UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

Controlling T Lymphocyte Activation with a Molecular Rheostat

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biology

by

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2008

The Dissertation of Juris Andris Grasis is approved, and it is acceptable in quality and form for publication on film and electronically:

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2008

DEDICATION

To my parents, Andris Girts Grasis (Andy) and Patricia Mahoney Grasis (Pat),

for your devotion to your children.

EPIGRAPH

Don’t try to gain the world

And lose your soul

Wisdom is much better

Than silver and gold.

Bob Marley (Zion Train)

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LIST OF ABBREVIATIONS

a.a., amino acid(s)
Ab, antibody (mAb, monoclonal Ab)
ADAP, adhesion- and degranulation-promoting adaptor protein
Ag, antigen
AIDS, acquired immune deficiency syndrome
AP-1, activator protein 1
APC, antigen presenting cell
Arp2/3, actin-related proteins 2/3
ATP, adenosine triphosphate

BAL, bronchoaveolar lavage
BCL-X_L, basal cell lymphoma, extra-large
BM, bone marrow
bp, base pair
BSA, bovine serum albumin
Btk, Bruton's tyrosine kinase

cAMP, cyclic adenosine 3', 5'-monophosphate
C.A., constitutively active
CBP, Csk-binding protein (also called PAG)
CD, cluster of differentiation
cDNA, complementary DNA
CMV, cytomegalovirus
CPP, cell-penetrating peptide
CRAC, Ca^{2+}-release-activated Ca^{2+} channel
CRIB, Cdc42/Rac interactive binding domain (also called GBD)
CsA, cyclosporine A
CSF, colony stimulating factor
Csk, c-src tyrosine kinase
CTL, cytotoxic T lymphocyte
CTLA, cytotoxic T lymphocyte associated antigen
CypA, cyclophilin A
CXCL, chemokine (C-X-C motif) ligand

d, deoxy or distilled
DAG, diacylglycerol
DC, dendritic cell
DMEM, Dulbecco's modified essential medium
DMSO, dimethylsulfoxide
DN, double negative ($CD4^-CD8^-$)
DNA, deoxyribonucleic acid
DNase, dioxyribonuclease

DP, double positive ($CD4^+CD8^+$)

EAE, experimental autoimmune encephalomyelitis
ED_{50}, 50% effective dose
ELISA, enzyme-linked immunosorbent assay
ELISPOT, enzyme-linked immunospot
Emt, expressed in mast and T cells (also called Itk)
ENU, *N*-ethyl-*N*-nitrosourea (known mutagen)
ER, endoplasmic reticulum
ERK, extracellular signal-regulated kinase
ERM, ezrin, radixin and moesin
EVH1, Ena/VASP homology domain 1 (also called WH1)
EVL, Ena/VASP-like protein

F-actin, filamentous actin
FACS, fluorescence-activated cell sorter
FBS, fetal bovine serum (also FCS)
FcεRI, high-affinity receptor for IgE
FCS, fetal calf serum (also FBS)
FITC, fluorescein isothiocyanate
FRET, Förster resonance energy transfer
FYB, Fyn-binding protein (also called ADAP)

g, gram
g, unit of gravity
G-actin, globular actin
Gads, Grb2-related adaptor downstream of Shc
GATA-3, GATA sequence binding protein 3
GBD, GTPase-binding domain (also called CRIB)
GDP, guanosine diphosphate
GEF, guanine-nucleotide exchange factor
GFP, green fluorescent protein (e, enhanced)
GM-CSF, granulocyte/macrophage colony stimulating factor
GRB, growth factor receptor-bound protein
GST, glutathione S-transferase
GTP, guanosine triphosphate
GTPase, guanosine triphosphate phosphatase

h, hour
HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HIV, human immunodeficiency virus
HLA, human histocompatibility leukocyte antigens
HPLC, high performance liquid chromatography
HRP, horseradish peroxidase

ICAM, intercellular adhesion molecule
ICCS, intracellular cytokine staining
ID_{50}, 50% inhibiting dose
IFN, interferon
Ig, immunoglobulin
IκB, inhibitor of kappa light chain gene enhancer in B cells
IKK, i-kappa-b kinase
IL, interleukin
i.p., intraperitoneally
IP, immunoprecipitate
IP_3, inositol 1,4,5-trisphosphate
IP_4, inositol 1,3,4,5-tetraphosphate
ITAM, immunoreceptor tyrosine-based activation motif
ITIM, immunoreceptor tyrosine-based inhibitory motif
Itk, IL-2 inducible tyrosine kinase
ItpkB, insitol 1,4,5-triphosphate kinase B

JAK, Janus kinase
JNK, jun-N-terminal kinase
JTAg, Jurkat large T antigen

kb, kilobase
kD, kilodalton
kDa, kilodalton
KO, knockout

LAT, linker for activation of T cells
Lck, Lymphocyte specific protein tyrosine kinase
LCMV, lymphocytic choriomeningitis virus
LFA-1, leukocyte function-associated antigen-1
LN, lymph node
LPS, lipopolysaccaride

m, meter
M, molar
2-ME, 2-mercaptoethanol (also β-mercaptoethanol)
MAPK, mitogen-activated protein kinase
MHC, major histocompatibility complex (peptide containing, pMHC)
min, minute
MTOC, microtubule organizing center
myr, myristoylation
μg, microgram
μl, microliter

n, number in study or group
N, normal
N.A., not applicable
Nck, non-catalytic region of tyrosine kinase
N.D., not determined
NFAT, nuclear factor of activated T cells
NFκB, nuclear factor of kappa light chain enhancer in B cells
NK, natural killer cell
NKT, natural killer T cell
NP-40, Nonidet P-40
n.s., not significant
N-WASp, neuronal-Wiskott-Aldrich syndrome protein

O.D., optical density
OVA, ovalbumin

p, probability
PAGE, polyacrylamide gel electrophoresis
PAK, p21-activated kinase
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PE, phycoerythrin
PH, pleckstrin homology domain
PI, phosphatidyl inositol
PIP_2, phosphatidyl inositol (4,5) bisphosphate
PIP_3, phosphatidyl inositol (3,4,5) triphosphate
PI_3K, phosphatidylinositol 3-kinase
PKA, protein kinase A
PKB, protein kinase B (also known as Akt)
PKC, protein kinase C (you're kidding, right?)
PLCγ1, phospholipase C, gamma 1
PMA, phorbol myristate acetate
PMSF, phenylmethylsulfonyl fluoride
PRR, proline rich region
PTD, protein-transduction domain
PTEN, phosphatase and tensin homolog
PY, phosphotyrosine

r, recombinant
R, receptor
RasGRP, Ras guanyl releasing protein
RIBP, Rlk- and Itk-binding protein
Rlk, resting lymphocyte kinase
RNA, ribonucleic acid
RNAi, RNA interference

ROCK, Rho-associated kinase
RORγt, retinoic acid-related orphan receptor gamma in T cells
RTK, receptor tyrosine kinase
RT-PCR, reverse transcription polymerase chain reaction (q, quantitative)

s, seconds
SD, standard deviation
SDFα1, stromal-cell derived factor 1 alpha (also CXCL12)
SDS, sodium dodecyl sulfate (also lauryl sulfate)
SEM, standard error of the mean
SH, Src homology (1, 2, or 3) domain
SHIP, SH2-containing inositol phosphatase
siRNA, small interfering RNA
SIT, allergen-specific immunotherapy
SLAP-130, SLP-76 associated protein of 130 kDa (also called ADAP)
SLP-76, SH2 containing leukocyte phosphoprotein of 76 kDa
SMAC, supramolecular adhesion complex, proximal (p), central (c), and distal (d)
SOCE, store-operated calcium entry
SP, single positive ($CD4^+CD8^-$ or $CD4^-CD8^+$)
STAT, signal transducer and activator of transcription
STIM1, stromal interaction molecule 1

TBST, tris-buffered saline with Tween 20
TCR, T cell receptor
Tec, tyrosine kinase expressed in hepatocellular carcinoma
TGF, transforming growth factor
T_H, T helper cell, (p) precursor, (1, 2, or 17)
TN, triple negative ($CD3^-$ $CD4^-$ $CD8^-$)
T_{Reg}, regulatory T cell
Tris, tris(hydroxymethyl)amniomethane
Tsk, T cell specific kinase

U, unit

V, volt
VCA, verprolin-homology, cofilin-homology, and acidic domain
VSV, vesicular stomatitis virus
VV, vaccinia virus

W, watt
WASp, Wiskott-Aldrich syndrome protein
WAVE, WASp-family verprolin homologous protein
WB, western blotting
WH1, WASp homology domain 1 (also called EVH1)
WIP, WAVE interacting protein

WISH, WASp interacting SH3 domain
WT, wild-type

ZAP-70, zeta-associated protein of 70 kDa

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ACKNOWLEDGEMENTS

Time spent in graduate school would be considered a poor life choice if it were not for the help of others. I would like to thank the following in helping me through it:

To my committee members, Dr. Stephen Hedrick from UCSD, Dr. Michael David from UCSD, Dr. Robert Zeller from SDSU, and Dr. Christopher Glembotski from SDSU. Also, though now in retirement but present at the proposal examination, Dr. Bartholomew Sefton from the Salk Institute. Thank you all for you time and your constructive advice. I apologize for not meeting with each of you more, but the times I did meet with you was invaluably time well spent.

To my mentor and stalwart educator, Dr. Constantine Tsoukas from SDSU, the molding that took place while under your tutelage will pay dividends for a lifetime. Thank you for the patience, the encouragement, and the support through the years in matters pertaining to science and beyond.

To my immediate family, Mom (Pat) and Dad (Andy), my sisters Amanda (PJ) and Maija (Mai), and my brother Gonzo (G), thank you for sticking with me through all the ups and downs of the time spent here. To my extended family, Aunt Erin and Uncle Al, Caitlin and Cara, Uncle Mark and Molly, Uncle Tim, Uncle Maris and Aunt Pat, Mari, Juan and my fascinating niece Jovia, I thank each of you for significantly contributing to my success.

To Gloria, my Muse and my Foil, who stood by my side through all the aforementioned strikes and gutters and experienced first-hand the mania that creates.

To the various lab members who made LS406 home over the years, Keith Ching, Cecille Browne, Krystal Herman, Amy Bathke, Dave Guimond, Roman Levytskyy, Sunny Levy, Patty Berringer, Zach McDonald, Roma Munday, Gerardo Perez, Jules Chen, and anybody else I might have missed, thanks for the learning experiences.

To the unofficial biology department golf team, Matt Giacolone, Marc Rideout, and Nate Spann, thank you for the time spent chasing the little white ball and for helping to keep me sane.

To my old roommates, Dan Baker, Eric Chaplin, and Nate Sheehey, you helped make San Diego home and gave me the grounding to do this thing.

To my good friends, Mark Eischeid, Ben Bartley, and Derek Hutton, I value the friendships we've kept through the years and Spoon!

To all of those who encouraged my progress as a scientist, Forest Rohwer, Anca Segall, John Miyake, Kathie McGuire, Cathie Atkins, Steve Barlow, Dennis Young, Malcolm Wood, and Scott Kelley, thank you for the support, real and perceived.

To the bands who kept my mind occupied on positive things, Radiohead, Portishead, PJ Harvey, the Black Keys, the White Stripes, the Dirtbombs, Electric Six, Zero 7, the Pixies, Bob Marley and the Wailers, the Skatalites, the National, and all the other (1000+) artists in my music library, thank you for soothing the savage beast.

To my 1991 Honda Civic, for safely helping me to navigate the highways and byways of California and Arizona with all the exploration that took place within.

To alcohol, the cause of and solution to, all of the tiny voices in my head.

Portions of Chapter 2 were published as: Grasis, J.A., C. D. Browne, and C. D. Tsoukas. (2003) Inducible T cell tyrosine kinase regulates actin-dependent cytoskeletal events induced by the T cell antigen receptor. J. *Immunol.* 170:3971-3976.

Portions of Chapter 4 have been submitted as: Grasis, J.A., K. Herman, and C. D. Tsoukas. (Submitted) Inhibition of Itk Activation and Cytokine Production by Cell Permeable Peptides. *J. Immunol*.

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2. Tsoukas, C. D., **J. A. Grasis,** C. D. Browne, and K. A. Ching. (2006) Inducible T Cell Tyrosine Kinase (ITK) Structural requirements and actin polymerization. *Adv Exp Med Biol*. 584: 29-41. Review.

3. **Grasis, J. A.,** C. D. Browne, and C. D. Tsoukas. (2003) Inducible T cell tyrosine kinase regulates actin-dependent cytoskeletal events induced by the T cell antigen receptor. *J. Immunol.* 170: 3971-3976.

4. Tsoukas, C. D., **J. A. Grasis,** K. A. Ching, Y. Kawakami, and T. Kawakami. (2001) Itk/Emt: a link between T cell antigen receptor-mediated Ca^{2+} events and cytoskeletal reorganization. *Trends Immunol.* 22: 17-20.

5. Ching, K. A., **J. A. Grasis,** P. Tailor, Y. Kawakami, T. Kawakami, and C. D. Tsoukas. (2000) TCR/CD3-Induced activation and binding of Emt/Itk to linker of activated T cell complexes: requirement for the Src homology 2 domain. *J. Immunol.* 165: 256-262.

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2. Huang, Y. H., **J. A. Grasis**, A. T. Miller, S. Soonthornvacharin, C. D. Tsoukas, M. P. Cooke, and K. Sauer. (2006) IP_4 is essential for TCR signaling during positive selection. *Keystone Symposia In Lymphocyte Activation and Signaling 2006.* Poster.

3. **Grasis, J. A.,** P. Bettinger, and C. D. Tsoukas. (2004) Inducible T cell tyrosine kinase regulation of Wiskott-Aldrich syndrome protein. *Tyrosine Phosphorylation and Cell Signaling Conference 2004.* Poster.

4. **Grasis, J. A..** (2003) Itk regulation of actin polymerization in T lymphocytes. *San Diego Actin Forum 2003*. Selected Talk.

