The Pennsylvania State University

The Graduate School

AGRICULTURAL SCIENCES

ROLE OF TYROSINE KINASE ITK IN SKIN ALPHA BETA T CELLS

A Thesis in

Pathobiology

by

Yuting Bai

Submitted in Partially Fulfillment

of the Requirements

for the Degree of

Master of Science

May 2011

The thesis of Yuting Bai was reviewed and approved* by the following:

Avery August

Distinguished Adjunct Professor of Immunology (Penn State)

Professor and Chair Department of Microbiology & Immunology (Cornell University)

Thesis adviser

Margherita Cantorna

Professor of Molecular Immunology

Na Xiong

Assistant Professor of Molecular Immunology & Infectious Diseases

Anthony Schmitt

Assistant Professor of Molecular Immunology & Infectious Diseases

Director of Pathobiology Graduate Program

* Signatures are on file in the Graduate School.

Abstract

Itk, a member of the Tec family of tyrosine kinases, plays an important role in T cell development and differentiation. Itk regulates the development of αβ T cells such that positive selection and negative selection in the thymus are defective in absence of Itk. Itk is also important in the development and homeostasis of innate-like γδ T cells in the skin. In this thesis, we focused on skin αβ T cells and investigated the role of Tec kinase family members, Itk and Txk, in αβ T cell homoeostasis in skin intraepithelial T lymphocytes. For this purpose, we characterize αβ T cell populations in Itk deficient mice compared to WT mice. We found that the total cell number and the percentage of $CD4^+$ T cells in Itk KO mice are significantly higher than those in WT mice. No significant difference was found in skin $CD8^+$ T cells. We also used mice that express human Itk or murine Txk only in T cells on the $Itk^{-/-}$ background to determine whether this would rescue the difference we observe between WT and Itk KO mice. We found that T cell specific expression of both human Itk, and mouse Txk could restore the $CD4^+$ population (percentage) in Itk KO mice. We also compared WT and Itk KO mice on a Balb/c background. The data did not show significant differences in the percentage of $CD4^+$ and $CD8^+$ T cells in these mice. In addition, we used CD44 and CD122 surface marker to characterize the phenotype of $CD4^+$ and $CD8^+$ T cells in skin and found that both WT and Itk KO have high CD44 expression, resembling activated or memory T cells. Thus this suggests that Itk may play an impotent role in development and or homeostasis of skin αβ T cells.

TABLE OF CONTENTS

LIST OF FIGURES

Acknowledgments

I would first like to give my thanks to my advisor, Dr. Avery August. He gave me great support and confidence in my graduate life. I appreciate the excellent environment and freedom that he provided to me. He is very patient and has given me a lot of help in my projects and my thesis.

Thanks to other thesis committee members: Dr. Margherita Cantorna, Dr. Na Xiong, for their input of time, direction and thoughtful suggestions throughout my thesis work.

I would also like to thank my past and current lab members: Meg Potter, Margaret Kensinger, Jianfang Hu, Elizabeth Walsh, J. Luis Morales, Qian Qi, Fei Huang, Weishan Huang, Man Kit Law, Arun Kumar, Sonia Mohinta, Chavez Carter, Kindra Stokes, Amie Wood, Tina Chew. They have been extremely supportive, in coordination on experimental schedules, and being good listeners to my troubles and findings. Thanks for the members in the lab of Henning and ASI building of Penn State University, especially, Dr. Xiong's lab, Dr. Cantorana's lab, Dr. Schmitt's lab, Dr.Paulson's lab, and Dr. Hankey's lab. Thanks for the member in the lab of Cornell University College of Veterinary Medicine. They generously allow me to use their facility and give me technical assistance and support.

I am especially thankful for my family, my parents, my husband and daughter, my brothers, for their great love and supports both in my life and study. I would like to thank all my friends for their support and encouragement.

Finally, I would like to give my heartfelt thanks to all of the people that directly and indirectly contributed to my accomplishments during my studies. I wish all of them health, happiness, peace in their life.

ACKNOWLEDGMENT

I would first like to give my thanks to my advisor, Dr. Avery August. He gave me great support and confidence in my graduate life. I appreciate the excellent environment and freedom that he provided to me. He is very patient and has given me a lot of help in my projects and my thesis.

Thanks to other thesis committee members: Dr. Margherita Cantorna, Dr. Na Xiong, for their input of time, direction and thoughtful suggestions throughout my thesis work.

I would also like to thank my past and current lab members: Meg Potter, Margaret Kensinger, Jianfang Hu, Elizabeth Walsh, J. Luis Morales, Qian Qi, Fei Huang, Weishan Huang, Man Kit Law, Arun Kumar, Sonia Mohinta, Chavez Carter, Kindra Stokes, Amie Wood, Tina Chew. They have been extremely supportive, in coordination on experimental schedules, and being good listeners to my troubles and findings. Thanks for the members in the lab of Henning and ASI building of Penn State University, especially, Dr. Xiong's lab, Dr. Cantorana's lab, Dr. Schmitt's lab, Dr.Paulson's lab, and Dr. Hankey's lab. Thanks for the member in the lab of Cornell University College of Veterinary Medicine. They generously allow me to use their facility and give me technical assistance and support.

I am especially thankful for my family, my parents, my husband and daughter, my brothers, for their great love and supports both in my life and study. I would like to thank all my friends for their support and encouragement.

Finally, I would like to give my heartfelt thanks to all of the people that directly and indirectly contributed to my accomplishments during my studies. I wish all of them health, happiness, peace in their life.

Chapter 1

Introduction

1.1 **Role of Itk in T cell development and differentiation**

1.1.1 **T cell development and differentiation**

T cells play a central role in the adaptive immune system and can be distinguished by the expression of the T cell receptors (TCR). The thymus is the organ that supports the development of T cells, at which stage they are called thymocytes. T cell precursors migrate into the thymus from bone marrow, These T cell precursors, which lack expression of coreceptors, CD4 and CD8, are called double negative (DN) $CD4^-CD8^-$ cells. The DN cells progress through DN1 to DN4. During the DN stage, most of the DN thymocytes express pre-TCR-α on DN3 stage and pair with a rearranged TCR-β chain. They will further proceed to the αβ T cell development pathway (1-3). A small percentage of DN2/DN3 thymocytes, on the other hand, make successful γ and δ TCR rearrangements become γδ TCR expressing T cells. During late DN3 and DN4 stages, the pre-TCR-α is lost and a low level of mature of αβ TCR assembled with CD3 chains along with the CD3/ζ chain is display on the cell surface. Those thymocytes also begin to express the coreceptors CD4 and CD8 to develop into double-positive (DP; $CD4^+CD8^+$) thymocytes (4). During this process DP thymocytes must express TCR that can recognize self major histocompatibility (MHC) self peptide complex to escape a default fate of programmed cell death. 90% of DP thymocytes express TCRs with poor or strong affinity to self peptide MHC ligands and therefore die by neglect or by negative selection, respectively (Figure1-1).

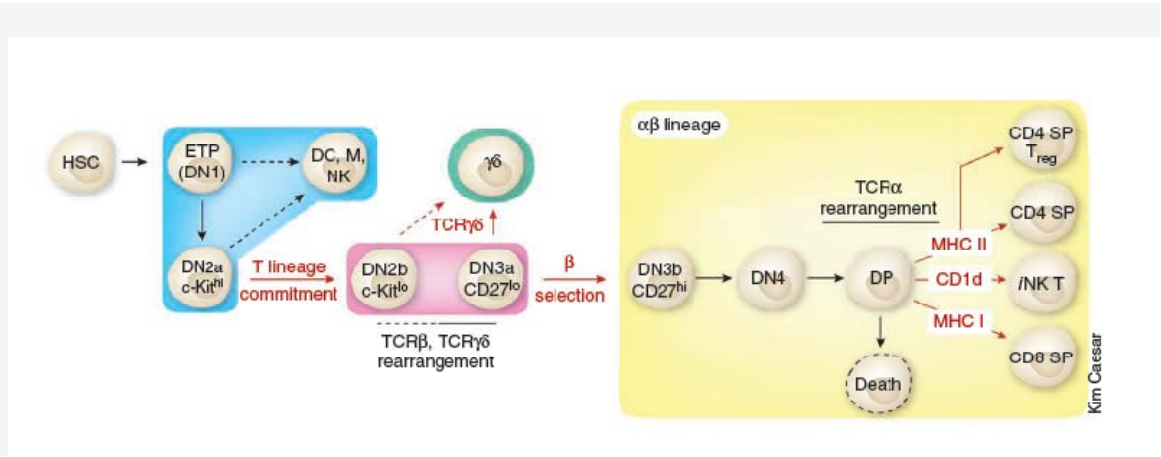


Figure 1-1: Overall Scheme of T cell development. The early T cell progenitors (ETP) derive from hematopoietic stem cells (HSCs) in the bone marrow. Upon entry into the thymus, the coreceptors are not expressed during the DN early stage. Rearranging γ and δ TCR during DN2/DN3, a small percentage of thymocytes become γδ T cells. Expression of the pre-TCR leads the cells to progress from DN to DP stage. DP thymocytes must express a TCR that can recognize MHC-self peptides complex to escape a default fate of programmed cell death.

