, 62, 218]. Our data, however, do not rule out a contribution for such IL-4 secreting γδ T cells in the presence of normal Th2 cell development and secretion of Th2 cytokines by these cells. Our data also indicate that the consequence of targeting Itk for diseases such as allergic asthma should be approached with a nuanced understanding of its effects in all of the cells types in which it is expressed.
Figure 6-1. Increased γδ T cells in mice lacking Itk. A) Flow cytometric analysis of γδ T cells in the thymus, spleen and lymph node of WT and Itk−/− mice. B) The percentage and absolute number of γδ T cells were calculated from thymus and spleen. C) Flow cytometric analysis of CD4 and CD8 populations of CD3+γδTcR+ cells in the thymus, spleen and lymph nodes of WT
and Itk⁻/ₐ mice. D) The percentage of CD4⁺ γδ T cells, as well as the absolute number of CD4⁺ γδ T cells in the thymus and spleen of WT and Itk⁻/ₐ mice (n>10 mice, p*<0.01).
Figure 6-2. Surface phenotype of CD4⁺γδ T cells from WT and Itk⁻/⁻ mice. A) Thymocytes from WT and Itk⁻/⁻ mice were gated on either CD3⁺γδTcR⁺ cells (top) or CD4⁺CD3⁺γδTcR⁺ cells and CD4⁻CD3⁺γδTcR⁺ cells (bottom), and analyzed for the expression of CD44 and CD24. B)
Splenocytes from WT and Itk\textsuperscript{−/−} mice were gated on either CD3\textsuperscript{+}\textgammadelta TcR\textsuperscript{+} (top) or CD4\textsuperscript{+}CD3\textsuperscript{+}\textgammadelta TcR\textsuperscript{+} cells and CD4\textsuperscript{−}CD3\textsuperscript{+}\textgammadelta TcR\textsuperscript{+} cells (bottom) and analyzed for the expression of CD62L and CD44. NK1.1 is expressed, but is not required for the development of CD4\textsuperscript{+} \gamma\delta T cells in WT and Itk\textsuperscript{−/−} mice. C) Thymocytes and splenocytes from WT and Itk\textsuperscript{−/−} mice were analyzed for the percentage of NK1.1\textsuperscript{+}\gamma\delta T cells by flow cytometry (n=7-8 mice, p*<0.01). D) \gamma\delta TcR\textsuperscript{+}CD3\textsuperscript{+} cells in the thymus and spleens of WT, Itk\textsuperscript{−/−}, T-bet\textsuperscript{−/−}, or T-bet/Itk DKO mice were analyzed for the expression of CD4 and NK1.1 by flow cytometry. Data are representative of two independent experiments.
Figure 6-3. Enhanced development of \( \gamma \delta \) T cells in the absence of Itk is bone marrow intrinsic. Thy1.1^WT or Thy1.1^Itk^−/− bone marrow were intravenously injected to irradiated Thy1.2 WT and Itk^−/− mice. 8 weeks later, the percentage of CD3^+\( \gamma \delta \)TCR^+ cells of donor WT and Itk^−/− cells (A) and the percentage of CD4 and CD8 population on donor \( \gamma \delta \) T cells (B) were
analyzed. Data are representative of two independent experiments. C) 1:1 mixtures of bone marrow from CD45.1/Thy1.2 congenic WT mice and CD45.2/Thy1.2 congenic Itk\textsuperscript{-/-} mice were intravenously injected to irradiated Thy1.1 WT mice. 8 weeks later, the percentage of CD3\textsuperscript{+}\gamma\delta TcR\textsuperscript{+} cells of donor WT and Itk\textsuperscript{-/-} were analyzed. Data are representative of three mice.
Figure 6-4. Increased CD4$^+$ γδ T cells in the absence of Itk is independent of MHC class I or II expression. γδ T cells in the thymus and spleens of β2m$^{-/-}$, β2m/Itk DKO, MHCII$^{-/-}$ and MHCII/Itk DKO mice were analyzed for the expression of CD8 and CD4. Data are representative of two independent experiments.
Figure 6-5. γδ TcR expression of γδ T cells from WT and Itk^−/− mice. Thymocytes and splenocytes from WT and Itk^−/− mice were gated on γδ T cells and analyzed for the expression of A) Vγ2 vs. γδ TcR (left), Vγ2 vs. CD4 (right); B) NK1.1 vs. Vδ6.2/3 (left), CD4 vs. Vδ6.2/3 (right); or C) Vγ1.1 vs. CD4. Data are representative of two independent experiments.
Figure 6-6. Itk null CD4⁺ γδ T cells develop independent of the γδ TcR they express. A) γδ T cells in the thymus and spleens of WT and Itk⁻/⁻ mice were analyzed for the expression of Vγ1.1 and Vδ6.2/3. Data are representative of three independent experiments. B) γδ T cells in the thymus and spleens of KN6 transgenic mice, or KN6 transgenic mice lacking Itk were analyzed for the expression of CD4 and CD8. Data represent two independent experiments.
Figure 6-7. Itk null CD4$^+$ γδ T cells secrete IL-4 and induced B cell class switch to IgE. A) Sorted CD4$^+$CD3$^+$γδTCR$^+$ cells and CD4$^+$CD3$^+$γδTCR$^+$ cells from WT and Itk$^{-/-}$ mice were stimulated with anti-γδTCR for 3 days. Supernatants were then analyzed for IL-4, IFNγ and IL-17. Data are representative of three independent experiments. Serum concentrations of IgE antibody in unimmunized mice. B) Sera from the indicated mice were analyzed for IgE concentration. C) Rag$^{-/-}$ mice were reconstituted with different groups of B cells and T cells as indicated. Sera were analyzed for IgE 4-weeks after transfer.
Figure 6-8. Itk<sup>−/−</sup> γδ T cells carry increased IL-4 mRNA. A) CD3<sup>+</sup>γδTCR<sup>+</sup> cells were sorted from the thymus of WT and Itk<sup>−/−</sup> mice and mRNA for T-bet, eomesodermin, GATA-3, IFN<sub>γ</sub> and IL-4 were analyzed.
IL-4 were analyzed by quantitative real-time PCR. B) CD3$^{+}$γδTCR$^{+}$ cells from the spleens of WT and CD4$^{+}$CD3$^{+}$γδTCR$^{+}$ cells and CD4$^{+}$CD3$^{+}$γδTCR$^{+}$ cells from Itk$^{-/-}$ mice were sorted and mRNA for T-bet, eomesodermin, GATA-3, IFNγ and IL-4 were analyzed by quantitative real-time PCR. Data were analyzed using the comparative threshold cycle ΔΔCT method, normalized to the expression of GAPDH and the relative gene expression levels were determined as fold increase compared to WT γδ T cells, which was set as 1. Data are representative of two independent experiments.
Chapter 7