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9. Ching, K. A., **J. A. Grasis,** Y. Kawakami, T. Kawakami, and C. D. Tsoukas. (2000) Emt/Itk-LAT association required for TCR/CD3 activation of Emt/Itk. *Keystone Symposia in T Lymphocyte Activation, Differentiation, and Death 2000*. Poster.

10. **Grasis, J. A.,** Ching, K. A., Y. Kawakami, T. Kawakami, and C. D. Tsoukas. (1999) Role of SH2 and SH3 domains of Emt/Itk in TCR/CD3 signaling. *Keystone Symposia in Oncogene Networks in Signal Transduction 1999.* Poster.

11. Sandstrom, D. J., **J. A. Grasis,** and L. L. Restifo. (1997) Epidermal tendon cells require Broad Complex function for correct attachment of the indirect flight muscles in *Drosophila melanogaster*. *The Society for Neuroscience Annual Meeting 1997.* Poster.

12. Sandstrom, D. J., **J. A. Grasis,** and L. L. Restifo. (1996) Broad-Complex transcription factors regulate thoracic muscle attachment in *Drosophila*. *The Society for Neuroscience Annual Meeting 1996.* Poster.

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ABSTRACT OF THE DISSERTATION

Controlling T Lymphocyte Activation with a Molecular Rheostat

by

Juris Andris Grasis

Doctor of Philosophy in Biology

University of California, San Diego, 2008

San Diego State University, 2008

Professor Constantine Tsoukas, Chair

Professor Christopher Glembotski, Co-Chair

The Tec family non-receptor tyrosine kinase, Interleukin-2 Inducible Tyrosine Kinase (Itk), is an enigmatic enzyme that plays a key role in T cell receptor (TCR)-initiated signaling. In a kinase-dependent fashion, Itk directly and significantly affects the regulation of Phospholipase C gamma-1 (PLCγ1) and the consequent mobilization of

Ca^{2+}. In a kinase-independent manner, Itk indirectly and significantly participates in the regulation of T cell cytoskeletal reorganization necessary to generate a productive T cell response. However, neither of these Itk functions lead to the complete activation of a T cell. Rather, Itk serves as an important mediator of T cell signals, enabling a tempered response to antigen necessary for the development and differentiation of a naïve T cell. The following dissertation includes a review of the scientific literature covering Itk, the characterization of the Itk protein domains necessary for its activation and localization through TCR engagement, the involvement of Itk in the modification of actin-mediated events emanating from the TCR, the discovery of a soluble ligand affecting Itk recruitment, and the development of an inhibitor specific for Itk activity. The collective incorporation of the data presented herein provides evidence that Itk serves as a modulator, or rheostat, critically fine-tuning the T cell response to antigen.

1 Review of Itk Literature

1.1 Abstract

The non-receptor tyrosine kinase Itk plays a key role in TCR-initiated signaling that directly and significantly affects the regulation of PLCγ1 and the consequent mobilization of Ca^{2+}. Itk also participates in the regulation of cytoskeletal reorganization as well as cellular adhesion, which is necessary for a productive T cell response. The functional cellular outcome of these molecular regulations by Itk leads it to be an important mediator of T cell development and differentiation. This review encompasses the structure of Itk, the signaling parameters leading to Itk activation, and Itk effects on molecular pathways leading to functional cellular outcomes. The incorporation of these factors leads one to believe that Itk serves as a modulator, or rheostat, critically fine-tuning the T cell response.

1.2 Introduction

Normal T lymphocyte activation occurs through its antigen receptor leading to various signaling cascades and ending in a certain function. These activating signaling cascades are exquisitely balanced, as different signals can lead to many different functional outcomes. Inappropriate signaling or skewed signals can cause many problems for the T cell and for a functional immune system. Aberrant T cell function can lead to many devastating diseases such as leukemia and autoimmune disorders.

When a T cell encounters an antigen presenting cell (APC), it must be able to discriminate whether or not the APC is healthy. This occurs through physical interaction between surface molecules of the APC and T cell, reading (if you will) the health of the APC, and translating that information into a response. This requires many coordinated molecular interactions from the cell surface, through the cytoplasm, on to the nucleus, and in some instances, back out through the cell surface again (1).

At the cell surface, interaction between the peptide-loaded major histocompatibility complex (pMHC) on the APC and the T cell receptor (TCR) on the T cell is preceded by the clearance of other "taller" surface molecules that promote adhesion between the two cells. This adhesion process pulls the two cells together, and consequently, moves the "shorter" pMHC closer to the "shorter" TCR (2, 3). This cellular interaction leads to the first domino to fall in the T cell signal transduction activation pathway. Co-receptors CD4 on helper T cells or CD8 on cytotoxic T cells, interacts with its corresponding APC pMHC of class II or class I, respectively. This

interaction causes the dissociation of the tyrosine phosphatase CD45 from CD4/CD8, which enables the homodimerization of CD45 molecules leading to their activation (the ligand for CD45 is still unknown) (4). Active CD45 causes dephosphorylation of CD4/CD8 bound Lck on its inhibitory tyrosine (tyrosine 505). Once uninhibited, Lck autophosphorylates itself within its activation motif (tyrosine 394). Further, active CD45 dephosphorylates membrane bound Cbp/PAG causing its associated molecules, Csk and PEP, to be released into the cytoplasm, away from the cooperative inhibition of Lck (5). Csk and PEP are negative regulators of Lck; Csk is a tyrosine kinase that phosphorylates the negative regulatory site of Lck (tyrosine 505), while PEP is a phosphatase that dephosphorylates the activation tyrosine of Lck (tyrosine 394). With activated Lck in tow, CD4/CD8 co-receptor interacts with its cognate pMHC, pulling activated Lck into proximity of the TCR/CD3 complex. Lck then phosphorylates the intracellular tyrosine activation motifs (ITAMs) within the CD3 complex. Phosphorylation of the CD3 ITAMs promotes the docking of other molecules to the CD3 complex, namely the zeta-associated protein of 70 kilodaltons (ZAP-70) which is then phosphorylated by Lck for its consequent activation (6, 7). Activated ZAP-70 then phosphorylates the linker for activated T cells, or LAT, a transmembrane and palmitoylated attached adaptor that bridges the initial TCR signal to many of the downstream signaling events (8).

Phosphorylation of LAT is paramount for subsequent signaling due to the critical nature of the proteins binding to it (9). Another cytoplasmic adaptor molecule, the SH2 domain containing leukocyte phosphoprotein of 76 kilodaltons (SLP-76), binds to LAT through Gads where it becomes phosphorylated by ZAP-70 (10). SLP-76 binds to and inducibly recruits many other cytoplasmic molecules to the activated LAT complex,

including Itk, PLCγ1, ADAP, and Nck (11). LAT recruited Itk becomes transphosphorylated at the membrane by Lck on its activation tyrosine (tyrosine 511), and then autophosphorylates itself on tyrosine 180 for full activation (12). Activated Itk phosphorylates PLCγ1 on tyrosines 775 and 783 leading to the activation of this lipase (13-15). PLCγ1 is critical in two signaling pathways, culminating in the activation of transcription factors important for genetic regulation.

Activated PLCγ1 cleaves membrane phosphatidyl inositol (4,5) bisphosphate (PIP_2) into two products, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses into the cytoplasm to bind its cognate IP_3 receptors (IP_3R) on the endoplasmic reticulum (ER). Calcium levels in the cytoplasm are sustained through the activation of capacitative Ca^{2+} channels on the plasma membrane (such as STIM1, explained in detail later) and through the activation of a indirectly Ca^{2+} triggered phosphatase, calcineurin (16). Cytoplasmic Ca^{2+} binds calmodulin, which causes the dissociation of calmodulin from calcineurin, freeing this phosphatase to dephosphorylate cytoplasmic localized nuclear factor of activated T cells (NFAT). NFAT then translocates into the nucleus to perform its transcription factor duties of genetic regulation (17).

The other half of PLCγ1 activity is the generation of DAG. DAG itself is integral in activating two more transcription factor pathways. First, DAG activates the serine/threonine protein kinase C theta (PKCθ), which phosphorylates i-kappa-b kinase (IKK) (18). In turn, active serine/threonine kinase IKK phosphorylates the inhibitor of kappa light chain gene enhancer in B cells (IκB) causing its polyubiquitination and consequent proteosomal degradation. When phosphorylated, IκB dissociates from its inhibitory target, the nuclear factor of kappa light chain enhancer in B cells (NFκB),

releasing NFκB into the nucleus to perform its transcription factor duties (19). DAG's secondary activation role is the phosphorylation of Ras guanyl releasing protein (RasGRP), a GTP exchange factor (GEF) (20). RasGRP activates Ras, leading to the downstream activation of mitogen activated protein kinase (MAPK) pathways. The MAPKs jun-N-terminal kinase (JNK) and p38 phosphorylate Jun and Fos respectively, causing them to translocate into the nucleus and combine to form the AP-1 transcription factor important for genetic regulation (21).

The TCR activated, LAT bridged, SLP-76 signalosome also brings in two more players important for the sustained activation of the T cell necessary for its full response. These two molecules are the adhesion- and degranulation-promoting adaptor protein (ADAP) and non-catalytic region of tyrosine kinase (Nck). ADAP is an adaptor protein that leads to the intracellular activation of extracellular integrin adhesion molecules, in a process termed as "inside-out" signaling (22, 23). Integrin activation helps to maintain T cell contact with the APC for an extended period to allow full T cell response to the presented antigen (24). Nck is another adaptor that brings the cytoskeleton organizing molecule, Wiskott-Aldrich syndrome protein (WASp), to the proximity of the SLP-76 signalosome (25, 26). WASp is indirectly activated through the GEF Vav1. Vav1 exchanges GTP for GDP for Rho GTPases, particularly that of Cdc42, which when in the GTP-bound conformation fully activates WASp by binding WASp's CRIB domain. This causes WASp to destabilize its autoinhibitory condition and take on an open conformation allowing the Arp2/3 complex to bind and seed actin polymerization (27). Therefore, active WASp causes actin to be reorganized at the T cell junction adjacent to

the APC, enabling the T cell to stay stably in contact with the APC for the duration of its response.

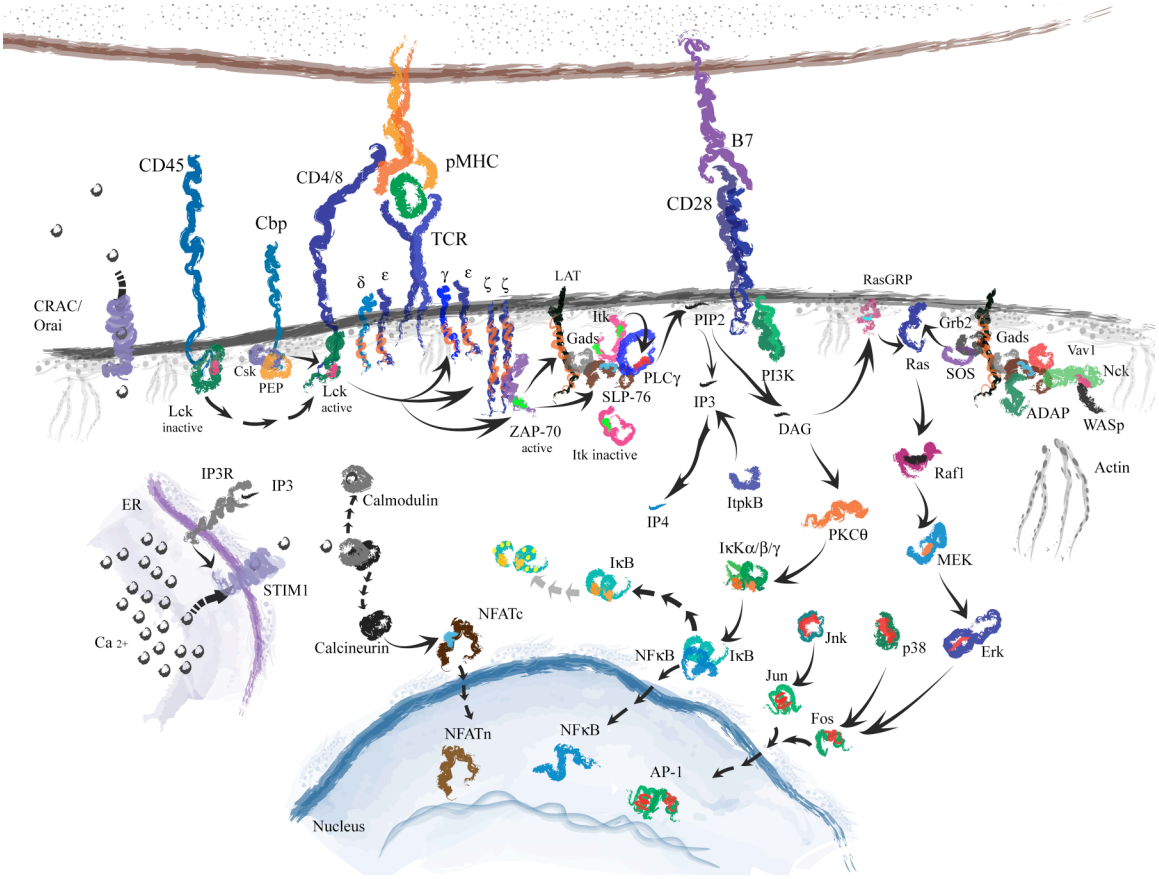

Figure 1.1. *T cell receptor activated signal transduction pathways.*

Cartoon diagram of the critical protein interactions necessary for the activation of a T cell when engaging an antigen presenting cell.

The focus of this review will be on Itk, an enigmatic kinase that serves as a rheostat for many of the signaling pathways needed for a full and effective T cell response. Itk is needed for the regulation of Ca^{2+} flux, actin polymerization, and integrin binding. Consequently, it has a profound role in the development of T cells, cytokine expression, and the clearance of pathogens. The mention of other lymphoid signal

transduction molecules, particularly that of other Tec family kinases, will only be mentioned as a reference to Itk in order to highlight the importance of Itk (28, 29).