(From A. C. Carpenter & R. Bosselut. 2010. Decision checkpoints in the thymus. *Nat Immunol.* 11:666-73)

1.1.2 T cell receptor induced signaling pathways

When the TCR is activated, a Src family tyrosine kinase, Lck, is activated, leading to the phosphorylation of immunoreceptor tyrosine-based activation motif (ITAMs) on the intracellular side of the TCR-CD3 complex. This leads to the recruitment of Syk family kinase member ζ-chain associated protein kinase (Zap-70) and subsequent phosphorylation of the adaptor proteins, such as LAT and SLP-76. The Tec kinase family member, Itk is recruited to the membrane though its PH domain (5-7) and interacts with the phosphorylated SLP-76/LAT adapter complex via its Src homology (SH3 and SH2) domains (3, 4, 8). This permits the phosphorylation of Itk on its activation loop (Y^{511}) by Lck (9). Upon activation, Itk further phosphorylates phospholipase Cγ1 (PLCγ1), which leads to its activation, hydrolysis of PIP2 into IP3, which is required for Ca^{2+} mobilization. PLCγ1 also releases DAG, which activates PKC and Ras GRP, thereby leading to activation of MAPK pathways, as well as the downstream transcription factors such as NF-κB, etc. The latter factors regulate genes involved in cytokine signaling, survival and differentiation (2, 10, 11). Subsequent to Ca^{2+} mobilization, the transcription factors nuclear factor of activated T-cell (NFAT) is activated and induces the activation of a number of genes essential for lymphocyte activation, such as IL-2. Thus, Itk can affect multiple processes important for T cell lineage development, activation and homeostasis. Impairments in Itk activation can affect several downstream events in T lymphocytes (Figure 1-2).

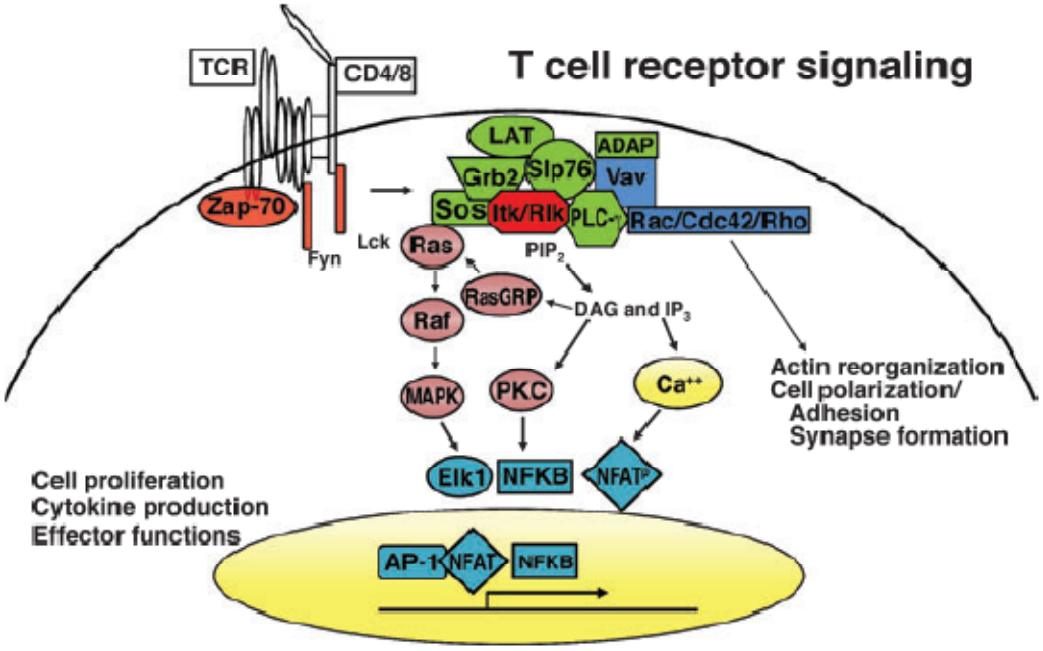


Figure1-2. Role of Itk in T-cell receptor (TCR) signaling. After TCR activation, one of the first events is the phosphorylation of the ITAMs on the intracellular side of the TCR-CD3 complex by Lck. ZAP70 is recruited to the TcR-CD3 complex and becomes active, which promotes recruitment and phosphorylation of downstream adaptor or scaffold proteins. Phosphorylation of SLP-76 by ZAP70 promotes recruitment of Vav, and the adaptor proteins Nck and GADS and Itk. Itk then phosphorylates PLCγ1 which leads to its activation. Activation of PLCγ results in hydrolysis of PIP2 and generation of second message DAG and IP3. DAG activates PKC, which in turn activates Ras, a GTPase that activates Raf leading to recruitment of the MAP kinase cascade. IP3 release calcium from intracellular stores in the ER and activates the Ca^{+} pathway to further activate the transcription factors.

(From J. A. Readinger, K. L. Mueller, A. M. Venegas, R. Horai, P. L. Schwartzberg. 2009. Tec kinases regulate T-lymphocyte development and function: new insights into the roles of Itk and Rlk/Txk. Immunological Reviews, 228: 93-114)

1.1.3 **Itk kinase domain structure**

The Tec family kinases are the second largest of non-receptor tyrosine kinases next to the Src family kinases and share a similar domain organization. This family has five members: Tec, Bruton's tyrosine kinase (Btk), Inducible T-cell kinase (Itk), resting lymphocyte kinase (Rlk/Txk), and bone marrow-expressed kinase (Bmx/Etk) (12). In T cells, three Tec kinases are expressed, Itk, Rlk / Txk, and Tec. Itk is composed of five main domains: from N-terminus to C-terminus, a pleckstrin homology (PH) domain, following by a Tec homology (TH) domain which contains a Zn^{2+}-binding Btk homology (BH) motif and one proline-rich region (PRR), a homology 3 (SH3) and a Src homology 2 (SH2) domain, and a carboxyl- terminal kinase domain. Btk and Tec have an additional secondary PRR next to their BH motifs. Bmx does not have a PRR, while Txk has a cysteine-string motif, instead of a PH domain (Figure1-3).

Figure 1-3: Structure of Itk structure. The Itk modular structure includes N-terminal Pleckstrin homology (PH) domain; Tec homology (TH) domain contains BH and PRR domains, Src homology 3 (SH3) domains, Src homology 2 (SH2) protein interaction domain, C-terminal kinase catalytic domain.

(From A. H. Andreotti, P. L. Schwartzberg, R. E. Joseph, et al. 2010. T-Cell Signaling Regulated by the Tec Family Kinase, Itk. Cold Spring Harb Perspect Biol; 2:a002287)

1.1.4 **Role of Itk in T cell development**

TCR signaling plays a critical role in the development of T cells within the thymus. Previous studies showed 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 (13. 14). The thymocytes of defected Itk have impaired calcium influx and reduced activation of ERK1/2 upon TCR stimulation (13). Optimal calcium signaling is important for both positive selection and negative selection, and ERK signaling pathway is required for positive selection (15). Several recent studies show the role of Itk in $CD4^+$ and $CD8^+$ cells development. Itk has been shown to be required for the development of conventional or naïve phenotype ($CD44^{low}$) $CD8^+$ and $CD4^+$, but not for the development of innate or memory phenotype ($CD44^{high}$) $CD8^+$ and $CD4^+$ T cells (16-20). In the absence of Itk, around 85% of $CD8^+$ T cells exhibit memory phenotype ($CD44^{hi}$ and increased expression of CD122). These memory phenotype CD8 T cells carry large amounts of preformed message for IFNγ and rapidly secrete large amounts of IFNγ upon activation (16, 18, 19, 21). Compared to the severe development 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 (22). The total number of $CD4^+$ T cells in Itk KO mice is decreased by roughly 60% in spleen and 40% in draining lymph nodes. The total number of $CD8^+$ T cell in spleen is slightly decreased and total $CD8^+$ T cell number in the draining lymph node remains similar in Itk KO mice compared to WT mice. Itk is also found to be required for the development and function of invariant nature killer T (NKT) cells (23, 24).