Discussion and future directions

7.1 Discussion

7.1.1 Comparison of BiFC and FRET

We show here that an intramolecular monomeric conformation of Itk exist when Itk is in the inactive state in vivo, with the PH domain less than 80 Å from the C-terminus. Zn$^{2+}$ coordinating residues in the TH domain, not the proline rich region control this intramolecular interaction. We also found that Itk forms clusters in the cell membrane when Itk is activated and the localization of Itk in the cell membrane is specific.

In these studies, we mainly used the BiFC assay to determine the conformation of Itk in-vivo and used FRET assay to confirm our conclusion. These two methods are widely used in the research of protein-protein interaction in-vivo and could visualize protein interactions directly in living cells. Each of them has its own advantages and limitations.

One advantage of the BiFC approach is that the reconstituted fluorescent protein has strong intrinsic fluorescence, which is stable and can be observed over long time in living cells without significant change. Compared to FRET assay, which is sensitive to the conformational change of the protein, BiFC does not require the information of the structure of the proteins and can be used to detect weak interactions between proteins [219]. In addition, the operation of BiFC assay is simple and the requirement for equipment is low, with standard flow facility and fluorescence microscopy. Furthermore, recent development of BiFC techniques allow the
visualization of multiple protein interactions based on the formation of distinct reconstituted fluorescent proteins [220]. The biggest limitation of the BiFC approach is that fluorophore maturation requires relatively long times, usually several hours [219]. In our case, low temperature (30°C) is required for the maturation of YFP fluorophore requires, which may not exactly mimic the physiological environment of the living cells. Recent developments in BiFC methods have led to improvements in the YFP fluorophore maturation at 37°C, however the long maturation time is still needed [220]. In addition, once the fluorescence protein is reconstituted, it is irreversible [219]. All of these limitations prevent the application of BiFC in real-time detection of rapid changes in protein-protein interactions.