1.3 Identification of Itk

Interleukin-2 inducible tyrosine kinase was discovered and cloned by three independent research groups. Stephen Desiderio's group was the first to identify the gene and named it Itk (IL-2 Inducible Tyrosine Kinase)(30). This was quickly followed by Leslie Berg's group (T-cell specific tyrosine kinase, Tsk)(31) and Toshiaki Kawakami's group (Expressed in Mast and T cells, Emt)(32). This nearly simultaneous cloning of the gene and subsequent naming by three different groups led to some conflicting nomenclature initially, but the gene is now accepted to be named Itk. This kinase is a 72-kDa protein expressed in the thymus, spleen, and lymph nodes. More specifically, Itk is expressed in T cells, Natural Killer cells, Natural Killer T cells and Mast cells. It has been chromosomally localized to 5q31-32 in humans (33). This localization is unique to Itk as other Tec family kinases are seemingly duplicated; Tec and Resting Lymphocyte Kinase (Rlk) both are on the 4p12 locus (34), while Bruton's Tyrosine Kinase (Btk) and Bone Marrow Kinase (Bmx) are both on the X chromosome (albeit separated somewhat, Btk on Xq22 and Bmx on Xp22 (35)). Evolutionarily, the Tec kinases have been found in flies (*Drosophila melanogaster*), zebrafish (*Danio rerio*), skates (*Raja eglanteria*), and in sea urchins (*Anthocidaris crassispina*) (36). Two different forms of Itk have been cloned in the mouse, which have six amino acids either included or deleted (37). The shorter version is exclusively detected in human cells. Itk has substantial effects on T cell development and function, due in large part to its disproportionate expression levels compared to other Tec kinases. As measured by qRT-PCR, naïve mouse T cells contain 100-fold more copies of Itk compared to Tec, and 3-fold more copies than that of Rlk

(38). Upon T cell stimulation, Itk expression is increased, especially in T_H2 cells, while Rlk expression is reduced and becomes relegated to T_H1 cells (39). Tec expression in T cells increases only after several days of stimulation (40).

1.4 Protein Structure of Itk

Itk is a modular protein, consisting of five distinct structural domains. At its amino terminus, Itk contains a Pleckstrin Homology (PH) domain, which allows the protein to bind phosphorylated lipids on the membrane. Adjacent to this is the Tec Homology (TH) domain from which comes Itk's familial association. This domain contains a poly-proline rich region (PRR) necessary for binding Src Homology 3 (SH3) domains. The adjoining domain is the SH3 domain, which binds to PRR both in cis- (to itself) and in trans- (to other proteins). The following Src Homology 2 (SH2) domain enables the protein to bind tyrosine-phosphorylated substrates. At its carboxyl terminus, Itk possesses an Src Homology 1 (SH1) or kinase domain, which specifically phosphorylates tyrosine residues, particularly that of PLCγ1 (41). Itk, as well the Tec kinases, seems to be evolutionarily similar to the Src family kinases in their structural similarities. However, the Tec kinases lack the N-terminal myristoylation sequence constitutively localizing Src kinases to the membrane and lack the C-terminal negative regulatory tyrosine residue present on Src kinases.

1.4.1 The Pleckstrin Homology (PH) domain (amino acids 5-112)

PH domains are protein-lipid interaction domains of about 100 amino acids in size, forming a β-barrel from two β-sheets and a carboxyl-terminal α-helix (42, 43). A unique feature of the Tec family tyrosine kinases is the presence of a PH domain. Rlk is an exception as it contains a cysteine palmitoylation motif and no PH domain (44). The

PH domain contains the propensity to bind phospholipids in many forms, including phosphatidylinositol-3,4,5 triphosphate (PIP_3), $PI(4,5)P_2$ and PI(4)P, with Itk binding to PIP_3 at the highest affinity. Binding of Itk through its PH domain to the plasma membrane is inducible through the activity of PI_3K, which phosphorylates PIP_2 to PIP_3. Addition of PI_3K inhibitors leads to a lack of Itk localization to the plasma membrane and consequently, its activity (45-47). Therefore, Itk is targeted to the membrane and activated through a PI_3K-dependent mechanism. Interestingly, localization to the plasma membrane through the PH domain is not enough for Itk's activation. Itk with the PH domain deleted and replaced with a myristoylation sequence enables Itk to go to the membrane, but it does not become tyrosine phosphorylated upon TCR stimulation (45). These data indicate that the PH domain contains some other vital role to Itk's activation other than simple membrane localization. PH domain binding to PIP_3 localizes Itk in proximity of its activating kinase, Lck, thereby increasing Itk's kinase activity, similar to how the PH domain of Btk does to increase its activity (45, 46, 48). Mutation of the PH domain of Itk at amino acid 29 (arginine to cysteine, R29C) leads to its lack of membrane localization and its inactivation (45). Also, this domain is integral for binding to the nuclear membrane when the molecule is shuttled to the nucleus (49). Although selective PH domain inhibitors of Btk have been reported, such as the quinone epoxide terreic acid (50), no selective PH domain inhibitors of Itk have been reported. Recently, the PH domain of Itk was shown to bind to inositol-1,3,4,5-tetrakisphophate (IP_4) *in vitro* with a functional *in vivo* consequence (51). Molecularly, this includes Itk's disruption of its inducible localization to the T cell/APC contact site and for its activation. Cellularly, this creates a phenotype where thymocytes undergoing positive selection become unable to

progress beyond the $CD4^+CD8^+$ double positive stage of development. This necessitates Itk's involvement in T cell development, which requires an alternative role for Itk's PH domain.

1.4.2 The Tec Homology (TH) domain (a.a. 115-163)

Itk's TH domain contains a familial Tec motif of a conserved 27 a.a. sequence at the amino-terminus of the domain (52) as well as a proline rich region (PRR) at a.a. 153-163. This domain is integral to the activity of Itk as it is thought that this domain binds to its own SH3 domain in an auto-inhibited state (53). Disruption of this association, either by competitive interaction with another peptide sequence or by mutation, was thought to relieve this auto-inhibition and activate the kinase. However, deletion of only the PRR has an opposite effect, it reduces Itk's basal activity by 50% (54). As of yet, no protein has been shown to functionally bind to the TH domain of Itk. Those proteins that have been shown to bind *in vitro* have later been shown to bind indirectly *in vivo*, either through another Itk structural domain or through another molecule entirely (55). Given the paradoxical nature between the structure and the function of this domain, more work should be done to identify what the TH domain is doing in Itk.

1.4.3 The Src Homology 3 (SH3) domain (a.a. 174-230)

SH3 domains allow for protein-protein interactions to proline peptide sequences (either (R/K)XXPXXP or PXXPXR motifs, where X is any amino acid) facilitating protein function or cellular localization. Not only is the SH3 domain of Itk involved with

the binding of internal and external PRRs, but it is also involved in the negative regulation of its own catalytic activity. To wit, elimination of the SH3 domain or mutation of a Tec family conserved tryptophan (W208K) that is required for SH3 binding to PPR causes a spontaneous activation of the enzyme, presumably through the release of auto-inhibition (56). Paradoxically, auto-phosphorylation on tyrosine 180 leads to the activation of Itk (12, 57), so elimination of this domain should theoretically lead to an inactive condition. However, elimination of this domain and mutational disruption most likely leads to a conformational release of auto-inhibition, allowing the kinase domain to become exposed and catalytically active. It is also possible that the phosphorylation within this domain prevents the binding of a repressor protein. There are a plethora of proteins that bind the SH3 domain of Itk *in vitro* (58), including WASp, Sam68, Cbl, SLP-76 and Vav1, many of which have not been confirmed *in vivo* (refs.). The interaction between Itk's SH3 domain and the nuclear import chaperone karyopherin α (Rag cohort 1α, Rch1α) leads to Itk's transport into the nucleus (49). This interaction requires Rch1α's proline 242, as a mutant Rch1α (P242A) decreased nuclear localization of Itk and diminished IL-2 production. As described later, this is important as Itk not only serves as a cellular membrane proximal kinase linking early signal transduction events, but it also serves to regulate transcriptional activation of certain proteins in the nucleus.

1.4.4 The Src Homology 2 (SH2) domain (a.a. 237-329)

SH2 domains allow for inducible protein-protein interactions with phosphotyrosine-containing peptide sequences (59, 60). Itk's binding to tyrosine phosphorylated substrates provides the mechanism not only for it to inducibly interact with protein partners, but has shined light on its own localization and consequent activation. Mutation of the SH2 domain of Itk, either through deletion or through point mutation of the tyrosine binding pocket, leads to the inactivation of Itk (56). Itk has been reported to bind to SLP-76 (55, 61), LAT (46, 56) and Vav1 (62) through its SH2 domain, although it has not yet been determined which of these molecules the SH2 domain of Itk has a higher affinity for. Itk also binds to PLCγ1 through it's SH2 domain, though it is not resolved as to whether it is direct (41), or indirect through SLP-76 (13, 63). Importantly, it has been shown that Itk is negatively regulated through its SH2 domain by the prolyl-isomerase cyclophilin A, whose binding to Itk can be competed against by Cyclosporin A leading to Itk's activation (64). Cyclosporin A is a cyclic decapeptide produced by the soil fungi *Tolypocladium inlatum*, and is used as an immunosuppressant particularly for organ transplantation (65-67). Peptidylprolyl isomerases, such as cyclophilin A, convert proline peptide bonds to cause a cis- or trans-conformational switch. Cyclophilin A binds to a proline residue in the SH2 domain of Itk (P287), promoting the cis-intramolecular interaction with the SH3 domain. This proline residue switch is unique to Itk, as it is not a shared phenomenon with the other Tec kinases (68). When cyclophilin A is relieved of binding, an isomeric switch within the SH2 domain causes Itk to take on a trans-conformation, which allows it to bind to other proteins. These data imply that cyclophilin A is a negative regulator of Itk activity.

Consistent with this idea, T cells deficient for cyclophilin A display an overall pattern of increased signaling in response to TCR stimulation, with an increase of PLCγ1 phosphorylation in particular (69).

1.4.5 The Src Homology 1 (SH1) domain (a.a. 363-612)

The sting for Itk's kinase activity resides within the SH1 domain. Herein lies its ATP binding pocket, allowing it to enzymatically transfer the terminal ATP phosphate to its protein target(s). Also within this domain is tyrosine 511 which needs to become trans-phosphorylated by Lck for the initial activation of Itk (70). X-ray crystal structures of the kinase domain of Itk have been resolved both in the non-phosphorylated and in the phosphorylated states (71). Interestingly, phosphorylation of tyrosine 511 did not induce a conformational change in this domain indicating that although phosphorylation of this residue is required for the activity of this enzyme, it does not do so through a phosphorylation dependent conformational switch. Rather, phosphorylation of the kinase domain could lead to some secondary function. Further, the isolated kinase domain of Itk is similar to the other Tec kinases in that they are kinetically inactive. This is in stark contrast to any other SH1 containing kinase. This indicates that other domains of Itk help to regulate the kinase.

1.5 Upstream Regulators of Itk

The activation of Itk is a complex orchestration of events. This kinase is activated through a myriad of surface receptors, including the TCR/CD3 signaling complex, co-receptors, chemokine receptors and hetero-trimeric G-protein-coupled receptors (GPCRs). Prior to the activation of the T cell, Itk resides in the cytoplasm in a closed, auto-inhibited state. This inhibitory conformation occurs through the cis- binding of its SH3 domain to the PRR of the TH domain (53). More recently, however, it was found that the SH2 and SH3 domains of Itk could dimerize with each other in a trans-head-to-tail manner that could preclude or mitigate the cis-binding of a single Itk molecule to itself (77). Determining the conformational state and activation of full-length Itk is critical to know, and will soon be solved with either NMR or X-ray crystallography using full-length Itk, or by using full-length Itk employing FRET technology in live cells. Itk associates with SLP-76 while in the cytoplasm, and is shuttled to the membrane associated LAT signalosome by SLP-76 upon TCR/CD3 stimulation (55). While at the LAT signalosome, Itk becomes phosphorylated at tyrosine 511 of the SH1 domain by Lck (12, 70). Trans-phosphorylation activates Itk kinase activity, whereupon the kinase auto-phosphorylates itself at tyrosine 180 of the SH3 domain (12). Cis-phosphorylation fully opens the protein, allowing the PH domain to bind PI_3K phosphorylated lipids at the membrane and therefore act as a fully active kinase within the LAT signalosome. Cells lacking Lck or containing a mutant Itk phenylalanine substitution for tyrosine at position 511 leads to a kinetically inactive Itk (70). Further, mutation of Itk at tyrosine 180 to phenylalanine impairs Itk's activity in response to stimulation (12). This Itk

autophosphorylated tyrosine lies within the substrate binding sequence of the SH3 domain (53), leading one to speculate that this tyrosine regulatory site is not just important for enzymatic activity, but for protein-protein interactions as well. Similarly, Btk's autophosphorylation site at tyrosine 223 can only bind to the Wiskott-Aldrich Syndrome protein (WASp) while in the non-phosphorylated state and can only bind to Syk while in the phosphorylated state (78). It would be reasonable to speculate that Itk acts in a similar fashion.