1.1.5 **Role of Itk in T cell differentiation**

Itk signaling plays a critical role in regulating T cell differentiation and T cell effector function (12). There are three major lineages of effector T cells, Th1, Th2 and Th17. Multiple cytokines and transcription factors are involved in Th- cell development. These subsets are defined mainly by their unique cytokine profiles. For example, Th1 cells express interferon-γ (IFN-γ), IL-2 and lymphotoxin. Th2 cells produce IL-4, IL-5, IL-10 and IL-13. Th17 cells secrete IL-17, IL-21, and IL-22 (25). Unlike Th1 cells, which coexpress Itk and Rlk, Th2 cells express only Itk. The status of Rlk expression in Th17 cells is currently unknown (12). Itk deficiency affects the development of various T cell populations differentially (20, 26-29). Other studies highlighted the importance of Itk signaling in the generation of Th2 response. In the absence of Itk, NFAT activity is greatly reduced; leading to impaired production of IL-4 by Th2 cells (Fig1-4).

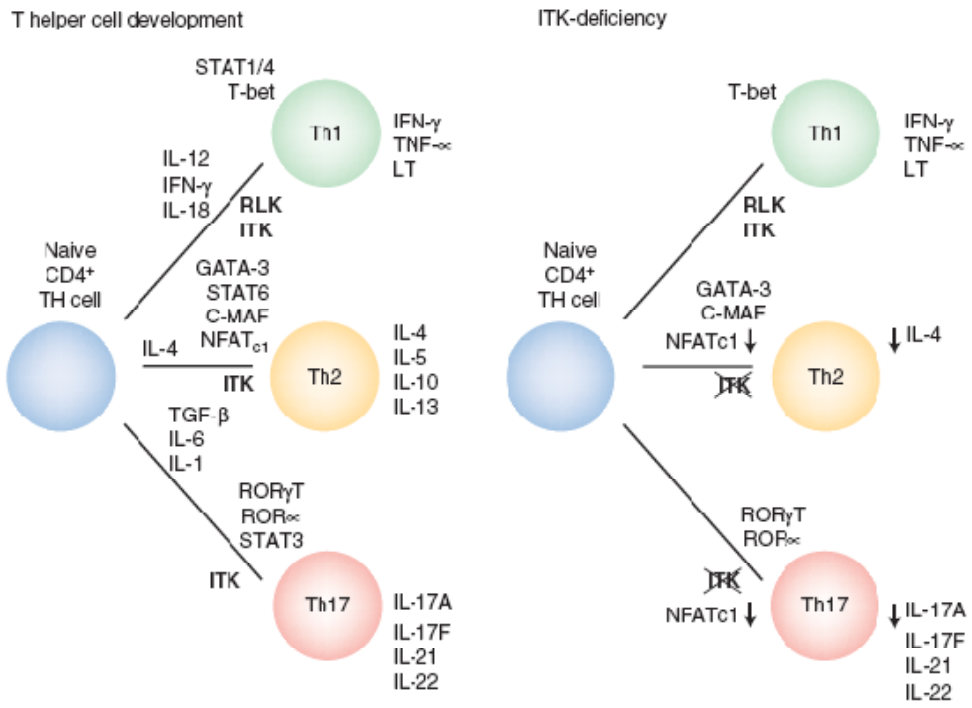


Figure 1-4. Itk function in T helper cell differentiation. Left, naïve $CD4^+$ T helper (Th) cells develop to three major lineages: Th1, Th2 and Th17. This is regulated by multiple cytokines and transcription factors. Right, absence of Itk results in impaired function of Th2 and Th17 cells, which produce low levels of IL-4 or IL-17.

(From A. H. Andreotti, P. L. Schwartzberg, R. E. Joseph, et al. 2010. T-Cell Signaling Regulated by the Tec Family Kinase, Itk. Cold Spring Harb Perspect Biol; 2:a002287)

1.2 αβ and γδ T cells in skin

1.2.1 Skin structure and cellular effectors

The skin is an important immune organ between the body and the environment. It provides a first line of defense to protect the body from injury and infection. There are two primary layers in mammalian skin. The epidermis has a simple histology and Langerin-expressing LCs populate the outer epidermis layer. The underlying dermis is anatomically more complicated, with greater cell diversity. It contains many specialized immune cells, including dendritic cells (DCs), $CD4^+$ T helper (Th) cells, γδ T cells and NKT cells (Figure 1-5).

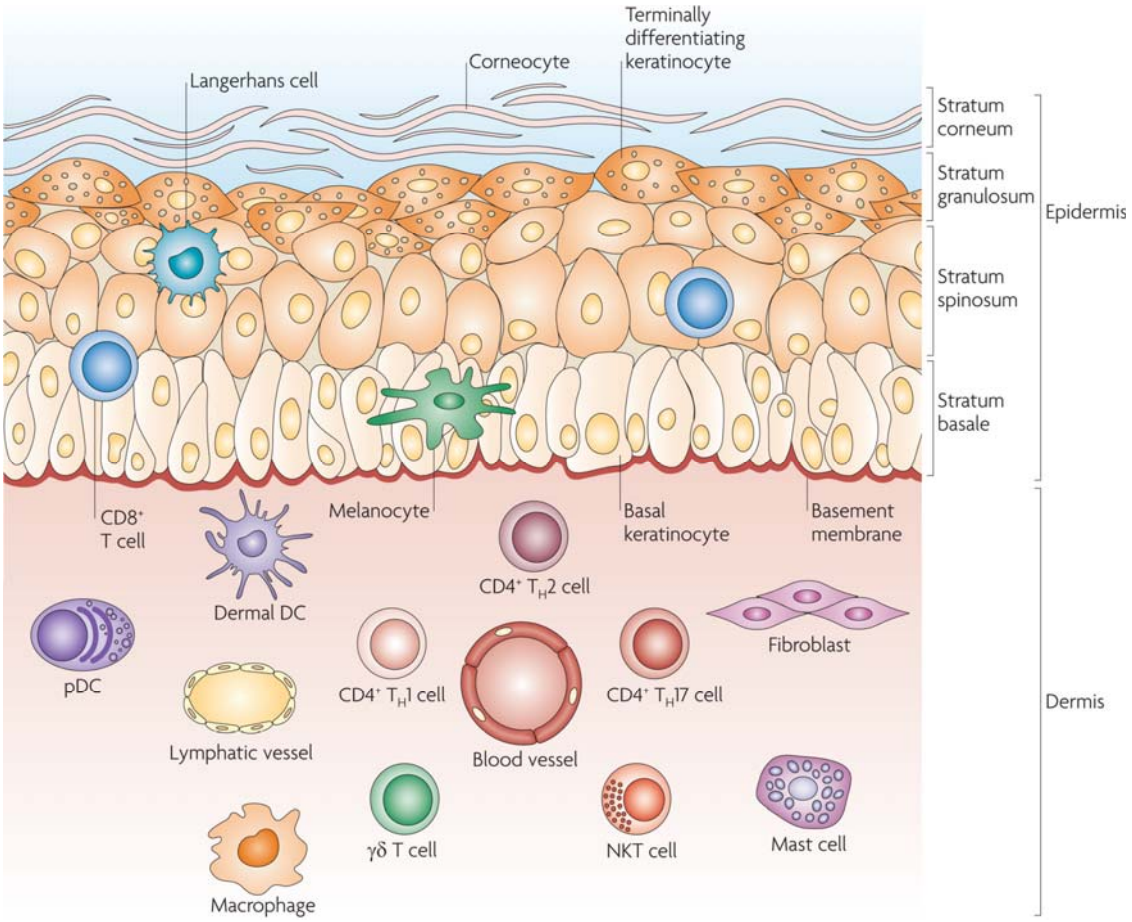


Figure 1-5. The general structure of skin is composed of the outer epidermis layer and the underneath dermis layer. The epidermis contains the stratum basale, the stratum spinosum, the stratum granulosum and the stratum corneum. Specialized cells in the epidermis include melanocytes and Langerhans cells, $CD8^+$ cytotoxic T cells can be found in the stratum basale and stratum spinosum. The dermis is made of collagen, elastic tissue and reticular fibers. It contains many specialized immune cells, including DCs, $CD4^+$ T helper (Th) cells, T cells and natural killer T (NKT) cells. Moreover, macrophages, mast cells, fibroblasts and nerve-related cell types are also present.

(From F. O. Nestle, P. Di Meglio, J.-Z. Qin, and B. J. Nickoloff. 2009. Skin immune sentinels in health and disease. *Nat Rev Immunol*. 9(10): 679–691)

1.2.2 Conventional T cells in the skin

It was shown that normal epidermis harbors a phenotypically heterogeneous population of T cells, most of which are γδ T cells; there are some memory αβ T cells in mice, and most of which are memory $CD8^+$ αβ T cells in human (30, 31). In the dermis, T cells are preferentially clustered around postcapillary venules and are often situated just beneath the dermal–epidermal junction or adjacent to cutaneous appendages (32). Previous studies have found the three main types of $CD4^+$ Th cells, Th1, Th2 and Th17 cells, in the skin during various inflammatory diseases. Th1 and Th17 cell responses were associated with autoimmunity and immune-mediated pathologies, such as psoriasis, whereas Th2 cell responses were linked to allergic diseases, such as asthma and atopic dermatitis. Recent reports have shown that Th17 cells are essential for the first line defense against various fungal and bacterial infections (33).