FRET, in contrast, is widely used to detect the interaction dynamics between two proteins and the exact distance between two proteins can be calculated by FRET software [221]. These features make FRET a better assay to detect the real-time interaction among proteins. The drawbacks of FRET are that it could not detect the weak interactions and generally needs the overexpression of the fusion proteins. In addition, FRET assay is technically demanding and requires advanced fluorescence microscope.

The combination of these two methods in studies could provide more information and be complimentary to each other. In our studies, we mainly used BiFC assay, which requires only standard flow cytometric machine and fluorescence microscope and is easy to master. We also used FRET assay to confirm the conclusion from BiFC assay, which is that Itk exists in an intramolecular folded conformation in the cytoplasm. Thus the conclusion is more convincing.

### 7.1.2 The conformation of Itk and other Tec family kinases

According to our data, we suggest that when Itk is in an inactive state, Itk could exist as an intramolecular folded conformation, and the BH motif in the TH domain is important for
maintaining this conformation. Combining the NMR structures of Itk PRR, SH3 and SH2 domain, the crystal structure of Itk kinase domain and the crystal structure of Btk PH and BH motif (which should be very similar to Itk), we draw a model of the structure of Itk in the inactive state. (Fig. 7-1)

Since the BH motif is important for maintaining the intramolecular folded conformation of Itk, it may interact with another region of Itk. Based on the size and sequence of each domain, SH2 and kinase domain are the most possible candidates. Further study is needed to find out the possible interactions.

The crystal structure of full length Tec family kinases has not been yet reported. According to the NMR studies of individual domains of Tec family kinases, different conformation and domain interactions have been proposed. Both intramolecular and intermolecular interactions between PRR and SH3 domain have been found in Btk and Tec [12, 222, 223]. The intermolecular interaction but not intramolecular interaction was also detected in Txk [224]. All of these studies focused on the potential interactions between PRR and SH3 domain, whereas it has not been examined whether the unique Tec family domain-BH motif has interactions with other domains or regions of Tec family kinases. Our work demonstrates the potential role of BH motif in the conformation of Itk. This may also apply to Btk, Tec and Bmx, all of which have the BH motif.

7.1.3 The role of Itk and Txk in the development of innate T cell populations

Unlike conventional αβ T cells, innate T cells are T cells containing immediate effector functions, which include NKT cells, γδ T cells, H2-M3- specific T cells, CD8αα T cells, innate-like CD4 and CD8 αβ T cells and etc [225]. The mechanisms of development of innate like T cells are not well understood as conventional T cells.
The role of Itk and Txk in the development of conventional or innate like CD4 and CD8 αβ T cells has been reported recently [58, 60, 61, 64]. Itk and Txk are only required for the development of conventional CD4 and CD8 αβ T cells, but not for the development of innate like CD4 or CD8 αβ T cells, suggesting the different signaling requirement in conventional and innate-like αβ T cells [58, 60, 61, 64]. Here we analyzed the role of Itk in the development of two other innate T cell populations: iNKT cells and γδ T cells. We and others showed that Itk and Txk are important for the development of iNKT cells [24, 62, 226]. In the absence of Itk and Txk, the frequency and numbers of iNKT cells decrease significantly, in addition to defects in the final maturation of iNKT cells. Furthermore, Itk regulates the development of iNKT cells partly independent of its kinase activity (Fig. 7-2). In contrast, Itk affects the development of γδ T cells in a different way. In the absence of Itk, the frequency and numbers of γδ T cells increase significantly, which is due to the increased population of CD4+ γδ T cells. The Itk−/− CD4+ γδ T cells exhibit a mature phenotype in the thymus and express activation markers in the peripheral lymphoid organs. All these data demonstrate that distinct innate cell populations have different developmental mechanisms or different signaling threshold for their development.