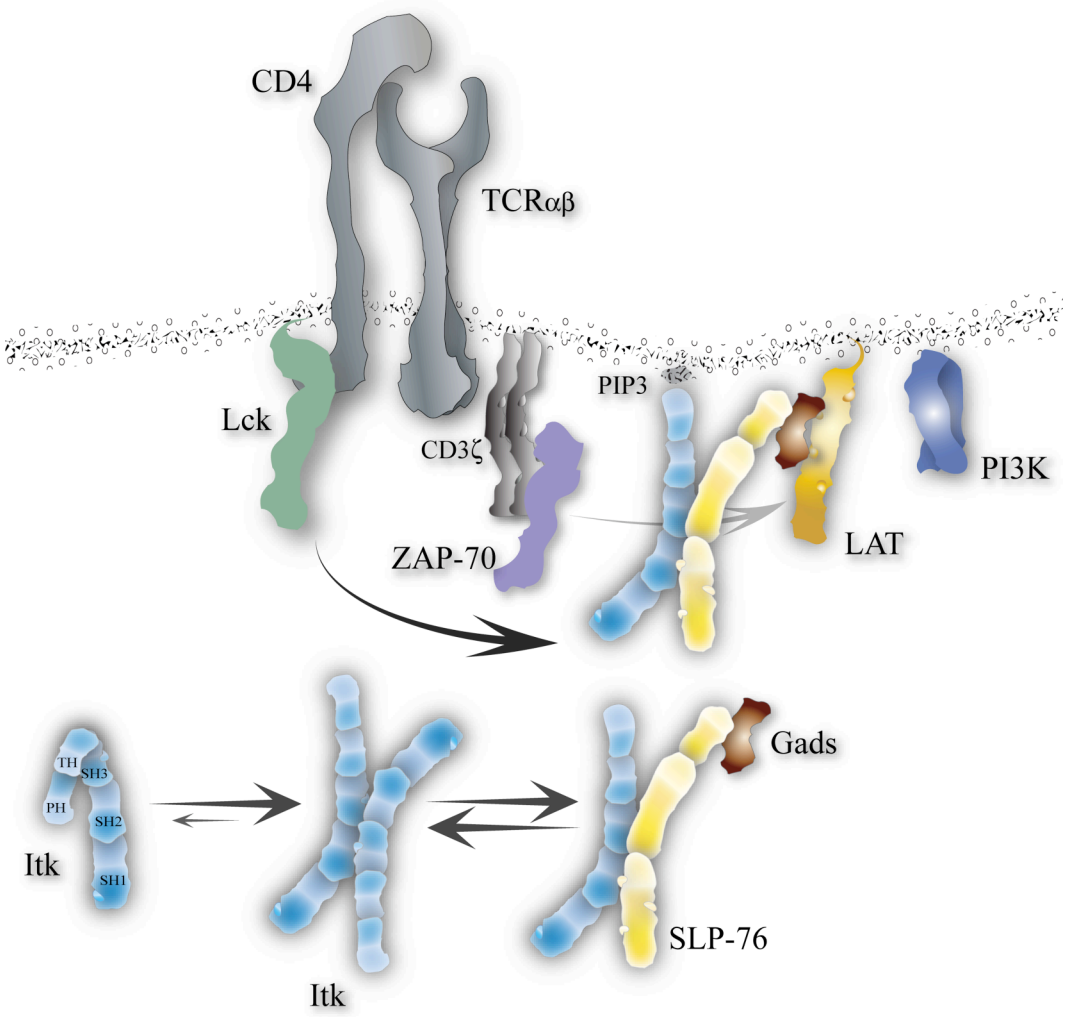

Figure 1.2. *Mode of Itk activation through the T cell receptor.*

While residing in the cytoplasm, Itk preferentially takes on an inhibited homodimer conformation and in some instances as an inhibited independent cis-conformation. Upon T cell engagement, Itk is able to bind SLP-76 through its SH3 domain and shuttled to the LAT signalosome. Once there, activated ZAP-70 phosphorylates SLP-76 on tyrosine 145 which Itk then binds to with its SH2 domain to solidify the connection at the signalosome. At which time Itk localizes to the plasma membrane by binding PI_3K generated PIP_3 with its PH domain and Itk then becomes tyrosine phosphorylated by Lck on tyrosine 511. This leads to Itk's catalytic activity that first phosphorylates itself on tyrosine 180 culminating in a fully activated enzyme.

1.5.1 T cell receptor activation

TCR engagement leads to the pivotal activation of two molecules that are integrally important for the activation of Itk, Lck and PI_3K. Activation of Lck through the dephosphorylation of its inhibitory tyrosine by CD45 causes a rapid phosphorylation of receptor components as well as associating molecules such as ZAP-70 and downstream adaptor molecules LAT and SLP-76. Recruitment of Itk to the TCR nucleated signalosome is mediated through SLP-76, which brings Itk from the cytoplasm to the plasma membrane where Itk PH domain can bind to PI_3K phosphorylated phospholipids (Grasis, unpublished observations). Recruitment by SLP-76 to the ZAP-70 primed LAT signalosome leads Itk to be trans-phosphorylated by Lck. This combination of events leads to the fully activated state of Itk, one that is relieved of auto-inhibited restraints, and one that can phosphorylate downstream targets.

1.5.2 Co-receptor activation

Upon CD28 stimulation, Itk binds to CD28 and becomes tyrosine phosphorylated by Lck (79-81). Itk's interaction with the cytoplasmic polyproline motif of CD28 through its SH3 domain facilitates both the activation of Itk and the tyrosine phosphorylation of CD28 on all four tyrosines found in its cytoplasmic tail (82-84). This activation of Itk helps to amplify TCR signals, which has a profound effect on Ca^{2+} mobilization (85). This association was originally proposed to illuminate Itk as a negative regulator of T cell signaling through CD28, as mice lacking Itk have an increased proliferative response to CD28 stimulation (86). However, more recent

findings indicate that Itk is not essential for CD28 signaling (87). This paper used purified $CD4^+$ T cells from $Itk^{-/-}$ mice and noted that these cells already have an activated or memory phenotype that becomes enhanced upon stimulation. They therefore found that CD28 signaling was normal in $Itk^{-/-}$ cells and that Itk does not act as a negative regulator of CD28 stimulation. Similar to the binding of Itk to CD28, Itk has been reported to bind to the PRR on the cytoplasmic portion of CD2, enabling Itk to become activated by Lck upon CD2 cross-linking (88, 89).

1.5.3 Chemokine and Hetero-trimeric G-protein coupled receptors

Both sets of binding G-protein subunits can functionally associate with Itk. The G-protein βγ subunits have been shown to bind to Itk through Itk's PH domain and can promote Itk activity (90). Chemokines are small proteins that are inducibly secreted by resident cells to promote the recruitment and migration of lymphocytes to an area of infection. Chemokine stimulation linking G-protein coupled receptors to actin polymerization events have shown that Itk can be mobilized to the plasma membrane and tyrosine phosphorylated upon Stromal cell-derived Factor 1-alpha (SDF-1α, also known as CXCL12) chemokine stimulation (91, 92). Itk's response to SDF-1α is sensitive to both PI_3K and pertussis toxin from the bacterium *Bordetella pertussis*, which inactivates $G\alpha_i$. Additionally, overexpression of $G\alpha_{transducin}$ leads to the dominant-negative inhibition of endogenous Gβγ subunits, which affects recruitment of Itk to the plasma membrane in HeLa cells. Since PI_3K can be activated through Gβγ, it follows that recruitment of Itk to the plasma membrane upon chemokine stimulation is dependent on a

Gβγ-PI_3K pathway. The Schwartzberg group also showed that mutant Itk can block the chemokine-induced polarization and activation of the Rho GTPases Cdc42 and Rac (91). Consistent with the molecular findings, $Itk^{-/-}$ mice are deficient in their ability to home to the lymph node, and as such, there are reduced numbers of T cells in the lymph nodes in comparison to the numbers of T cells in the spleen (91). Further, Itk deficient T cells display an impairment in their ability to migrate to the lungs in response to CXCR4 chemokine (92). This finding has been reiterated in mice treated with a kinase-domain Itk-specific inhibitor, whose T cells were also reduced in ability to home to the lungs (75).

1.6 Downstream Itk effectors

Intriguingly, Itk is a known activator of many different downstream targets leading not only to the immediate activation of certain signal transduction pathways, but also to transcriptional activation of certain genes corresponding to the development of T cell subsets.

1.6.1 PLCγ1 and calcium mobilization

As mentioned above, Itk is a critical activator of PLCγ1. T cells from $Itk^{-/-}$ mice display diminished tyrosine phosphorylation of PLCγ1 (93) and of tyrosine-783 in particular (63), the critical activating site for this lipase. Phosphorylation of PLCγ1 leads to the hydrolysis of PIP_2 into the secondary messengers IP_3 and DAG. IP_3 binding to the IP_3R on the ER causes a change in cytoplasmic Ca^{2+} concentration from 100 nM to 1 μM within seconds. The consequence of PLCγ1 inactivation is lack of downstream IP_3 binding to IP_3R preventing the release of intracellular Ca^{2+} stores (93, 94) and the inhibition of calcium release activated channels (CRAC) at the plasma membrane (94). CRAC-mediated capacitative influx of Ca^{2+} from extracellular sources prolongs the signaling necessary for later activation, including transcriptional activation. This sustained Ca^{2+} influx is necessary for long-term signaling in the T cell as well as the formation and the continued presence of the immunological synapse (95). Accordingly, Itk-deficient T cells are severely diminished in their short-term and long-term Ca^{2+} flux abilities. The lack of sustained Ca^{2+} flux leads to the downstream inactivation of Erk

(93), which manifests itself into a lack of NFAT translocation to the nucleus to initiate its transcriptional activity. Kinase-inactive mutants of Itk cannot phosphorylate PLCγ1 and therefore, downstream activation of Erk and of TCR-induced NFAT reporter activation is compromised (55). This inactivation is also due to the lack of PLCγ1 generated DAG, a necessary component for the activation of the DAG-binding domain containing protein RasGRP, which is required for Erk pathway activation in T cells (96, 97). There is some functional redundancy to the Tec kinases in T cells, as PLCγ1 tyrosine phosphorylation and subsequent Ca^{2+} flux is only reduced, and not ablated in cells lacking Itk (93, 94). Rlk or Tec knockouts do not display these phenotypes. However, double knockouts of Itk and Rlk diminish these two characteristics even further (93). Further, overexpression of Tec or Rlk in the absence of Itk can functionally rescue the Itk-deficient phenotype (40).

1.6.2 Actin reorganization

The Tec family kinases were initially implicated in the regulation of the actin cytoskeleton through work done in Drosophila. The Tec family kinase Tec29 was found to be required for the growth of ring canals, a necessary bridge between the nurse cells and oocyte during embryogenesis (98, 99). The ring canals in the Tec29 mutant egg chambers failed to form correctly, thereby failing to nourish the maturing oocyte. This data shows an evolutionarily conserved function for Tec kinases in actin regulation.

Within mammalian T cells, it was discovered by two groups that Itk was involved in TCR-induced actin polymerization (100, 101). These data showed that in T cells

lacking Itk, actin directed processes (such as lamellipodial formation and directed actin polymerization) were impaired. The significance of this deficiency is striking, as the defects in actin polymerization and polarization in $Itk^{-/-}$ T cells is complete, whereas the defect in Ca^{2+} flux is only reduced. Additionally, this defect is most likely entirely due to the absence of Itk, as T cells deficient for both Itk and Rlk are just as defective in actin polymerization as Itk singly deficient T cells (101). Further, in an overexpression system in Jurkat T cells, Itk leads to an increase in membrane ruffling (lamellipodia) (46). This regulation of actin is through the interaction of Itk with two molecules directly involved with actin polymerization, Vav1 and WASp (62, 102). Vav molecules are GTP exchange factors (GEFs) that exchange GDP for GTP to activate members of the Rho GTPase family, particularly that of Cdc42 in T cells. Vav1 deficient T cells not only display a defect in the ability to activate the Rho proteins, but also show very striking actin defects. These cells fail to polymerize actin, show impaired integrin adhesion and clustering, and display broader cellular defects than that of T cells lacking either Itk or WASp (103, 104). Initially, it was determined that Itk affected Vav1 localization, as T cells deficient in Itk failed to localize Vav1 when engaging antigen-pulsed APCs (101). This defect had a functional consequence, as activated Cdc42 no longer localized to the contact site as measured with a biosensor. Itk was discovered to constitutively associate with Vav1 (62), an association that was dependent upon Itk's SH2 domain. This interaction confirms a hypothesis that Itk has a kinase-independent function (105), thus broadening the role of this kinase beyond the simplistic idea that a kinase must only act as a kinase, that is, to phosphorylate targets. Notably, however, is the finding that Vav1 still becomes tyrosine phosphorylated in cells lacking Itk (although it has not been determined whether

tyrosine 174 phosphorylation is defective, the key activating tyrosine in Vav1 (106)), a finding compounded by the data that Itk activation is also abnormal in cells lacking Vav1 (107).

The Wiskott-Aldrich Syndrome protein (WASp) is the gene product whose deficiency leads to Wiskott-Aldrich Syndrome, a disease state that includes lymphopenia mediated by a defect in actin regulation. WASp is vital in actin regulation as it activates the Arp2/3 complex which helps to nucleate new actin filaments (108-112). WASp is indirectly dependent upon Vav1 for its activation as it needs to bind to GTP bound Cdc42 to release its auto-inhibition and activate the Arp2/3 complex, leading to the nucleation of new actin filaments (27). Itk has been shown to bind WASp, both *in vitro* (58) through its SH3 domain and *in vivo* (102) using full-length molecules, however the functional outcome of this interaction is muddied because Itk seems to be required for WASp's localization (101), but not for its phosphorylation (113), a requirement necessary for WASp's full activity (114, 115).

The intricate dance between Itk, Vav1, and WASp, as it relates to TCR-induced actin polymerization remains an interesting topic, as it seems that Itk affects both of these molecules in a kinase-independent manner. This does not, however, preclude that Itk can have a kinase-specific role for the continued activation of these molecules. If Vav1 is probed for its phosphorylation on its activating tyrosine 174 (116) and if WASp is probed for its phosphorylation on its activating tyrosine Y291 (114) in the presence or absence of Itk, one will get a better idea of Itk's role in a kinase-dependent or kinase-independent manner. Furthermore, it has been reported that the Tec family kinase Btk can not only bind actin directly through its PH domain (117), but it can also tyrosine phosphorylate

WASp as well (118, 119). Also, given that the tyrosine kinase Abl can bind and phosphorylate actin directly (120), and that the tyrosine kinase Fyn can phosphorylate WASp (113), it may be useful to pursue whether Itk can perform any of these duties as well.

One more factor in Itk's regulation of TCR-induced actin polymerization is that Itk's role in Ca^{2+} flux cannot be misunderestimated (all pun intended). Actin reorganization itself can be regulated by the Ca^{2+} sensitive protease calpain. Calpain cleaves actin binding proteins, which allows for reorganization of the actin cytoskeleton. It is not known whether Itk is directly involved in calpain activation, or whether calpain deficiencies are evident in cells lacking Itk (121-123).