1.2.3 Unconventional T cells in the skin

The different tissue-specific γδ T cells preferentially use different subsets of TCRs. In mice, a prototype of the tissue-specific T cells is skin-specific intraepithelial γδ T lymphocytes. In the epidermal epithelium almost all of γδ T cells express an identical γδ TCR composed of Vγ3–Jγ1Cγ1 and Vδ1–Dδ2–Jδ2Cδ chains (34, 35).

A recent study found that among different thymic γδ T cell subsets, fetal thymic precursors of skin-specific intraepithelial γδ T lymphocytes specifically acquire a unique skin-homing property after positive selection, suggesting an important role of the TCR

selection signaling in “programming” them for tissue-specific development (36).

1.2.4 Role of Itk in skin T cell development

It is reported that multiple TCR signaling molecules, like Lck, Syk, and ZAP-70 are important for skin-specific intraepithelial γδ T lymphocytes development in various knockout mice (36). For example, in ZAP-$70^{-/-}$ mice, thymic development of αβ T cells is completely arrested at the immature $CD4^{+}CD8^{+}TCR^{low}$ stage. The expression levels of γδ TCR is low and ZAP-$70^{-/-}$ skin-specific intraepithelial γδ T lymphocytes showed markedly reduced proliferation and no IL-2 gene expression in response to anti-CD3 or concanavalin A stimulation, suggesting that ZAP- 70 is important for TCR signaling and affected the proliferation and maturation of skin-specific intraepithelial γδ T lymphocytes in the epidermis (37). Itk is critical for the development of conventional naïve αβ T cells as well as lineage differentiation of TCR αβ T helper cells in the context of infection or antigen stimulation. However, its role in the skin T cells homing and maintenance is poorly understood. A recent study has shown that Itk is required to develop skin-homing skin-specific intraepithelial γδ T lymphocytes precursors in fetal thymus. The study mainly focused on innate γδ T cell homing to and maintenance in skin. Whether Itk plays a role in αβ T cells homeostasis in skin, however, is completely unknown.

1.3 **The purpose of the study**

The purpose of this project is to determine the role of Tec kinases, particularly Itk and Txk, in the development of TCR αβ T cells in skin. Since Itk is important for naïve $CD4^+$ and $CD8^+$ T cell development and skin-homing γδ T cell precursors in fetal thymus, we hypothesize it also plays a role in the homeostasis of αβ TCR T cells to skin. We therefore use mice lacking Itk, or carrying transgenes for Itk or Txk in the place of Itk in T cells to examine the homeostasis of αβ T cells in skin intraepithelial lymphocytes.

Chapter 2

Materials and Methods

2.1 Animals

C57Bl/6 (WT), Itk null mice ($Itk^{-/-}$), Tg(CD2-Txk)$Itk^{-/-}$, Tg(CD2-hItk)/$Itk^{-/-,}$ Balb/c (WT), 4get (IL-4-IRES-eGFP), and 4get$Itk^{-/-}$. Tg(CD2-Txk)$Itk^{-/-}$ and Tg(CD2-hItk)/$Itk^{-/-}$ mice were generated by breeding $Itk^{-/-}$ mice with Tg(CD2-Txk) or Tg(CD2-hItk) mice, respectively. 4get$Itk^{-/-}$ mice were generated by breeding $Itk^{-/-}$ mice with 4get mice. Tg(CD2-hItk)/$Itk^{-/-}$ were generated by cloning a human Itk cDNA into a transgenic expression cassette driven by the CD2 promoter and CD2 enhancer (38). These mice were backcrossed >5 generations. All the mice used were 6 to 8 weeks of age. All mice were bred and housed in specific pathogen-free mouse facility at the Pennsylvania State University or Cornell University and all experiments were approved by the IACUC at both universities.

2.2 Cell preparations

To isolate lymphocytes from mouse skin, hair was removed from the skin with Nair (Church & Dwight, Princeton, NJ). The treated skin was minced and digested with collagenase for 1–2 hours with gentle shaking to dissociate the cells. Lymphocytes were enriched from the cell preparations using Percoll gradients (40%/80%). Briefly, 6 mL of 80% percoll placed into a 15 mL centrifuge tube. Cells were then resuspended in 6 mL of 40% percoll, then overlaid and spun at 2000 X g for 20 minutes at room temperature. The cells at the interface between 40% and 80% of percoll were collected and washed once with medium.

2.3 Antibodies and Flow Cytometry

Cells were preincubated with Fc-block (2.4G2, BD Biosciences Inc.) for 10 minutes, and then incubated with the fluorescent antibodies for 30 minutes at 4°C, following by two washes in 2%FBS/PBS. The samples were analyzed using a flow cytometer (BD LSR II). Anti-B220-FITC, TCRβ-FITC, CD4-PE-Cy7, TCRδ-PE, CD122-EF450 antibodies were purchased from eBiosciences. Anti-CD8-ECD, CD4-ECD antibodies were purchased from Invitrogen Inc. Anti-CD44-PE-Cy5, CD-44-V500 antibodies were purchased from BD Biosciences Inc., and anti-B220-AF647, TCRβ-AF700 antibodies were purchased from Biolegend Inc.

2.4 Statistical analysis

Data was analyzed by Prism. The statistic significance was tested by Student *t* test, and $p < 0.05$ was considered statistically significant.

Chapter 3

Results

3.1. Increased $CD4^+$ T cells in the skin of $Itk^{-/-}$ mice

To study the role of Itk-mediated signaling in skin αβ T cell development, we first assessed the skin αβ T cells population in 6- to 8-wk-old $Itk^{-/-}$ and wild type (WT) mice by flow cytometry. As shown in figure 1a and b, $Itk^{-/-}$ mice had increased percentages of $CD4^+$ skin T cells compared to the WT controls, WT mice $CD4^+$ skin T cells percentage is 7.594% ± 1.537, and $Itk^{-/-}$ mice $CD4^+$ skin T cells percentage is 24.23% ± 4.257 (N=5. p=0.0063, Fig. 3-1d). By contrast, $CD8^+$ skin T cells in $Itk^{-/-}$ mice was 8.114% ± 0.6928 compared with wild type 10.06% ± 3.839 (N=5, p=0.6309, not significant, Fig. 3-1d). This result suggests that Itk-mediated signaling may play an important role in skin αβ T cells development or homeostasis, especially for $CD4^+$ T cells (Fig. 3-1).

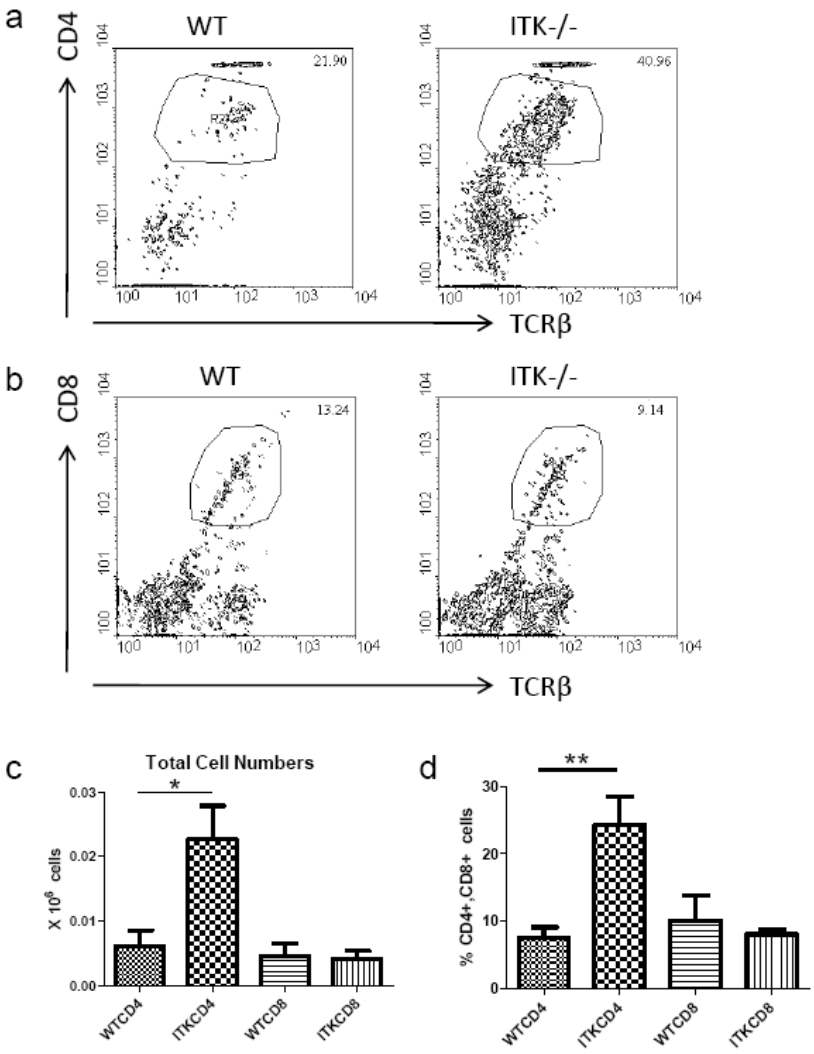


Figure 3-1: Itk plays an important role in skin αβ T cells development. (a,b) Skin T cell preparations from 6-8-week-old WT and Itk$^{-/-}$ mice were stained with antibodies against CD4, CD8 and TcRβ and then analyzed by flow cytometry. (c, d) The percentage of CD4^{+} and CD8^{+} T cells and total cell number. Data were obtained from three independent experiments, *$p<0.05$, **$p=0.0063$, $n=5$.