Itk−/− innate like CD4 and CD8 T cells show high transcription level of eomesodermin, which are suggested to play role in the development of innate like CD4 and CD8 T cells [58, 60, 61, 64]. Here we and others show that T-bet expression level is decreased significantly in Itk−/− iNKT cells, which may contribute to the developmental defect of NKT cells in these mice [24, 62]. We also show here that Itk−/− iNKT cells have dramatically increased mRNA level of eomesodermin compared to WT iNKT cells. The ratio of T-bet/eomesodermin are rescued with the expression of kinase domain deleted Itk. Itk−/− γδ T cells exhibit high mRNA level of T-bet but low mRNA level of eomesodermin. Together, these data suggest that Itk regulates the expression of T-bet and eomesodermin in different ways in distinct innate populations, and indicate that the
ratio of these two T-box transcription factors may be important for the development of innate T cell populations.

In the absence of Itk, the function of innate like CD4 and CD8 T cells is still intact [58, 60, 61, 64]. However, both IL-4 and IFNγ secretion of Itk−/− iNKT are defective in response to TcR stimulation or α-GalCer stimulation, suggesting that Itk play important role in iNKT cell function [24, 62]. Itk−/− γδ T cells show normal IL-4 and IL-17 secretion, but have defect to produce IFNγ upon γδTcR stimulation. These data implicate that innate cell populations have different signaling requirements for their function.
7.2 Future directions

7.2.1 Dynamics of Itk during TcR stimulation

A lot of studies on the role of Itk in T cell signaling have focused on the activation of Itk and downstream signaling pathways it regulates [12]. Little is known about the temporal and subcellular localization of Itk upon and after activation, such as when Itk localizes to cell membrane and get activated, how long the activation of Itk lasts, when Itk is inactivated and leaves cell membrane, the real-time movements of Itk during this process. Studying the dynamics of TcR-proximal signaling protein usually requires fluorescence microscopy in which T cells are stimulated on coverslip coated with antibody to TcR. But it is difficult to tell the exact time when the signaling proteins localize in the cell membrane.

The work presented here shows that Itk only forms dimer/clusters when it is localized in the cell membrane. Thus, by using FRET pair of CFP-Itk and YFP-Itk, the dynamics of the subcellular localization of Itk upon TcR stimulation could be studied under fluorescence microscope based on the FRET values of Itk pair. Since the cell membrane localization is required for the activation of Itk, it will also give us the temporal and spatio information about the activation status of Itk.

7.2.2 The role of Txk in iNKT cell and γδ T cell development and function

Because of the functional redundancy among Tec family kinases, the role of Txk in T cell signaling is studied mostly by comparing Itk+/−/Txk+/− T cells to Itk−/− T cells. When compared to Itk−/− T cells, Itk+/−/Txk+/− T cells have a more severe defect in TcR induced PLC-γ1 activation and subsequent calcium influx, suggesting a role for Txk in TcR signaling [42]. Mice defective in both Itk and Txk showed exacerbated defects in the development of conventional CD8 αβ T cell,
as well as positive and negative selection, demonstrating that Txk also has functions in the development of T cells [60, 61, 227]. Furthermore, a recent study from our lab revealed that Tg(CD2-Txk)/Itk<sup>−/−</sup> transgenic mice, which overexpress Txk to the similar level of Itk in Th2 cells, rescue the defective Th2 response, strongly suggesting that different roles of Itk and Txk on Th2 cell function may result from their differential patterns of expression, but no intrinsic distinct function [57].

The role of Txk in the development of iNKT cells has been studied by comparing Itk/Txk DKO mice to Itk null mice [24]. However, whether Txk has the similar function in iNKT cell development, homeostasis and function as Itk and could replace the role of Itk is still not clear. In order to answer this question, both Tg(CD2-Txk)/Itk<sup>−/−</sup> transgenic mice and Tg(CD2-Itk)/Itk<sup>−/−</sup> transgenic mice could be used. In the Tg(Lck-Itk)/Itk<sup>−/−</sup> transgenic mice, the expression of Itk is lower than that in the endogenous level, which is about 25-30% of Itk in WT mice [64]. The frequency, numbers, maturation status, function of iNKT cells could be determined in these two types of mice and compared with WT and Itk<sup>−/−</sup> mice. In addition to answering the question above, we could also find out whether lower expression of Itk will affect iNKT cell development and function. This will give us a thorough understanding of the role of Itk and Txk in iNKT cells and the molecular mechanism involved.