The effects of Itk seen on actin polymerization in T cells also dismisses the idea that the cellular defects seen in $Itk^{-/-}$ cells is due merely to the molecular inactivation of PLCγ1. The decrease in actin polymerization can affect signal strength and signal duration, as well as affect integrin-mediated adhesion leading to the known defects in $Itk^{-/-}$ T cells including altered CD4/CD8 ratios, specific decrease in cytokine production, and in the proliferation of these cells. It is likely that the defect in cytoskeletal reorganization contributes to the decreased response of $Itk^{-/-}$ T cells as a result of inefficient immunological synapse formation (124).

1.6.3 Adaptor proteins

As mentioned above, Itk associates with a plethora of adaptor molecules, most notably, SLP-76. Itk needs SLP-76 for its phosphorylation and for its localization to

T cell/APC contact site (Grasis, unpublished observations, and (13, 63)). The interaction between Itk and SLP-76 is pivotal, as it helps to integrate Itk's functional relationship with many molecules, including LAT, PLCγ1, and Vav1. The interaction of Itk with SLP-76 is a complex undertaking, as it is surmised through *in vitro* studies that the interaction is a cooperative one involving SH3 domain of Itk binding to PRR of SLP-76, and then SH2 domain of Itk binding to phosphorylated tyrosine 145 of SLP-76, once it has been phosphorylated by ZAP-70 (55). This multivalent binding of Itk to SLP-76 provides the means for Itk to associate with another adaptor molecule necessary for its activation, LAT. TCR-induced SLP-76 signalosome bridging to the LAT signalosome is mediated by the adaptor protein Gads (125). SH3 domain of Gads binding to a PRR motif within SLP-76 separate from that of Itk indirectly connects SLP-76 to LAT when the latter becomes phosphorylated and the SH2 domain of Gads can bind that site. Although Itk does not bind Gads directly, Itk activation is therefore dependent on Gads ability to unite SLP-76 and LAT. Itk has been reported to bind LAT directly *in vitro* using GST-fusion proteins of the Itk SH2 domain (126). This is supported by the evidence that Itk needs to be localized to the LAT signalosome for its activation (56). Further, the association of Itk with LAT may have functional conformational consequences for Itk's activation (127). This would imply LAT involvement with Itk to be more than that of localization, and might have an additional role. Therefore, the question remains whether the binding to Itk to SLP-76 or LAT is more important for Itk activation. The answer probably is both. SLP-76 and LAT are integral for the activity of a T cell, so consequently both are necessary for the activation of Itk. Lastly, Itk has also been reported to bind another adaptor molecule, Rlk/Itk binding protein (RIBP) (128,

129), which has been reported to negatively regulate Itk function, but the mechanism of this has yet to be determined.

1.6.4 Regulation of cellular adhesion

In order for T cells to initiate and maintain contact with an APC, functional adhesion through cell surface proteins known as integrins are necessary. This integrin adhesion requires a functional actin cytoskeleton and also requires signaling through the TCR. Data from Jurkat T cells expressing mutant kinase domain Itk shows that Itk is integral in the activation of β1 integrins through the T cell receptor in an "inside-out" signaling function (130). Further, blocking TCR-induced T cell activation through inhibition of Itk leads to a block in β1 and β2-integrin adhesion, and it affects the recruitment of LFA-1 to the site of TCR stimulation (130). This observation was carried out further into primary mouse T cells by showing that TCR-induced upregulation of β2 integrin adhesion was also severely reduced in cells lacking Itk (131). Thus, the TCR-induced maintenance of the T cell interaction with APC is very much dependent on Itk's kinase activity. Although the exact molecular mechanism as to how this regulation through Itk occurs remains unknown, many signaling molecules involved in actin reorganization are involved in integrin adhesion as well. Molecules such as PI_3K, Vav1, PKCθ, and ADAP are all involved in integrin signaling, with only ADAP currently unknown as to being a participating partner with Itk (although given its extraordinary complicity with integrin signaling and its association with SLP-76, it would be a natural target to explore for Itk's regulation of integrin signaling).

1.6.5 Transcriptional regulation

$Itk^{-/-}$ mice T cells display a lack of NFATc translocation to the nucleus when the T cell becomes activated (132), as a result of diminished Ca^{2+} movement in cells lacking Itk. This is an expected result because this transcription factor is Ca^{2+} sensitive due to the phosphatase that dephosphorylates NFAT, calcineurin, becomes activated through binding free cytoplasmic Ca^{2+} (133). NFAT is regulated in a highly dynamic Ca^{2+} dependent manner because as intracellular Ca^{2+} levels drop, NFAT quickly becomes phosphorylated and exits from the nucleus. Consequently, for NFAT-dependent transcription to occur, quick pulses of ER-derived Ca^{2+} are not enough to sustain transcription. Rather, a prolonged surge of Ca^{2+} from extracellular sources is needed for NFAT to stay active and promote transcription (134). NFκB appears not to be defective in Itk-deficient cells, an interesting outcome as this transcription factor needs PKC to activate it, which it cannot do without sufficient DAG present (132). Though not measured directly, DAG activity is diminished as PKCθ-mediated activation of Erk is reduced in $Itk^{-/-}$ cells. The activation of AP-1 is also reported to be dependent on Itk activity, as its Erk dependent ability to bind DNA is reduced (135). Itk is also known to directly phosphorylate the transcription factor T-bet on tyrosine 525 (39, 136), which may cause the most direct means in controlling T_H1 development. When T-bet cannot become phosphorylated, it loses the ability to bind and downregulate GATA-3 expression, which allows GATA-3 to promote a T_H2 response. As mentioned earlier, Itk can localize to the nucleus upon activation, phosphorylating T-bet at this site, although the exact cellular location of this phosphorylation event is yet unknown, as well as the functional significance of Itk localization to the nucleus. The functional consequence of

Itk's phosphorylation of T-bet, however, will be explained in the context of T cell differentiation.

1.7 *In Vivo* Role of Itk

At first glance, the physiological defects in cells lacking Itk would be considered modest since a deficiency in Itk has not manifested itself in any known human disease. However, mice lacking Itk display intriguing and beguiling results when it comes to cellular function. When compared to other signaling molecules involved in TCR-induced T cell signal transduction, Itk defects mirror some, though not all the defects seen with nearby molecules. The degree of Itk defects is also variable, placing Itk more as a modulator of T cell signal pathways, rather than a bridge through which all signals must pass. Attached is a table relating the *in vivo* effects of Itk knockout mice with other knockout mice of related molecules (Lck (137), ZAP-70 (138, 139), Rlk (93), Tec (140), Itk/Rlk double knockout (93), LAT (141-143), SLP-76 (144, 145), PLCγ1 (146), WASp (147, 148), PKC-θ (149-153), Vav1 (103, 104, 154-158), ItpkB (159, 160), Gads (161), CypA (69, 162)).

Table 1.1 *Comparison of phenotypes between TCR-inducible signaling molecules.*

Table depicting developmental, biochemical, and phenotypic defects observed in mice deficient for the indicated gene in the first column. A "Yes" response denotes confirmation of the defect, while "N.A." indicates "not applicable" and "N.D." indicates "not determined". References for the defects are listed in the last column.

	Developmental Defects		**Biochemical Defects**								***In Vivo* Phenotype Defects**					**Ref.**
KO Mouse	Pre-TCR	Stage Affected	PLCγ1 Phos	Ca++ Flux	Erk Phos	NFAT Activity	IL-2 Prod.	Proliferation	Actin Poly.	Adhesion	Activated Phenotype	AICD	T_H2 Response	Pathogen Clearance	Eosinophilia	
Itk	Mild	DP - SP	Yes	Mild	Yes	Yes	Yes	Mild	Yes	Yes	Yes	Yes	Yes	Yes	Mild	39, 93, 163, 166, 183
Rlk	No	No	No	No	No	No	Mild	No	No	No	N.D.	N.D.	N.D.	N.D.	N.D.	93
Tec	No	No	N.D.	N.D.	N.D.	N.D.	No	No	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	140
Itk/Rlk	Yes	DP - SP	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	N.D.	93, 167
Lck	Yes	DN1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N.D.	N.A.	N.A.	N.A.	N.A.	N.A.	137
ZAP-70	Yes	DN3 - DN4	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N.D.	N.A.	N.A.	N.A.	N.A.	N.A.	138, 139
LAT^{Y136F}	Yes	DN3 - DN4	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Increase	N.D.	Yes	141-143
SLP-76	Yes	DN1	Yes	Yes	Yes	Yes	N.D.	Yes	Yes	N.D.	N.A.	N.A.	N.A.	N.A.	N.A.	144, 145
Vav1	Mild	DP - SP	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N.D.	N.D.	Yes	N.D.	N.D.	103, 104, 154-158
ItpkB	Yes	DP - SP	Yes	No	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	159, 160
Gads	Yes	DN3 - DN4	Yes	Yes	Yes	Yes	Yes	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	161
PLCγ1	Mild	DP - SP	Yes	Yes	N.D.	Yes	Yes	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	146
WASp	Mild	No	N.D.	Mild	No	Yes	Yes	Yes	Yes	No	Yes	N.D.	Yes	N.D.	N.D.	147, 148
PKCθ	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	N.D.	N.D.	Yes	Yes	Yes	N.D.	149-153
Cyp A	No	No	No	No	No	Yes	Yes	Yes	N.D.	N.D.	N.D.	N.D.	Yes	N.D.	N.D.	69, 162

1.7.1 Development and differentiation

$Itk^{-/-}$ mice display decreased numbers of maturing thymocytes, reduced proliferative responses and show a profound defect in $CD4^+$ T cell development (163). $Itk^{-/-}$ mice have half the number of $CD4^+$ T cells, which when related to Itk molecular signaling, may be due not only to signal strength, but to signal duration as well (124).

Proteins upstream and downstream of Itk, Lck and Erk1/2 respectively, have both been shown to be important in lineage commitment (164). Therefore, it makes sense that Itk would have defects in this area as well. However, both $CD4^+$ and $CD8^+$ T cells do emanate from the $Itk^{-/-}$ thymus, but at altered ratios due to lower $CD4^+$ numbers and normal $CD8^+$ numbers (though this does not mean that the $CD8^+$ cells are normal, as explained later). Thymic development in both fetal and adult $Itk^{-/-}$ mice is normal when analyzed at $CD4^-CD8^-$ (double-negative, DN) and $CD4^+CD8^+$ (double-positive, DP) stages, although there is evidence that the time of progression from DN to DP stages is longer in $Itk^{-/-}$ thymocytes when compared to wild-type cells (165). There is a reduction seen in peripheral $CD4^+$ T cells (163), which is due to an impairment in positive selection in class II MHC TCRs in $Itk^{-/-}$ T cells (166). There is little defect seen in positive selection using the conventional H-Y TCR system (93, 163). Class I MHC H-Y TCRs in $Itk^{-/-}$ T cells result in decreased efficiency of negative selection (167). Alternatively, $CD8^+$ T cell peripheral numbers are higher than controls, indicating that negative selection in the absence of Itk has failed. Both positive and negative selection need a functional calcium flux leading to the activation of Erk1/2 for positive selection (168-170) and p38 activation for negative selection (171). $Itk^{-/-}$ T cells do not possess an optimal calcium flux and fail to activate Erk1/2, but p38 activation is still intact. Furthermore, in the recent Huang et al. paper, positive selection is affected while negative selection is spared when Itk becomes inactive due to the lack of IP_4 (51). These data indicate that Itk would have a more profound impact on positive selection rather than negative selection. This is confirmed when in the absence of Itk the deletion of self-reactive thymocytes in strong stimulus conditions is impaired, but not under weak

stimulus conditions. Only when Itk-deficient thymocytes continue to progress to a later stage of development do they succumb to negative selection signals (166).

Although the total numbers of $CD8^+$ T cells from Itk-deficient mice appear to be normal, conventional $CD8^+$ T cells are severely diminished. Instead, an innate or memory phenotype exists causing the $CD8^+$ T cell numbers to appear normal, but in reality have a different phenotype (172, 173). $CD8^+$ thymocytes from $Itk^{-/-}$ mice have a memory cell surface phenotype ($CD44^{hi}$, $CD62L^{hi}$, $CD122^{hi}$) and include natural killer cell markers, such as NK1.1. Additionally, $Itk^{-/-}$ $CD8^+$ T cells spontaneously express IFNγ *ex vivo* and up-regulate anti-apoptosis genes such as BCL-X_L upon IL-15 stimulation, which is similar to memory cells. Furthermore, independent thymic development of $CD8^+$ T cells in $Itk^{-/-}$ mice is observed regardless of the type of cell presenting MHC class I, whereas conventional $CD8^+$ thymic derived T cells are only selected through interaction with thymic epithelial MHC class I presentation. Finally, $Itk^{-/-}$ $CD8^+$ T cells are dependent on the presence of IL-15 for their survival, again similar to a memory cell phenotype (174). The existence of these innate $CD8^+$ T cells emanating from $Itk^{-/-}$ mice was done in the Schwartzberg lab where they showed these cells arise in $Itk^{-/-}$ fetal thymic organ cultures (172). Molecularly, a marker for memory $CD8^+$ T cells, eomesodermin, is upregulated in the absence of Itk (173). This is interesting because eomesodermin is known to upregulate the expression of both the memory cell marker CD122 and the expression of IFNγ (175). Therefore, Itk-deficient mice provide an interesting insight into the development of $CD8^+$ memory T cells.

T helper cell differentiation is a necessary and crucial developmental process that selectively hones the T cell response towards certain pathogens. Primarily these

differentiated cells can be divided into two types, T helper 1 (T_H1) and T helper 2 (T_H2). This delineation is evolving to include T helper 17 (T_H17) and regulatory T cells (T_{Reg}), though the effects of Itk on these two subtypes are currently unknown.