3.2. T cell specific expression of Itk rescues skin αβ T cells $CD4^+$ in Itk KO mice

In order to determine whether the change in homeostasis of $CD4^+$ and $CD8^+$ αβ-T cell population in the skin of Itk KO mice is intrinsically regulated by T cells, we used Tg(CD2-hItk)/$Itk^{-/-}$ mice in which human Itk is expressed specifically in T cells in the Itk KO background. We found that the $CD4^+$ T cell percentage in skin of Tg (CD2-hItk)/$Itk^{-/-}$ mice can be restored to the level similar to WT (Fig.3-2d), indicating that T cell-specific human Itk expression in Itk KO mice background rescues $CD4^+$ T cell homeostasis in the skin of Itk KO mice.

The percentage of $CD8^+$ T cells in the skin is decreased significantly in the Tg(CD2-hItk)/$Itk^{-/-}$ mice, however, no difference was found between WT and Itk KO mice. This data suggest that Itk in T cells plays a role in the percentage of $CD4^+$ T cells in skin. However, whether Itk in T cells plays a role in total number of $CD4^+$ T cell number in these mice is not clear. This may be due to technical variation in the extraction and isolation of skin lymphocytes between the various experiments and mice in this study. The protocol was improved at the later time therefore the yield of total skin lymphocytes in the Tg(CD2-hItk)/$Itk^{-/-}$ mice was significantly higher than WT and Itk KO mice. Therefore, although we show the total numbers of T cells in figure 3-2, the total numbers of $CD4^+$ and $CD8^+$ T cells in Tg(CD2-hItk)/$Itk^{-/-}$ mice are not comparable to those in WT and Itk KO mice due to the technical improvement of the extraction methods in Tg(CD2-hItk)/$Itk^{-/-}$ mice. For example, The total cell number of $CD4^+$ T cell are different between WT, $Itk^{-/-}$ and Itk-a, Tg(CD2-hItk) mice (Fig. 3-2c). We were therefore unable to directly

compare the numbers of these cells between these mice, however, the percentages of these cells was consistent in the different mice and isolation protocols.

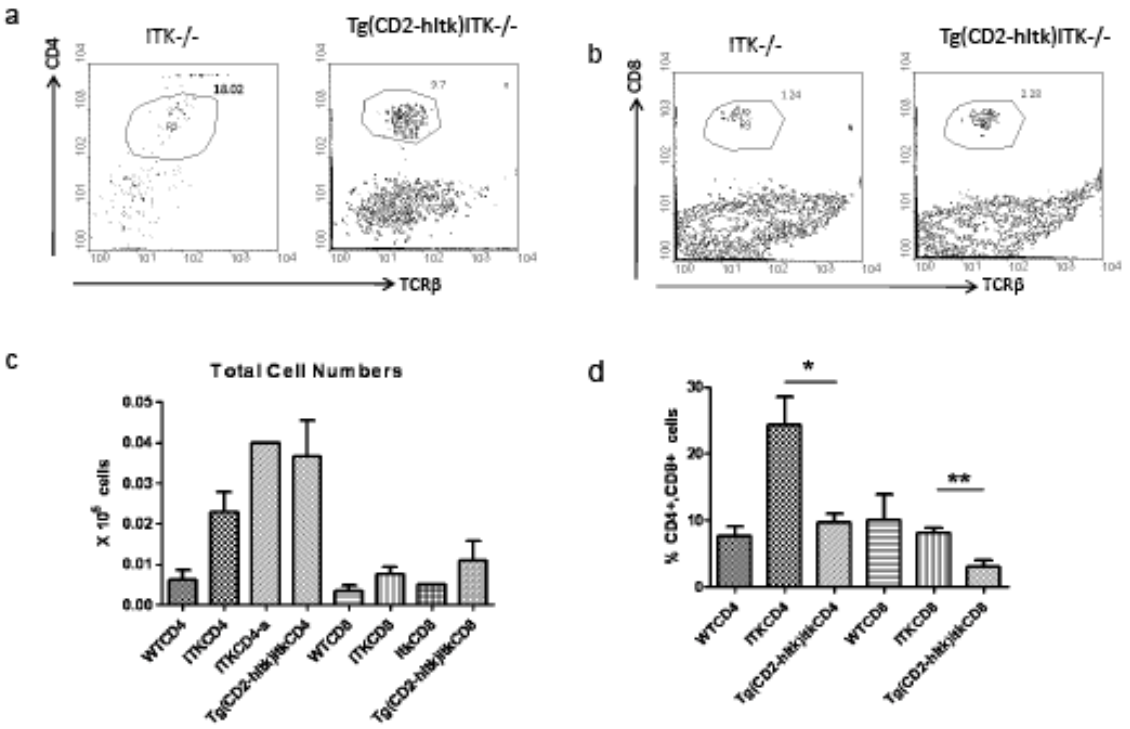


Figure 3-2: Tg(CD2-hItk) rescue the increased of $CD8^+$ but not $CD4^+$skin αβ T cells.

(a, b) $Itk^{-/-}$ and Tg(CD2-hItk)/$Itk^{-/-}$ mice skin T cells were stained with antibodies against CD4, CD8, TcRβ, and analyzed by flow cytometry. (c) The total cell number of $CD4^+$ and $CD8^+$ T cells. (d)The percentage of $CD4^+$ and $CD8^+$ T cells. *$p<0.05$, **$p=0.005$. WT=5, $Itk^{-/-}$=5, Itk$CD4^+$- a=1, Tg(CD2-hItk)$Itk^{-/-}$=3.

3.3. Over expression of Txk can restored $CD4^+$ and $CD8^+$ cells in skin αβ T cells

Itk and Txk are both Tec family kinases, and they both regulate the development of $CD4^+$ and $CD8^+$ T cells (39, 40). To test whether over expression of Txk can restore the percentage of $CD4^+$ skin T cells in Itk KO mice, we used Txk transgenic mice Tg(CD2-Txk)$Itk^{-/-}$ which over express Txk on an Itk KO background. We found both the percentage and the cell total number of $CD4^+$ and $CD8^+$ T cells in Tg(CD2 Txk)/$Itk^{-/-}$ mice can be restored to WT levels (Fig. 3-3). Compare with WT and Tg(CD2 Txk)/$Itk^{-/-}$ mice, there is no significantly different in both the percentage and the cell total number of $CD4^+$ T cells($p > 0.05$. WT, 7.594% ± 1.537, N=5, Tg(CD2 Txk)/$Itk^{-/-}$, 4.996% ± 1.514, N=5. Cell total number: $p>0.05$. WT, 0.0062 X 10^6 ± 0.002396/mouse N=5, Tg(CD2 Txk)/$Itk^{-/-}$, 0.00082 X 10^6 ± 0.00032/mouse N=5). There is a similar result looking at the $CD8^+$ T cells in skin between WT and Tg(CD2 Txk)/$Itk^{-/-}$ mice: $p>0.05$. WT, 10.06% ± 3.839, N=5, Tg(CD2 Txk)/$Itk^{-/-}$, 4.636% ± 1.669 N=5; $p>0.05$. Cell total numbers are WT, 0.00346 X 10^6± 0.001401/mouse, N=5, Tg(CD2 Txk)/$Itk^{-/-}$, 0.00078 X 10^6 ± 0.0003353/mouse, N=5, Fig. 3-3b,c).