Our work here demonstrates the role of Itk in γδ T cell development and function. Whether Txk also plays a role in γδ T cell development and function is unclear. The Txk/Itk DKO mice, Tg(CD2-Txk)/Itk<sup>−/−</sup> transgenic mice and Tg(Lck-Itk)/Itk<sup>−/−</sup> transgenic mice could be used to solve this problem.

These studies will give us a further understanding of the role of Txk in iNKT cells and γδ T cell development and function.
7.2.3 Mechanism of the increased CD4$^+$ γδ T cells in the absence of Itk

Our data show that Itk null mice exhibit increased amount of CD4$^+$ γδ T cells. Why deficiency in Itk leads to increases in this particular subset of γδ T cells still need to be investigated.

Studies have shown that negative selection is required for the development of γδ T cells [133, 134]. The subset of CD4$^+$ γδ T cells in WT mice is very small. This may be due to the negative selection, which eliminates most of the CD4$^+$ γδ T cells because of the strong TcR signal strength. In the absence of Itk, the γδ TcR signaling strength is proposed to be weaker. It is possible that the TcR signal of CD4$^+$ γδ T cells in the absence of Itk is weaker such that much more CD4$^+$ γδ T cells survived in Itk$^{-/-}$ mice (Fig.7-3). In order to test this hypothesis, we are now crossing Itk$^{-/-}$ mice with KN6 γδ TcR transgenic mice in C57/BL, Balb/c and β2M$^{-/-}$ background. The CD4$^+$ γδ T subset in KN6 transgenic mice and Itk null KN6 mice of different backgrounds will be determined. If the increased CD4$^+$ γδ T cell population is found in mice with weaker transgenic TcR signal, it will confirm the hypothesis that Itk involves in the development of CD4$^+$ γδ T cells through negative selection. In addition, more CD4$^+$ γδ T cells are provided in KN6 transgenic mice, which will help the biochemical analysis on the signaling pathway of WT and Itk$^{-/-}$ γδ T cells. In order to determine whether Itk also regulate the homeostasis of CD4$^+$ γδ T cells, the proliferation and apoptosis of CD4$^+$ γδ T cells in the WT and Itk null mice in the thymus and peripheral lymphoid organs will also be analyzed.

We analyzed several transcription factors in WT and Itk null γδ T cells. Although differences between WT and Itk null CD4$^+$ γδ T cells and CD4$^-$ γδ T cells were found, their roles in the development and function of CD4$^+$ γδ T cells are not clear. In order to find out which transcription factors that Itk regulates in γδ T cell development, an OP9-DL1 cell culture system could be used. The OP9-DL1 culture system is an in-vitro model for T cell development in which
delta-like 1 is expressed by the OP9 stromal cell line and activate the Notch pathway [228]. This system has been used to study the differentiation of various mature T subsets from progenitors, including γδ T cells [228, 229]. Itk^−/+ or WT bone marrow cells will be cultured with OP9-DL1 cell line to see whether CD4^+ γδ T cells still appear in an increased level in the absence of Itk. If the OP9-DL1 system could repeat the development of Itk^−/+ CD4^+ γδ T cells, we could then further check the role of different signaling molecules downstream of Itk in this process.

These studies should give us a better picture of the molecular mechanism of Itk in γδ T cell development.
Figure 7-1. Model of the conformation of Itk in the inactive state
Figure 7-2. Itk and Txk are important for the final maturation of iNKT cells. Itk regulates the final maturation of iNKT cells in both kinase independent and kinase dependent manner. Txk also regulates the development of iNKT cells.
Figure 7-3. A possible model of the role of Itk in γδ T cell development. In the absence of Itk, the TcR signaling strength in γδ T cells is proposed to be weaker, which may help more CD4⁺ γδ T cells escape negative selection.
References

123


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