During T_H1 differentiation, IL-12, IL-27, or IFNγ signaling through STAT4 and STAT1 induces the expression of the transcription factor T-bet, which can directly drive T_H1 development by increasing IFNγ production and inhibiting IL-4 production. T_H2 differentiation, by contrast, is dependent on the IL-4-induced activation of STAT6 leading to the activity of the transcription factor GATA-3, which is induced in T_H2 cells causing an increase in IL-4 production and a decrease in IFNγ production. T_H2 differentiation was recently described to occur in two steps when examined in mice expressing an IL-4 dual reporter gene (176). The first is the T_H2 competent stage where cells initially upregulate IL-4 and GATA-3 gene expression. The second is the T_H2 effector stage where cells support the production and release of T_H2 cytokines necessary for effector function. This two-step T_H2 differentiation model was extended by use of an IL-4-GFP reporter system crossed into Itk-deficient mice (177). In these studies, it was found that in the absence of Itk, T_H2 differentiation could proceed normally, but when these differentiated cells needed to produce and secrete T_H2 effector cytokines, they could not do so even with repeated stimulation. Additionally, under non-skewing conditions (no exogenous IL-4 added to the developing cultures to promote T_H2 differentiation) $Itk^{-/-}$ cells display defects in T_H2 competent cells, as they preferentially develop into IFNγ producing T_H1 cells (39). This is an interesting result, as IL-2 receptor signaling is imperative for IL-4 transcription (178), and since $Itk^{-/-}$ mice fail to produce substantial amounts of IL-2 upon stimulation, IL-4 production could be impaired due to

the lack of autocrine IL-2 signaling necessary to stabilize transcription factor access to the *Il4* gene leading to Th2 differentiation (179). The relative ratios of Itk and Rlk during T helper cell differentiation can also be useful in determining which pathway the cell will take. Naïve $CD4^+$ T cells express 3-5 times more Itk than Rlk when measured by qRT-PCR. When the T cell becomes activated through its T cell receptor, Rlk becomes down-regulated, leaving 100 times more Itk than Rlk present. While in this activated state exposure to cytokines secreted by resident non-T cell leukocytes provides a skewing environment that drives T helper cell differentiation. T_H1 effector cells are skewed by the presence of IL-12, IL-27, and IFNγ, whereas for T_H2 effector cell differentiation, IL-4 needs to be present. Importantly, T_H1-specific factors provide negative feedback signals that repress T_H2 development and vice versa, which further ensures the polarization of each T helper phenotype.

T_H1 and T_H2 effector cells are also characterized by the expression of two transcription factors, T-bet in T_H1 cells, GATA-3 in T_H2 cells. T-bet is required for the differentiation of naïve T helper cells into T_H1 cells and expression of IFNγ (180), while GATA-3 regulates chromatin remodeling at the locus that encodes the cytokines IL-4, IL-5, and IL-13, and consequently promotes T_H2 differentiation (181, 182). Itk plays an active role in the expression of these transcription factors, as it phosphorylates T-bet on tyrosine 525 (39, 73). Phosphorylation of T-bet leads to its proteolytic breakdown, leaving only GATA-3 to be expressed, and therefore, a T_H2 effector cell differentiated phenotype.

1.7.2 Cytokine Production

IL-2 production in T cells lacking Itk is decreased in response to stimulation through the T cell receptor, which also affects these cells and their ability to proliferate (93, 163, 183). Exogenous IL-2 enables these cells to overcome this proliferative defect, a response that originally led to the name of this kinase (30, 93).

Activation of naïve $CD4^+$ lymphocytes causes their differentiation into either T_H1 or T_H2 effector cells, which produce distinct sets of cytokines. T_H1 cells preferentially produce IFNγ, TNFα, and IL-2, while T_H2 cells primarily produce IL-4, IL-5, and IL-13. The balance between these two sets of $CD4^+$ effector cells allows for the tuning of the immune response to the state of infection. Conversely, when this balance is upset, self-reactive disease states can ensue. An excess of T_H1 cells can lead to autoimmune states, while an excess of T_H2 cells can lead to hypersensitivity. Therefore, T helper cell differentiation and cytokine expression regulation is of paramount importance for an adequate immune response. $Itk^{-/-}$ mice display a peculiar cytokine production profile, as the $CD4^+$ T cells emanating from these mice produce relatively normal levels of T_H1 cytokines (e.g. TNFα and IFNγ), while T_H2 cytokine expression (IL-4, IL-5, and IL-13) is diminished (132, 184). IL-4 expression can be rescued by the retroviral expression of Itk in Itk-deficient cells, indicating that Itk is the sole reason for the defect in these cells and not something due to the development of these cells in the absence of Itk (132). Strangely, IL-2 cytokine production is also reduced in these cells, blurring the lines of T_H1 and T_H2 differentiation. Since $Itk^{-/-}$ cells have an impaired NFAT transcriptional activity, it is therefore expected that IL-4 and IL-13 cytokine production would be hindered as both of those cytokines are dependent upon NFAT activity (185). There is

some discrepancy, however, between cytokine production in $Itk^{-/-}$ mice generated on different strain backgrounds. Schaeffer et al. have shown that $Itk^{-/-}$ mice display five times the amount of IgE and IgG1 in serum (when the cells are not stimulated, indicating a T_H2 bias), an IL-4-dependent antibody subclass, when compared to wild-type mice (135).

1.7.3 Pathogenic Clearance

During the course of a viral infection $CD8^+$ T cells differentiate into activated killer cytotoxic T lymphocytes (CTL) whose main functions are the lysis of infected cells and the secretion of antiviral cytokines, such as IFNγ and TNFα. $CD8^+$ T cell function in cells lacking Itk has yielded perplexing results, as $Itk^{-/-}$ mice were initially thought to have only a mild defect in their response to viral infection (186). When infected with either lymphocytic choriomeningitis virus (LCMV), vaccinia virus (VV), or vesicular stomatitis virus (VSV), CTL responses were reduced two to six-fold. However, the kinetics of viral clearance was the same in $Itk^{-/-}$ mice as in wild-type mice. Further, in the case of anti-VSV-antibodies, the serum titers of these antibodies were normal in $Itk^{-/-}$ mice when compared to wild-type mice when both had been infected with VSV. A very interesting recent finding is the determination that Itk can affect the replication of human immunodeficiency virus (HIV). Through use of Itk-specific siRNA or by using an Itk chemical inhibitor, production of HIV protein p24, actin-dependent viral entry, and HIV transcription were all reduced (187). Although this research does not imply Itk in the

direct clearance of HIV virally infected cells by CTLs, it does show that by inhibiting Itk activity, one can ameliorate a viral infection.

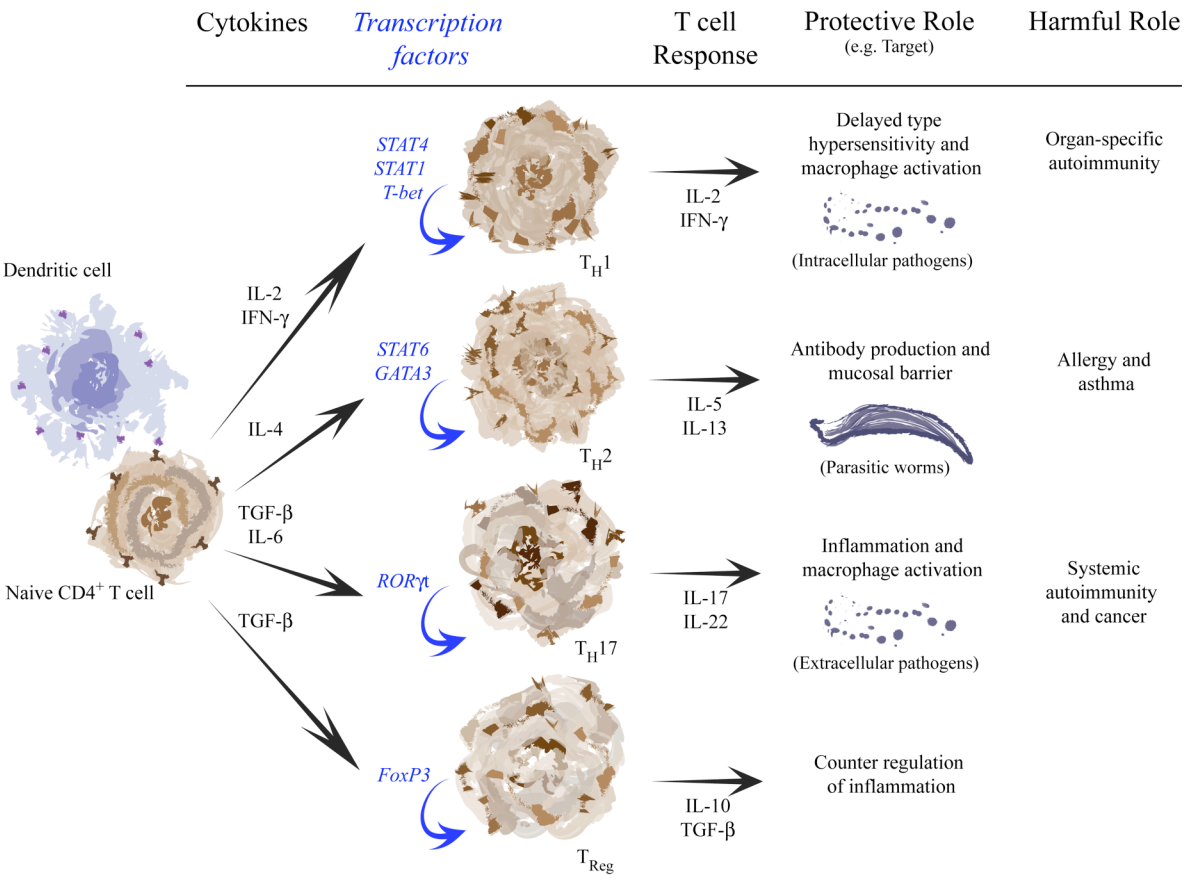

Figure 1.3. *T cell differentiation and their responses to pathogens.*
Cartoon diagram displaying the interaction of a naïve $CD4^+$ T cell with a dendritic cell APC leading to differentiation into T helper subsets. The development of these subsets allows for protective immunity against specific pathogens.

T helper cell differentiation is crucial for the clearance of pathogens. T_H1 cells are important in promoting responses to clear intracellular pathogens, whereas T_H2 cells support productive humoral immune responses against extracellular pathogens. $Itk^{-/-}$ mice display an increased susceptibility to parasitic infection, as displayed in their inability to clear the intracellular pathogen *Toxoplasma gondii* (*T. gondii*) (93), a parasite

that normally induces a protective T_H1 response in wild-type mice. This is odd because *T. gondii* requires a fast and robust IFNγ response by the host to prevent lethality, which $Itk^{-/-}$ mice produce. This may be due to the mouse strain used for these studies since there are differences in T_H1 and T_H2 differentiation among mouse strains (188, 189). During a *Leishmania major* (*L. major*) infection wild-type C57Bl/6 mice are able to produce T_H1 cytokines resulting in the clearance of the parasite, whereas wild-type BALB/c mice primarily produce T_H2 cytokines, which causes them to succumb to the infection. During a infection that requires a T_H1 response by the host to clear the infection, $Itk^{-/-}$ mice on C57Bl/6 background elicit a protective T_H1 response (132). However, BALB/c mice deficient for Itk are unable to normally produce a T_H1 response, and could not produce a T_H2 response due to the absence of Itk, resulting in the inability of the mice to clear the infection. The discrepancy between these results may be because *T. gondii* infection requires a rapid T_H1 response, while *L. major* can be cleared by a slow, less robust response. Therefore, the kinetics of infection and response may dictate the lethality of the pathogenic infection.

In an attempt to address this issue, the Fowell group backcrossed the $Itk^{-/-}$ mice on IL-4-GFP reporter mouse background (190) to examine an IL-4 producing T_H2 response during *L. major* infection. IL-4 producing $CD4^+$ T cells were present at the site of infection, indicating that differentiation and migration to site of infection were not a problem. However the amount of IL-4 produced, particularly upon restimulation, was insufficient in the $Itk^{-/-}$ mice when compared to the wild-type mice (177). This elegantly determines that Itk is not necessarily required for differentiation, but is required in the production of this cytokine.

Meanwhile, T_H2 effector function for protective immunity of extracellular pathogens is clearer. Infection with *Nippostrongylus brasilinensis* (*N. brasilinensis*) causes a protective T_H2 response in BALB/c mice, while $Itk^{-/-}$ BALB/c mice were unable to clear the nematode due to a suboptimal T_H2 response (132). Similarly, $Itk^{-/-}$ mice on a C57Bl/6 strain background were unable to mount a protective T_H2 response to the helminth *Schistosoma mansoni* (*S. mansoni*) due to a severe reduction in T_H2 cytokine production of IL-4 and IL-5 (135).

1.7.4 Allergy induced asthmatic response

The connection between Itk and allergic responses was first made in a clinical study on patients with atopic dermatitis, a disease state characterized by an itchy rash and inflammation caused by an excess of T_H2 cell response (191). There is an increased incidence of Itk expression in these patients correlating to the severity of the disease state. This corresponds well with elevated Itk expression levels in mouse T_H2 cells (39). Furthermore, the linkage between atopy and Itk can be traced genetically to the Itk genomic locus as there are single nucleotide polymorphisms at this locus in atopic patients (192). T_H2 cellular responses are involved in the pathology of allergic asthma, as the number of T_H2 cells recruited to the lungs (an immune privileged site) is increased, as well as the expression of T_H2 cytokines and the consequent movement of responding cells to the lungs resulting in increased inflammation and mucous production (193, 194). Itk's first direct molecular and cellular correlation to asthma was made by Mueller and August where they induced airway hyperresponsiveness in mice and found that $Itk^{-/-}$ mice

displayed a reduced number of T cells and eosinophils infiltrating the lungs when compared to similarly treated wild-type mice (184). This finding included a reduction in lung inflammation, diminished mucous production, and reduction in the T_H2 cytokine expression of IL-5 and IL-13. This was later extended by Ferrara, et al. in the finding that $Itk^{-/-}$ mice display a diminished tracheal response to allergen challenge using the same airway hyperresponsiveness model system (195). Mast cells degranulation was also found to be impaired in $Itk^{-/-}$ mice in the same model system (196). On a related issue, mice lacking the transcription factor T-bet, which Itk normally phosphorylates, spontaneously develop airway hyperresponsiveness, display enhanced inflammation and an increase in secreted T_H2 cytokines (197). Given these results, competitive inhibitors that selectively target Itk would be of beneficial therapeutic value in the treatment of asthma. One study has shown that by inhibiting Itk kinase activity, lung inflammation in mice can be ameliorated (75).