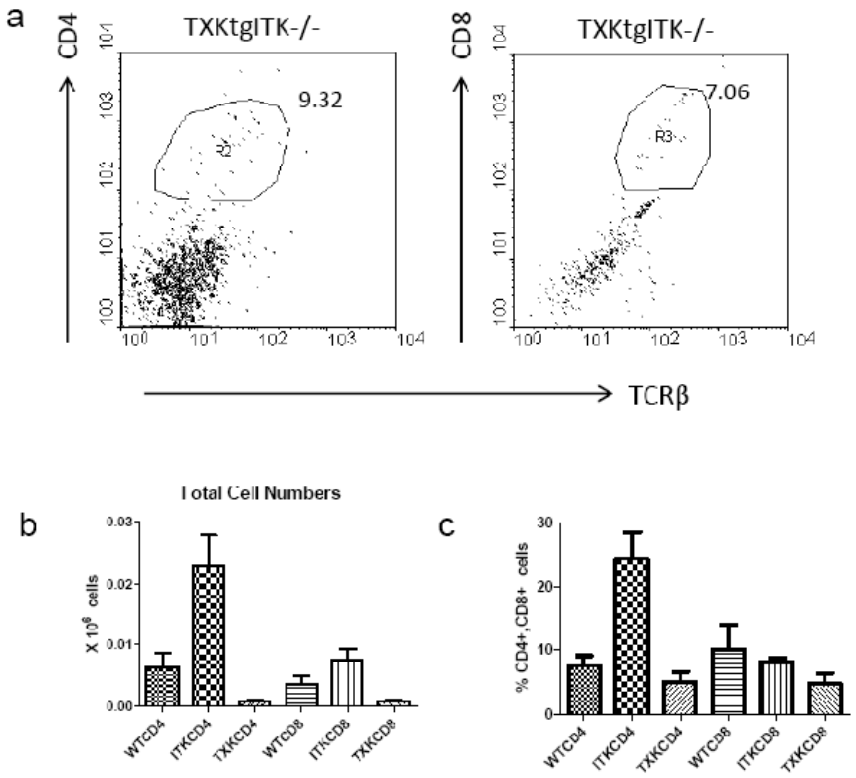


Figure 3-3: Over expression of Txk rescued CD4$^+$ skin αβ T cells but reduced CD8$^+$skin αβ T cells compared to WT and Itk KO mice. (a) Skin T cells were stained with antibodies against CD4, CD8, TcRβ, and analyzed by flow cytometry. (b, c) The percentage of CD4$^+$ and CD8$^+$ and total cell numbers of WT, Itk$^{-/-}$, Tg(CD2-Txk)/Itk$^{-/-}$ mice. n=5.

3.4. No defects in development of skin αβ T cells in Itk null mice on a Balb/c background

In addition to C57/BL6 mice, we also examined the cells in the skin in Balb/c background mice. We used WT Balb/c, IL-4-IRES-eGFP (4get) reporter mice, and IL-4-IRES-eGFP (4get) $Itk^{-/-}$ mice for this study. We found that there was no difference in percentage of $CD4^+$ T cells in the WT 4get and 4get/$Itk^{-/-}$ mice (WT, 17.78% ± 1.860, n=2 and $Itk^{-/-}$, 16.90% ± 1.401, n=11. Total cell numbers were WT, 0.0825 X 10^6 ± 0.0275/mouse, n=2 and 0.03927 X 10^6 ± 0.005848/mouse, n=11, Fig. 3-4 c, d). There was also no difference in $CD8^+$ T cells between 4get and 4get/$Itk^{-/-}$ mice (WT, 1.045% ± 0.0055, n=2 and $Itk^{-/-}$, 1.412% ± 0.1579, n=11. The total cell total numbers are: WT, 0.0045 X 10^6 ± 0.0015/mouse, n=2 and $Itk^{-/-}$, 0.0026 X 10^6 ± 0.0005/mouse, n=11), (Fig .3-4 c,d).Meanwhile there is similar result about $CD4^+$ and $CD8^+$ T cell in the skin between Balb/c and 4get/$Itk^{-/-}$ mice, ($CD4^+$ T cells:Balb/c, 13.82% ± 0.5208 N=8 and 4get/$Itk^{-/-}$, 16.90% ± 1.401 N=11. The total numbers are: Balb/c, 0.02825 X 10^6 ± 0.01037/mouse, N=8, and 4get/$Itk^{-/-}$, 0.03927 X 10^6 ± 0.005848/mouse, N=11. $CD8^+$ T cells: Balb/c, 1.788% ± 0.1591 N=8 and 4get/$Itk^{-/-}$, 1.142 %± 0.1579 N=11. The total numbers are: Balb/c, 0.003625 X 10^6± 0.001349/mouse, N=8 and 4get/$Itk^{-/-}$, 0.002636 X 10^6 ± 0.0005094/mouse N=11), (Fig. 3-4 c,d). While the difference in $CD4^+$ and $CD8^+$ T cell percentage between 4get and 4get/Itk KO mice is not significant, we found that the total number of $CD4^+$ and $CD8^+$ T cells in WT Balb/c and 4get/Itk KO mice was lower than that in 4get mice. The data from 4get mice is from only two mice in one time experiments, therefore, more experiments need to be done in 4get mice to determine if

there is a significant difference in both $CD4^+$ and $CD8^+$ numbers between these mice and others (Fig. 3-4).

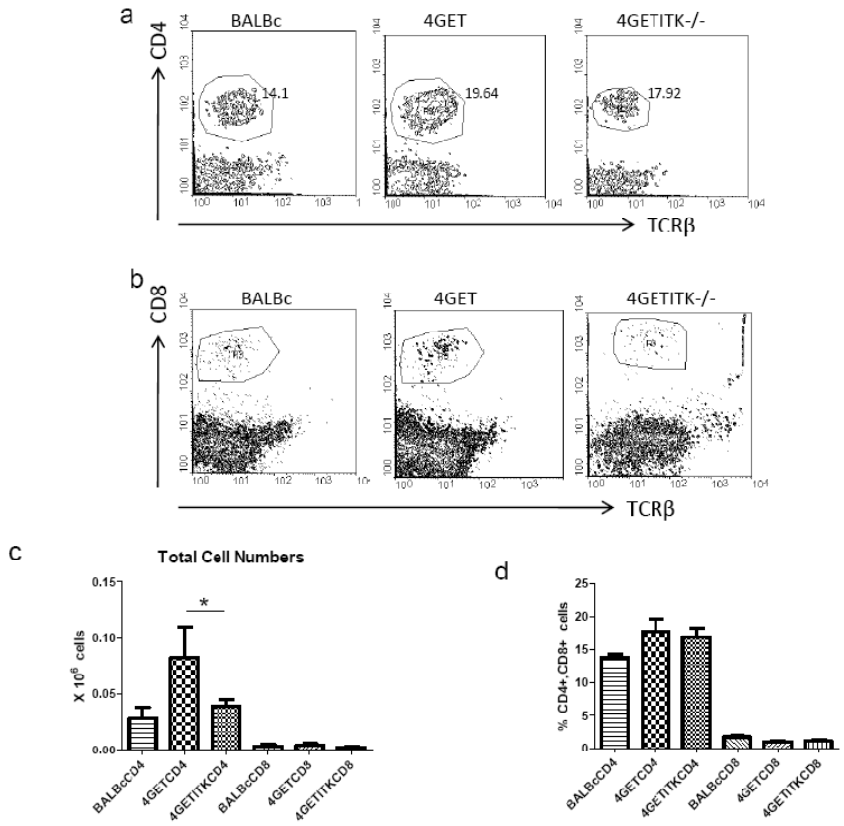
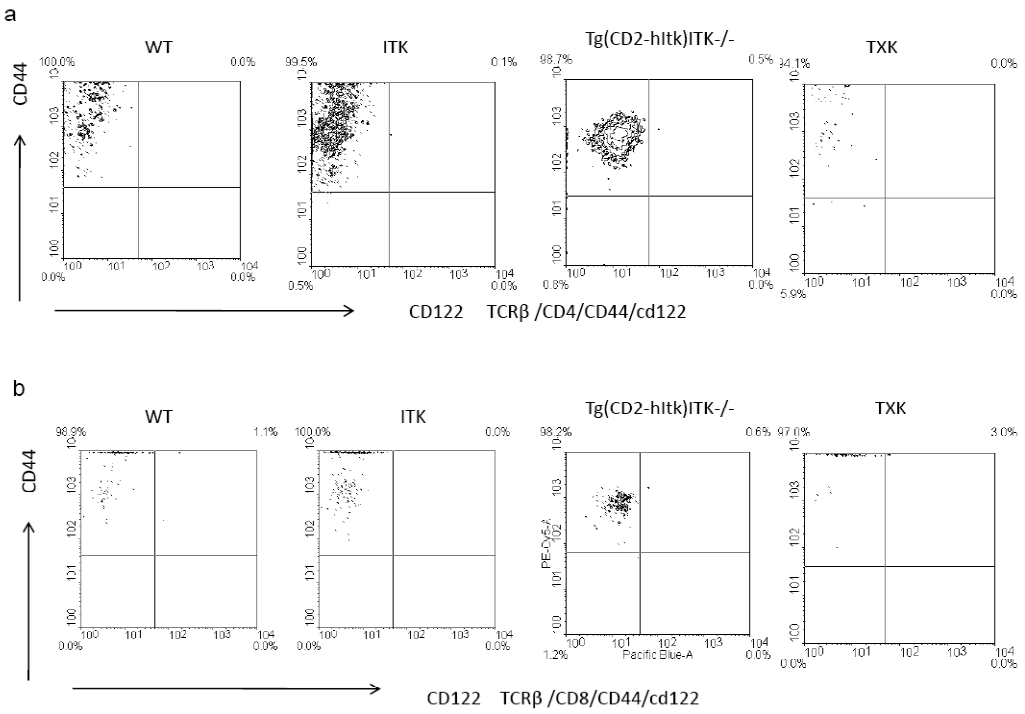


Figure 3-4: No defects in development of skin αβ T cells in Itk null mice on a Balb/c background. (a, b) Skin T cells from Balb/c, 4get, and 4get/$Itk^{-/-}$ (all on a Balb/c background) mice were stained with antibodies against CD4, CD8, TCRβ, and then analyzed by flow cytometry. (c, d) The percentage of $CD4^+$ and $CD8^+$ and total cell numbers. $p<0.5$ Balb/c=8 4get=2 4get/$Itk^{-/-}$=11.