1.7.5 Apoptosis

Activation induced cell death (AICD) is the process by which cells are instructed to commit suicide, or apoptose, in response to an extracellular signal. This process is genetically linked as most cells contain signaling pathways always on the ready for the signal to apoptose. Functionally, this process is crucial in the life of a lymphocyte, as stimulation induced clonal expansion leads to an enormous number of cells generated to combat an infection, the clearance of which leads to many cells that are no longer needed. Apoptosis serves as a means to eliminate extra cells, which can possibly be destructive (if

no more pathogen is present). During instructive thymic development, T lymphocytes that give too robust a signal to self-antigen during positive selection are signaled to apoptose in order to limit self-reactive T cells from entering the periphery. The apoptotic elimination of clonally expanded T cells after an infection is also necessary to prevent autoimmunity (198, 199). $Itk^{-/-}$ T cells have a decreased susceptibility to CD3-induced apoptosis (93). Thymocyte deletion, particularly that of double-positive T cells, is also defective in cells lacking Itk (167). Furthermore, Itk is involved with the expression of Fas-ligand as well as well as the interaction between Fas-receptor and its downstream cell death induction molecules (183). Itk, however, has not been shown to be involved in anti-Fas engagement leading to apoptosis. Whether the absence of Itk influences the lack of apoptosis of the large population of $CD8^{+}$ memory-like T cells remains to be seen.

1.7.6 In vivo comparison to other signaling molecules

Itk-deficient cells display many commonalities with cells deficient in other signaling molecules within the TCR-induced signalosome. $LAT^{-/-}$ mice do not develop mature T cells, although transgenic point mutant Y136F, which binds to PLCγ1 (200), mice have a similar phenotype to that of $Itk^{-/-}$ mice (141, 142). These mice have impaired Ca^{2+} mobilization, T_H2 cytokine production, and increased amounts of IgE in the serum. $JNK^{-/-}$ mice are also incapable of clearing a *L. major* infection, indicating that Itk could also act through the JNK pathway. These JNK-deficient mice have a reduced IL-4 yield, yet are able to produce IFNγ when stimulated *in vitro* under non-skewing conditions much like that of $Itk^{-/-}$ mice. Mice deficient in cyclophilin A (CypA) spontaneously

develop allergic disease marked by increases in IgE antibody titer in serum, as well as an increased infiltration of mast cells and eosinophils into the lungs (69). T_H2 cells emanating from these mice are hypersensitive to TCR stimulation, and although Itk activity has not been directly assessed in these cells, given CypA repressive function on Itk, it would be assumed that Itk could be hyperactive in these cells. Vav1 and PKCθ deficient T cells have a decrease in IL-2 production, and consequently, IL-4 production as well (150, 151, 157, 158, 201). Downstream, these cells are defective in Ca^{2+} mobilization leading to a defect in NFAT transcriptional activation, are similar to $Itk^{-/-}$ cells as they are defective in AP-1 transcription factor activation, and are defective in NFκB activity. Although Itk's role in the activation of this transcription factor has not yet been elucidated, it can be predicted to have an effect. Mice defective for the expression of NFAT1 have a decreased T_H2 cytokine expression profile (185).

1.7.7 Itk function in non-T cells

Although much of the research on Itk has been produced in T cells, Itk is expressed in Natural Killer (NK) cells, Natural Killer T (NKT) cells, and in Mast cells. Natural Killer cells are lymphocytes that can eliminate virally infected cells, cells presenting intracellular antigens, or transformed cells, independent of an antigen stimulus as in CTLs. The NK cellular response in $Itk^{-/-}$ or in $Itk^{-/-}Rlk^{-/-}$ double knockout cells was first reported to be normal, as interpreted from the robust response to LCMV infection in these mice (38, 186). However, recently it was determined that Itk both positively regulates NK cell cytotoxicity in response to FcR stimulation and negatively regulates

NK cell cytotoxicity upon NKG2D receptor activation (202). This indicates a more complex role for Itk in NK function, one that deserves further exploration. Mast cells are tissue resident granulocytes that are the effector cells in immediate hypersensitivity reactions such as allergic rhinitis. The role of Itk in Mast cells is blurred by the presence of Btk, where Btk seems to be the dominant Tec kinase in these cells. Itk-deficient Mast cells elicit little difficulty in producing a cellular response, while Btk-deficient Mast cells have much difficulty doing the same in most experimental cases of hypersensitivity (203). However, in the specific context of an acute allergic response in the lung, Mast cell degranulation in $Itk^{-/-}$ mice was significantly reduced, irrespective of late phase T_H2-dependent inflammatory defects (196). NKT cells are a rare heterogenous subset of T cells that are important for the initiation and regulation of an immune response through the ability to immediately secrete large quantities of cytokines, particularly IFNγ and IL-4. Itk has recently been shown to be important for the development and the homeostasis of NKT cells (204, 205). Interestingly, not only are NKT cell numbers reduced, but the continued homeostasis of these cells is dependent on Itk as NKT cells are diminished even further in $Itk^{-/-}$ mice as they age when compared to strain-matched control mice. Itk is involved in the production of both IFNγ and IL-4 in NKT cells (206), as Itk-deficient NKT cells fail to produce either cytokine thus making the cells functionally defective. This is an interesting finding because this is different from the T_H2 skewed phenotype seen in T cells from Itk-deficient mice where only IL-4 production is affected.

1.8 Future Directions

Itk can be an ideal therapeutic target for the regulation of T_H2 cell mediated diseases. Since Itk is the Tec kinase primarily expressed in T_H2 cells, the selective inhibition of its activity could affect T_H2 cell function, including cytokine expression, without affecting immune responses against other infections, such as viral or T_H1 cell-mediated pathogens. As such, Itk is a TCR proximal rheostat serving as a critical molecule that affects signaling and development in subtle ways, helping to evaluate the strength of the TCR signal in order to produce a T cell response that is appropriate for the presented situation.

An intriguing question for future consideration is whether Itk is involved in the regulation of T_H17 cellular function. T_H17 cells normally mediate the host defensive response to extracellular bacterial infections and are reportedly involved in the pathogenesis of autoimmune diseases through the overexpression of pro-inflammatory cytokines. The signature cytokines of T_H1 and T_H2 cells, IFNγ and IL-4 respectively, have both been shown to inhibit T_H17 differentiation (207, 208). Markedly, while IFNγ normally upregulates T-bet, the expression of T-bet is significantly lower in T_H17 cells (209, 210). Further, the promotion of T_H17 development leads to the pathogenesis of experimental autoimmune encephalomyelitis (211). The T_H17 signature cytokine IL-17 is overexpressed in the airways of asthmatics where it induces the production of IL-8 chemoattractant needed by neutrophils, which are phagocytic granulocytes involved in eliminating bacterial infections (212). These findings draw a connection between Itk, T_H17 cells, asthma and autoimmunity through the transcription factor T-bet. Since Itk

phosphorylates and affects the down-regulation of T-bet, Itk could be implicated in promoting the development of T_H17 cells. It would be interesting to speculate that by inhibiting Itk functional activity, and by proxy, promoting T-bet activity, T_H17 cellular development and T_H17 mediated autoimmunity could be suppressed.

Another re-emerging set of differentiated $CD4^+$ T cells that have garnered lots of interest lately are regulatory T cells, or T_{Reg} cells. T_{Reg} cells have a critical function in the regulation of peripheral tolerance. It may be that T_{Reg} and T_H17 cells are reciprocally regulated through the activation and deactivation of transcription factors, much like inverse relationship between T_H1 and T_H2 cells (213). The main cytokine produced by T_{Reg} cells is IL-10. This cytokine inhibits the expression of many pro-inflammatory cytokines and chemokines, helping to decrease the number of circulating leukocytes from entering the inflamed tissue. Notably, another significant defect in Itk-deficient T cells is the failure to produce IL-10 in response to activation (135). This was previously interpreted to be part of a diminished T_H2 function. However, given what is now known about the T_{Reg} signature cytokine being IL-10, it would be interesting to determine the function of these T_{Reg} cells in the absence of Itk. Speculatively, $Itk^{-/-}$ T cells could have a diminished T_{Reg} response leading to hyperinflammatory conditions.

The regulation of the T lymphocyte cytoskeleton by Itk is through an intricate dance between two additional cytoskeletal molecules, Vav1 and WASp. Although Itk may not be integral in the phosphorylation of WASp (113), it is intimately involved in locating WASp to the immunological synapse thereby affecting WASp activity (101). Recently, it has been discovered that WASp is important in the development and function of T_{Reg} cells, in both mice and in humans (214-217). These studies confirm that WASp-

deficiency curbed the differentiation of these cells, as well as abrogated their suppressive cellular activity, such as curbing the production of TGFβ and IL-10. Given the relationship between Itk adaptor function and the localization of WASp necessary for its activity, a similar interaction in T_{Regs} may indicate that Itk may very well be involved in the management of T_{Reg} activity, thus affecting autoimmune disorders.

Additionally, one of the original cellular defects seen in Wiskott-Aldrich Syndrome patients were T cells that displayed defective activation profiles due to the lack of expression of the sialylated co-receptor, CD43 (218, 219). Stimulation through this co-receptor leads to the activation of molecules involved in Itk activity, including the inducible association between Vav1 and SLP-76 (220). Stimulation through CD43 or through CD3ε leads to an interesting cellular phenomenon where CD43 migrates to the distal pole of cell, opposite to that of the immunological synapse (221, 222). This distal migration was later determined to be dependent on T cell signaling pathways, and not due simply to cellular adhesion interactions (223, 224). Finally, and very recently, it has been discovered that CD43 is involved in regulating T_H2 differentiation and cellular responses (225). Despite all the above demonstration of a plausible role for Itk in the molecular regulation of CD43-induced cellular effects, no research has been reported implicating Itk in these processes. Given the membrane location of CD43, it would be relatively easy to identify whether it is absent in $Itk^{-/-}$ lymphocytes as it is in $WASp^{-/-}$ cells, or if it is present whether its localization is perturbed as a result of loss of Itk activity. If it were found that Itk is involved in CD43 signaling, it would be a natural therapeutic target for patients with Wiskott-Aldrich Syndrome, as the activity of Itk might regulate and enhance the effectiveness of the T cells from these patients.

Itk has been strongly established to regulate Ca^{2+} flux in T cells, rightly thought to be an indirect effect due to its phosphorylation of PLCγ1, the lipase that directly leads to the release of Ca^{2+} from ER stores. However, this does not explain Itk effect on prolonged Ca^{2+} flux. One can speculate that this sustained effect is mediated through the relationship of Itk with immunologic synapse formation. This would be a simplistic resolution, however the recently characterized calcium-release-activated calcium (CRAC) channels would further explain the relationship between Itk and sustained Ca^{2+} signaling. How ER-released Ca^{2+} stores regulate CRAC channel operation was recently identified to be due to an ER-resident Ca^{2+} sensor named STIM1 (226). STIM1 knockdown leads to a complete abrogation of store-operated calcium entry in T cells. Furthermore, upon TCR-induced activation of the T cell, STIM1 co-localizes with a plasma membrane resident CRAC channel named Orai1 at the immunological synapse, which leads to a sustained Ca^{2+} flux for the T cell (227). Orai1 is a tetraspanin pore-forming subunit of the CRAC channel, which when absent or mutated also causes an abrogation of sustained Ca^{2+} flux (228, 229). This mutation (R91W) in Orai1 manifests in human disease, as one aspect for the development of SCID is due to the attenuation of Ca^{2+} flux after T cell activation (230). Given Itk's strong role in sustained Ca^{2+} flux, it would be of interest to determine whether Itk has an effect on the activity of either STIM1 or Orai1. Regardless of a direct or indirect relationship, it might still provide a more specific method for temporary therapeutic relief of some inflammatory responses, such as graft rejection in transplantation, psoriasis, arthritis, or colitis. Current therapies for these disorders involve the use of cyclosporin A and FK506 to inhibit calcineurin activity leading to reduced Ca^{2+} flux and reduced T cell activity. Unfortunately, since calcineurin is widely

expressed in most cell types, these treatments often lead to unwanted and sometimes severe side effects. If one could directly affect Ca^{2+} flux in T cells using Itk inhibitors, without affecting other cells, it would be of extremely beneficial for the treatment of these disorders.

The finding that inhibition of Itk activity can reduce the infectivity and replication of HIV leads one to think of Itk beyond that of typical immune responses such as pathogenic clearance and asthma. Itk exists at a pivotal position in T cell regulation; it can molecularly affect both Ca^{2+} flux and actin polymerization, while cellularly affecting the balance of a T helper response. The polarization of T_H1/T_H2 differentiation can manifest itself in many disease states and as such can be manipulated to combat those diseases.

For example, T_H1/T_H2 polarization is strongly linked to fibrogenesis. Once an infection has cleared and inflammation has subsided, the repair of damaged tissue is paramount for continued survival of the host. Repairing this tissue is done through the replacement of cells and replacement of connective tissue, or fibrosis. This healing process can become pathogenic if left unchecked, resulting in permanent formation of scar tissue. Further, fibroproliferative diseases account for nearly 45% of all deaths according to United States government (231). These diseases include interstitial lung disease, liver cirrhosis, kidney disease, and heart disease (232). The T_H1/T_H2 paradigm plays a role in this as T_H2 expressed cytokines have been shown to have a pro-fibrotic response, promoting scarring after tissue damage, while T_H1 expressed cytokines contain anti-fibrotic activity, preventing scar formation. In fact, current clinical therapies include the promotion of T_H1 cytokine expression (233) and the blockage of T_H2 cytokine

expression (234), in an effort to skew the T_H1/T_H2 balance and reduce fibrosis. The regulation of Itk activity fits well into this paradigm. Limiting Itk activity leads to the reduction in T_H2 cytokine expression and may even help in the increase of T_H1 cytokine expression. Therefore, if one can effectively target Itk activity, one would be able to specifically target a T cell response that can help fibroproiferative diseases without affecting the tissue itself.