3.5. CD4$^+$ and CD8$^+$ skin αβ T cells are skin-resident memory T cells

The sources of αβ T cells in skin are not clear. Most of the αβ T cells in the skin are memory T cells since they are activated and primed after antigen recognition and then later home to the skin (41, 42). The memory T cells in skin usually accumulate after inflammation and antigen exposure. Thus we further characterize these CD4$^+$ and CD8$^+$ T cells by analysis of CD44 and CD122 that are usually expressed on memory T cells. In this experiment, we found that regardless of whether they were Itk knockout or not, C57Bl/6 mice as well as Balb/c mice , the CD4$^+$ and CD8$^+$ T cells in skin are mostly CD44^{hi} , indicating that Itk does not affect the memory phenotype status of skin T cells (Fig. 3-5).



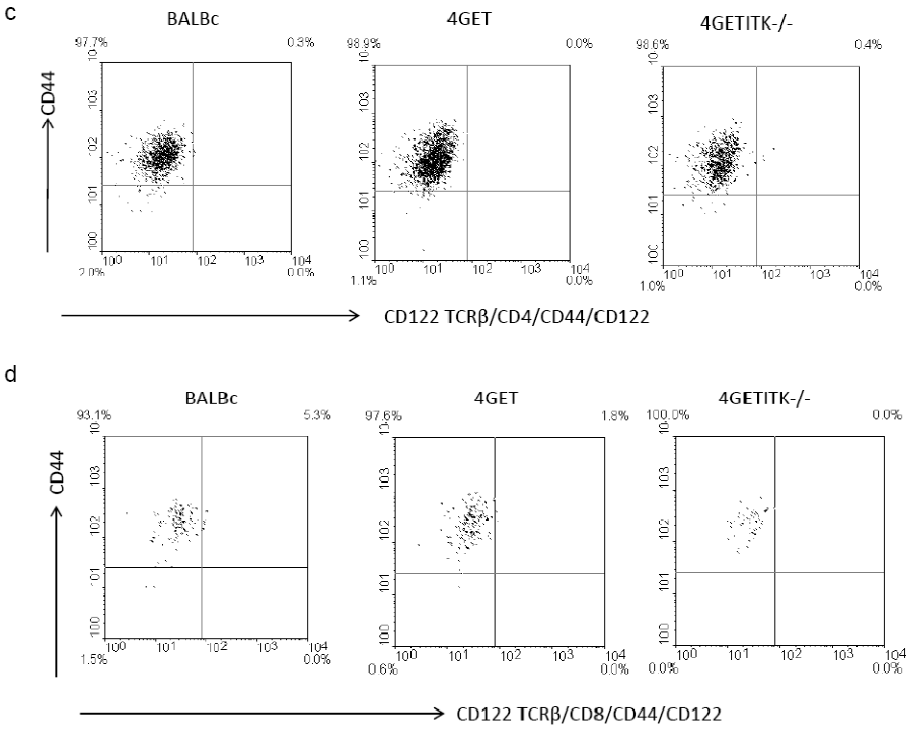


Figure 3-5: CD4$^+$ and CD8$^+$ skin αβ T cells are skin-resident memory T cells. Skin T cells were stained with antibodies against CD44 and CD122, and then analyzed by flow cytometry. The population is gated by CD4$^+$/TCRβ$^+$ and CD8$^+$/TCRβ$^+$ cells.

Chapter 4

Discussion

Increasing evidence suggest that various subsets of T cells in the skin are important components of the immune system critical for the first line of defense, but mechanisms regulating their homeostasis are poorly understood (36). In this study, we focused on skin αβ T cells, and investigated the role of Tec kinase family members, Itk and Txk, in αβ T cell homoeostasis in skin intraepithelial lymphocytes. Our rationale is that Tec family kinases are important for TCR signaling, and is important for T cell development in central and peripheral T cell development, as well as γδ T cell precursors in fetal thymus and their homing to skin. Therefore, it is likely that Itk also plays a role in regulating αβ T cell homeostasis in skin. In this study, we characterize αβ T cell populations in Itk deficient mice compared to WT mice. We found the cell total number and the percentage of $CD4^+$ T cells in Itk KO mice is significantly higher than those in WT mice. No significant difference was found in $CD8^+$ T cells in skin. In addition, we use mice that express human Itk only in T cells on the $Itk^{-/-}$ background (Tg(CD2-hItk)/$Itk^{-/-}$)to determine whether this would rescue the difference we observe between WT and Itk KO mice. Human Itk is 93% identical to mouse Itk and has the same function. We also used mice which express murine Txk specifically in T cells on an Itk KO background to determine if Txk could rescue the function of Itk in skin T cells. We found that T cell specific expression of both human Itk, and mouse Txk could restore the $CD4^+$ population (percentage) in Itk KO mice. From these data, we concluded that re-expression of human Itk in T cells and over-expression of Txk can both restore the change of $CD4^+$ T cell population in the skin in Itk KO mice.

In addition to C57Bl/6 mice, we also examined the skin T cells in Balb/c mice. We used 4get, and 4get/$Itk^{-/-}$ mice (IL-4 IRES GFP mice on a Balb/c background) in

addition to Balb/c mice. The IL-4-IRES-eGFP should not affect the phenotype of the mice. The data did not show significant differences in the percentage of $CD4^+$ and $CD8^+$ T cells in these mice. Total number of the $CD4^+$ and $CD8^+$ T cells in Balb/c and 4get/$Itk^{-/-}$ mice are also similar. Although a larger number of mice will be needed to verify the results and verify whether there is a difference of Balb/c and 4get mice, the current data did not show significant differences in $CD4^+$ and $CD8^+$ T cell populations in skin in Balb/c mice.

To further characterize the phenotype of $CD4^+$ and $CD8^+$ T cells in skin intraepithelial lymphocytes, we used CD44 and CD122 surface markers to determine their memory phenotype. We found that both WT and Itk KO have high CD44 expression, that resembles activated or memory T cells. The data supports the conclusion that Itk does not play a major role in determining the memory phenotype of αβ T cells in skin intraepithelial lymphocytes.

Taken together, we found there is a significant increase in total number and percentage of $CD4^+$ T cells in Itk KO mice. This suggests that Itk affects the homeostasis of $CD4^+$ T cells in skin intraepithelial lymphocytes. The role of Itk on T cell homoeostasis in skin intraepithelial lymphocytes is intrinsically regulated by T cells, since T cell expression of Itk on an Itk KO background (Tg(CD2hItk)/$Itk^{-/-}$) mice can restore the percentage of $CD4^+$ and $CD8^+$ T cells in Itk KO mice to the levels similar to those in WT mice. Interesting, we also found Txk over expression can restore the percentage of $CD4^+$ and $CD8^+$ T cells in Itk KO mice to close to the level of WT mice. This show Itk and Txk have redundant functions in homeostasis of skin intraepithelial lymphocytes.

The effect of Itk KO C57Bl/6 mice on $CD8^+$ T cells seems to be minimal. Although we found no significant change in percentage and total number of $CD8^+$ T cells in skin intraepithelial lymphocytes between WT and Itk KO mice, the percentages of $CD8^+$ T cells were lower in Tg(CD2hItk)/$Itk^{-/-}$ and Tg(CD2Txk)/$Itk^{-/-}$ mice compared to WT and Itk KO mice. However, further verification needs to be done.

In this study, we identified Itk as a critical signaling molecule that specifically regulates of the homeostasis of $CD4^+$ αβ T cells in skin. While the $CD4^+$ T cell numbers in spleen and lymph nodes of Itk KO mice are lower than in WT mice, the $CD4^+$ T cells in skin are higher in Itk KO mice than in WT mice. This suggests that the mechanism of $CD4^+$ T cell homeostasis in skin is probably not due to the total number of $CD4^+$ T cells in the lymphatic tissues of these mice. Further studies need to be done to understand how Itk in T cells affects the homeostasis of $CD4^+$ T cells in skin.

References

1. August A, Sadra A, Dupont B, and Hanafusa H. 1997. Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. *Proc Natl Acad Sci U S A. 94*, 11227-11232.
2. Liao XC, Littman DR. 1995. Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. *Immunity.*3: 757-769.
3. Wange RL. 2000. LAT, the linker for activation of T cells: a bridge between T cell-specific and general signaling pathways. *Sci STKE.* 19; 2000 (63): re1.
4. Kong G, Dalton M, Bubeck WJ, Straus D, Kurosaki T, Chan AC. 1996. Distinct tyrosine phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function. *Mol Cell Biol.* 16(9): 5026-35.
5. Yang WC, Ching KA, Tsoukas CD, Berg LJ.2001. Tec kinase signaling in T cells is regulated by phosphatidylinositol 3-kinase and the Tec pleckstrin homology domain. *J Immunol.* 166: 387-395.
6. Ching KA, Kawakami Y, Kawakami T, Tsoukas CD. 1999. Emt/Itk associates with active TCR complexes: role of the pleckstrin homology domain. *J Immunol.* 163: 6006-6013.
7. Ishiai M, Kurosaki M, Inabe K, Chan AC, Sugamura K, Kurosaki T. 2000. Involvement of LAT, Gads, and Grb2 in compartmentation of SLP-76 to the plasma membrane. *J Exp Med.* 192: 847-856.
8. Kahn ML, Koretzky GA. 2006. Integrins and ITAMs: more than just good

neighbors. *Nat Immunol*. 7(12): 1326-33.

9. Heyeck SD, Wilcox HM, Bunnell SC, Berg LJ. 1997. Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity. *J Biol Chem.* 272(40): 25401-25408.
10. Liu KQ, Bunnell SC, Gurniak CB, Berg LJ. 1998. T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. *J Exp Med.* 187: 1721–1727.
11. Fowell DJ, Shinkai K, Liao XC, Beebe AM, Coffman RL, Littman DR, Locksley RM. 1999. Impaired NFATc translocation and failure of Th2 development in Itk-deficient CD4+ T cells. *Immunity.* 11: 399-409.
12. Berg LJ, Finkelstein LD, Lucas JA, Schwartzberg PL. 2005. Tec family kinases in T lymphocyte development and function. *Annu Rev Immunol*. 23: 549-600.
13. Miller AT, Wilcox HM, Lai Z, Berg LJ. 2004. Signaling through Itk promotes T helper 2 differentiation via negative regulation of T-bet. *Immunity*. 21: 67-80.
14. Hao S, Qi Q, Hu J, August A. 2006. A kinase independent function for Tec kinase Itk in regulating antigen receptor induced serum response factor activation. *FEBS Lett.* 580(11): 2691-2697.
15. Starr TK, Jameson SC, Hogquist KA. 2003. Positive and negative selection of T cells. *Annu Rev Immunol.* 21: 139-176.
16. Sahu N, Venegas AM, Jankovic D, Mitzner W, Gomez-Rodriguez J, Cannons JL, Sommers C, Love P, Sher A, Schwartzberg PL, August A. 2008. Selective expression rather than specific function of Txk and Itk regulate Th1 and Th2 responses. *J Immunol*. 181(9): 6125-6131.

17. Hu J, August A. 2008. Naïve and innate memory phenotype. *J Immunol.*180: 6544–6552.

18. Broussard C, Fleischecker C, Horai R, Chetana M, Venegas AM, Sharp LL, Hedrick SM, Fowlkes BJ, Schwartzberg PL. 2006. Altered development for $CD8^+$ T cell lineages in mice deficient for the Tec kinases Itk and Rlk. *Immunity.* 25: 93–104.

19. Atherly LO, Lucas JA, Felices M, Yin CC, Reiner SL, Berg LJ. 2006. The Tec family tyrosine kinases Itk and Rlk regulate the development of conventional CD8+ T cells. *Immunity.* 25: 79–91.

20. Schaeffer EM, Broussard C, Debnath J, Anderson S, McVicar DW, Schwartzberg PL. 2000. Tec family kinases modulate thresholds for thymocyte development and selection. *J Exp Med.* 192: 987–1000.

21. Dubois S, Waldmann TA, Muller JR. 2006. ITK and IL-15 support two distinct subsets of CD8+ T cells. *Proc Natl Acad Sci U S A.* 103: 12075-12080.

22. Readinger JA, Mueller KL, Venegas AM, Horai R, Schwartzberg PL. 2009. Tec kinases regulate T-lymphocyte development and function: new insights into the roles of Itk and Rlk/Txk. *Immunological Reviews*. 228: 93–114.

23. Felices M, Berg LJ. 2008. The Tec kinases Itk and Rlk regulate NKT cell maturation, cytokine production, and survival. *J Immunol*. 180: 3007–3018.

24. Au-Yeung BB, Fowell DJ. 2007. A key role for Itk in both IFN gamma and IL-4 production by NKT cells. *J Immunol*. 179: 111–119.

25. Schwartzberg PL, Finkelstein LD, Readinger JA. 2005. Tec-family kinases: regulators of T-helper-cell differentiation. *Nat Rev Immunol*. 5(4): 284-295.

26. Schaeffer EM, Debnath J, Yap G, McVicar D, Liao XC, Littman DR, Sher A, Varmus HE, Lenardo MJ, Schwartzberg PL. 1999. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. *Science.* 284: 638–641.

27. Dombroski D, Houghtling RA, Labno CM, Precht P, Takesono A, Caplen NJ, Billadeau DD, Wange RL, Burkhardt JK, Schwartzberg PL. 2005. Kinase-independent functions for Itk in TCR-induced regulation of Vav and the actin cytoskeleton. *J Immunol*. 174(3): 1385-1392.

28. Prince AL, Yin CC, Enos ME, Felices M, Berg LJ. 2009. The Tec kinases Itk and Rlk regulate conventional versus innate T-cell development. *Immunol Rev*. 228: 115–131.

29. Readinger JA, Mueller KL, Venegas AM, Horai R, Schwartzberg PL. 2009. Tec kinases regulate T-lymphocyte development and function: new insights into the roles of Itk and Rlk/Txk. *Immunol Rev*. 228: 93–114.

30. Bos JD, Zonneveld I, Das PK, Krieg SR, van der Loos CM, Kapsenberg ML. 1987. The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J Invest Dermatol.* 88: 569–573.

31. Foster CA, Yokozeki H, Rappersberger K, Koning F, Volc-Platzer B, Rieger A, Coligan JE, Wolff K, Stingl G. 1990. Human epidermal T cells predominantly belong to the lineage expressing alpha/beta T cell receptor. *J Exp Med.* 171: 997–1013.

32. Bos JD, Kapsenberg ML. 1993. The skin immune system: progress in cutaneous biology. *Immunol Today.* 14: 75–78.

33. Weaver CT, Hatton RD, Mangan PR, Harrington LE. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol.* 25: 821–852.

34. Havran WL, Allison JP. 1990. Thy-1 dendritic epidermal cells of adult mice arise from fetal thymic precursors. *Nature.* 344: 68–70.

35. Xiong N, Raulet DH. 2007. Development and selection of γδ T cells. *Immunological Reviews*. 215: 15–31.

36. Xia M, Qi Q, Jin Y, Wiest DL, August A, Xiong N. 2010. Differential Roles of IL-2–Inducible T cell Kinase-Mediated TCR Signals in Tissue-Specific Localization and Maintenance of Skin Intraepithelial T Cells. *J Immunol.* 184: 6807-6814.

37. Endo Y, Ishikawa O, Negishi I. 2005. Zeta-chain-associated protein-70 molecule is essential for the proliferation and the final maturation of dendritic epidermal T cells. *Exp Dermatol*. 14: 188–193.

38. Hu J, August A. 2008. Naïve and Innate Memory phenotype CD4+ T-cells have different requirements for active Itk for their development. *J Immunol*. 180(10): 6544–6552.

39. Qi Q, Xia M, Bai Y, Yu S, Cantorna M, August A. 2010. Interleukin-2-inducible T cell kinase (Itk) network edge dependence for the maturation of *i*NKT cell. *J Biol Chem.* 286: 138–146.

40. Hu J, Sahu N, Walsh E, August A. 2007. Memory phenotype $CD8^+$ T cells with innate function selectively develop in the absence of active Itk. *Eur J Immunol.* 37: 2892–2899.

41. Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ. 2009. Skin immune sentinels in health and disease. *Nat Rev Immunol*. 9(10): 679–691.

42. Clark RA. 2010. Skin resident T cells: the ups and downs of on site immunity. *J Invest Dermatol*. 130(2): 362–370.