An extension of this anti-fibrotic response can be taken to that of atherosclerosis. This is a disease characterized by inflammation, lipid accumulation, cell death and fibrosis. Atherosclerotic lesions, or plaques, consist of a fibrotic cap whose rupture leads to the most severe of clinical events due to the release of thrombotic material within the plaque into the blood system which can cause sudden occlusion of the artery at the site of the plaque disruption (235). This ultimately leads to myocardial infarct and heart failure, and in the case of arteries perfusing the brain, ischemic stroke. Of the 10% $CD3^+$ cells present within the plaque, the overwhelming majority is expressing T_H1 cytokines, indicating that atherosclerosis is a T_H1 driven disease. Therefore, if T_H1 cytokines stimulate plaque formation and T_H2 cytokines can inhibit T_H1 cell responses, promotion of a T_H2 response can reverse the scale of the disease. This would require activating Itk to promote phosphorylation of T-bet leading to T_H1 down-regulation and T_H2 up-regulation. Indeed, in studies using T-bet-deficient mice, atherosclerosis was significantly reduced and there was a significant increase in the titer of the atheroprotective antibody E06 (236). Although the typical means of therapeutic regulation usually dictates limiting kinase activity, at minimum it would be interesting to

determine whether Itk could be involved in a debilitating disease process and whether altering Itk's active state affects the disease outcome.

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2 The Management of T Lymphocyte Actin Polymerization by Itk

2.1 Abstract

The Tec family kinase, interleukin-2 inducible T cell tyrosine kinase (Itk), is critical for both the activation and the development of T lymphocytes. In the following studies involving mutational analysis of Itk function, it was discovered that Src homology 2 (SH2) domain mutants of Itk rendered the enzyme inactive. Further, it inhibited the localization of Itk to a TCR/CD3 "cap", and regulated TCR/CD3-induced actin-dependent cytoskeletal events. In extension, $Itk^{-/-}$ murine T cells display significant defects in TCR/CD3-induced actin polymerization. Additionally, Jurkat cells deficient in the expression of the linker for activation of T cells (LAT), an adaptor critical for Itk activation, display impaired cytoskeletal events and the expression of SH3 mutant Itk transgene reconstitutes this impairment. Interestingly, expression of an Itk kinase-dead mutant transgene into Jurkat cells has no effect on cytoskeletal events. The molecular mechanism for this effect on the T lymphocyte was then explored. Collectively, these data suggest that Itk regulates TCR/CD3-induced actin-dependent cytoskeletal events, most likely in a kinase independent manner.

2.2 Introduction

In order for T lymphocytes to become fully activated, they must be able to synchronize the biophysical interaction between T cell antigen receptor (TCR) and peptide containing major histocompatibility complex (pMHC) along with T cell co-receptors, and link these interactions with signal transduction pathways leading to a functional outcome, such as cytokine expression. This synchronicity requires a dynamic cytoskeleton that combines integrin adhesion, immunological synapse formation, cellular polarization, receptor sequestration, as well as signaling.

Upon encountering an antigen-presenting cell (APC), the T cell samples the surface environment of the APC through the interaction between its TCR and the pMHC on the APC. This engagement leads to the movement of surface interactions on both the T cell and the APC. Adhesion molecule interactions pull the two cells together to form a tighter connection coined the immunological synapse (IS) (1-4). An ordered surface structure follows this connection where the adhesion molecules interaction form a peripheral ring to the central TCR-pMHC complexation. This surface configuration forms what is referred to as a SupraMolecular Activation Cluster, or SMAC (5). The peripheral adhesion stabilization is known as the pSMAC, while the central antigen-MHC interaction is known as the cSMAC. When T cells encounter stimulating antigen on an APC, the T cells polarize towards the APC, organized by actin, leading to recruitment of signaling molecules to the site of TCR stimulation. This recruitment results in immunological synapse formation, a process thought to not only stabilize the T cell

interaction with the APC, but to increase the density of the antigen receptors at the ligand interaction site (6). Further, IS formation causes a directional intracellular movement of signaling molecules that increases their concentration in the proximity of the antigen receptor. Formation of the immunological synapse is dependent on an active actin cytoskeleton, as disruption with cytochalasin D causes the synapse to fall apart (7-9). Cytochalasin D is a fungal toxin that binds to the barbed end of actin filaments, which prevents monomeric actin from being added to the growing actin chain. Further, disruption of TCR-mediated signaling events also leads to the disorganization of synapse formation (10, 11).

The SMACs, or immunological synapses, form a pattern of cell membrane molecules that cluster to distinct areas. The central SMAC (cSMAC) consists of TCR, CD2, and co-stimulatory receptor CD28, which is surrounded by a concentric peripheral cluster (pSMAC) that consists of adhesion molecules, such as CD11 and CD18 (otherwise known as LFA-1 and 2). The immunological synapse may function to release the steric hindrance between the T cell and the APC. Since the cell surface of the T cell is covered with molecules ranging as high (relatively speaking) as 40-45 nm, compared to the TCR and the other cSMAC molecules extending only 7-10 nm from the cell surface, there is the potential for the physical inhibition of contact between the TCR and the MHC. The synapse may the serve to segregate taller molecules away from the cSMAC, thus allowing close interaction between the TCR and pMHC (12-14). Furthermore, sequestration of surface molecules, as well as associated intracellular molecules may serve to increase signaling efficiency by creating signaling and non-signaling domains. For example, the c-Src tyrosine kinase (Csk), a negative regulator of lymphocyte specific

protein tyrosine kinase (Lck), phosphorylates Lck to make it catalytically inactive. The Csk binding protein (Cbp), when activated through TCR stimulation, becomes dephosphorylated and thereby releases Csk from the membrane, leaving Lck without this negative control. Since Cbp binds to the cytoskeleton ERM family of adaptors, this coordination between the cytoskeleton and signaling molecules allows for regulation of signaling events (15).

T cell receptor engagement with peptide MHC leads to a signaling cascade culminating with transcriptional activation (16). As the T cell receptor contains no enzymatic activity itself, it must recruit enzymes to promote activation. The T cell receptor can be divided into two functional components, the antigen binding αβ chain receptor and the CD3 signal-transducing complex. TCR-pMHC engagement leads to the recruitment of a proximal non-receptor tyrosine kinase, Lck, which phosphorylates immunoreceptor tyrosine activation motifs (ITAMs) contained within the chains of the CD3 signaling complex. Phosphorylation of the ITAMs leads to the recruitment of another proximal non-receptor tyrosine kinase, the zeta-associated protein of 70 kDa (ZAP-70). This protein also is phosphorylated and consequently activated by Lck. ZAP-70 activation leads to the phosphorylation of the critical bridging adaptor molecule, the linker for activated T cells, or LAT. Phosphorylation of LAT allows for the recruitment of many Src Homology 2 (SH2) containing molecules, which bind to phosphorylated residues on LAT, forming a link between the proximal TCR signals with that of downstream signaling events.

Recruitment of proteins to the membrane bound LAT adaptor complex forms what has come to be known as a signalosome, or a specifically recruited signaling

complex. Among the molecules recruited are the cytosolic adaptor molecules, SH2 containing leukocyte phosphoprotein of 76 kDa (SLP-76), growth factor receptor-bound protein 2 (Grb2), the Grb2 like adaptor molecule (Gads), and phospholipase C gamma 1 (PLCγ1). Phosphorylation and activation of PLCγ1 leads to the catabolism of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The molecule DAG activates protein kinase c-theta (PKC-θ) and Ras guanyl-releasing protein (RasGRP), while IP_3 binds to IP_3Rs on the endoplasmic reticulum leading to the intracellular release of Ca^{2+}. Binding of Grb2 to the LAT signalosome activates the Grb2 associated guanine nucleotide exchange factor (GEF) son-of-sevenless (SOS), which mediates the active exchange of GDP for GTP within the small G protein, Ras. Active Ras initiates the mitogen activated protein kinase (MAPK) signaling pathway, which in turn, activates many transcription factors, including AP-1, NFAT, and NFkB. The activation of these factors leads to transcriptional activation and a full T cell response.

Once SLP-76 becomes phosphorylated by ZAP-70, it complexes with a variety of molecules that have been reported to be indirectly involved in actin polymerization, including Vav1, Nck, Itk, and ADAP. Although thymocyte development in SLP-$76^{-/-}$ is completely blocked (making comparison difficult), platelet morphology, integrin signaling, and actin organization are all defective, indicating that SLP-76 plays a major role in cytoskeletal regulation. This was confirmed in a SLP-76 deficient T cell line as these cells failed to perform actin reorganization events (17). Vav1 activates the Rho GTPases Cdc42 and Rac through a SLP-76 dependent mechanism (18). Vav$1^{-/-}$ T cells are defective in synapse formation (8), and in an interesting twist, SLP-76 does not

become phosphorylated in Vav-deficient cells (19), thereby eliminating some of SLP-76's adaptor capabilities. The Rho family of GTPases consists of Rac, Cdc42, and Rho, all of which are localized to the cell membrane by prenylation. They are responsible for actin remodeling of the cytoskeleton, such as stress fibers regulation (Rho), lamellipodia formation (Rac), and filopodia formation (Cdc42) (20, 21). ADAP is an interesting adaptor that links the TCR and cytoskeleton signaling much like LAT does between TCR proximal and distal signaling events (22-24). ADAP-deficient T cells have defective proliferation, cytokine production, and an "inside-out" disconnect between TCR activation and integrin function (25-29). Finally, the adapter protein recruited to SLP-76, Nck, binds to the Wiskott-Aldrich Syndrome protein (WASp) and to WASp-interacting protein (WIP), which mediates their recruitment to the immunological synapse through SLP-76 (30-32). Jurkat cells transfected with Nck mutants unable to bind SLP-76 are unable to recruit WASp to the immunological synapse, therefore cells containing Nck mutants are deficient in actin polymerization (30).

The activation of T lymphocytes through the TCR/CD3 molecular complex is regulated by several protein tyrosine kinases, one of which is the Interleukin-2 inducible T cell tyrosine kinase (Itk). Itk is a protein of 72 kDa whose expression is restricted to T lymphocytes, natural killer (NK) cells, and mast cells. Itk-deficient mice display decreased numbers of mature thymocytes, reduced levels of PLCγ1 activation, inability to open membrane Ca^{2+} channels, suppressed cytokine production, reduced proliferative responses, and impaired resistance to infection.

The mechanism(s) that regulates TCR/CD3-induced actin-dependent events, including immunological synapse formation, is not clearly understood. In the present

investigation, the molecular conditions of Itk activation with relation to mutations within the molecule were first pursued. Further, its localization because of its mutation was also evaluated. Serendipitously, upon analyzing Itk localization with SH2 domain mutants, it was observed that Itk might have more functions than that related to its kinase activity. The evidence indicates that Itk is involved in the regulation of TCR/CD3-induced cytoskeletal events and actin polymerization. Since research findings have shown that TCR-induced signaling precedes synapse formation (33), it was then determined to pursue the molecular mechanism of Itk's regulation of cytoskeletal events. This was done using co-immunoprecipitation assays with molecules known to be involved in actin-related events. This includes determining whether Itk associates with Vav1 and with WASp. This work was extended to determine which domain of Itk might be critical for these interactions. Further, using a Förster Resonance Energy Transfer (FRET) biosensor specifically for WASp, it was sought to determine whether Itk has an effect on the localized activity of this molecule. All of these experiments were done under the concept that Itk's adaptor and enzymatic function is necessary for a mature formation of the T cell/APC immunological synapse.

2.3 Materials and Methods

2.3.1 Mice, cells, antibodies, and other reagents

The $Itk^{-/-}$ mice on the C57Bl/6 background have been previously described (34). They were provided by Dr. D. Littman (New York University School of Medicine, New York, NY) through Dr. T. Kawakami (La Jolla Institute for Allergy and Immunology, La Jolla, CA). C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were handled in accordance with the guidelines of the IACUC at San Diego State University, and were experimented upon between the ages of 6-12 weeks. Jurkat T cells (E6.1) were obtained from American Type Culture Collection; $SLP76^{-/-}$ (J14) and $LAT^{-/-}$ (JCaM2.5) Jurkat T cells were a kind gift from Dr. A. Weiss (University of California at San Francisco, San Francisco, CA), while Jurkat Large T Antigen (JTAg) cells were a generous gift from Dr. A. Altman (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Cells were cultured in RPMI 1640 culture medium containing 5% FBS in a 37°C humidified 5% CO_2 chamber and stimulated according to each individual figure. Recombinant Itk proteins were immunoprecipitated with the monoclonal antibody H902 that is specific for the gp120 tag (National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, MD). Anti-human CD3ε OKT3 was generated in-house from a hybridoma (American Type Culture Collection, Manassas, VA). Monoclonal anti-phosphotyrosine antibody (4G10) was provided by Dr. Bartholomew Sefton (Salk Institute, La Jolla, CA), anti-SLP-76 antibody was a gift from Dr. Gary Koretzky (University of Pennsylvania, Philadelphia, PA), and anti-Itk antibody

was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Normal rabbit IgG, and rabbit anti-mouse IgG were purchased from Jackson Immunoresearch (West Grove, PA). Protein G sepharose was obtained from Sigma-Aldrich (St. Louis, MO).

2.3.2 Itk constructs

Wild-type (WT) murine Itk (35) was cloned into the EcoRI-SpeI sites of the pME18s expression vector (36, 37). Itk constructs had previously been tagged with a peptide sequence (a.a. 319–333) of the HIVgp120 protein (38). Itk mutants were generated by a two-step PCR amplification of mutated cDNA sequences before substituting the mutated portion for the wild-type sequence in the pME18s vector. To generate the ΔSH3 mutant, two PCR reactions were performed using the wild-type cDNA as template. In one reaction, an appropriate 5'-primer and the mutagenizing 3'-primer (5'-TGGAGATTTTTCTACAATGACCAGGGTTTC-3') were used, whereas in the other reaction the mutagenizing 5'-primer (5'-GAAACCCTGGTCATTGTAGAAAAATCTCCA-3') and an appropriate 3' primer were used. Subsequently, another set of PCRs was performed using the products of the PCR reactions above as mixed templates and the 5' and 3' nonmutagenizing primers. The correct PCR product was cloned into the pCRII vector (Invitrogen, Carlsbad, CA) and was confirmed by sequencing. The 519-bp Eco47III fragment containing the mutated portion was isolated from this clone and used to replace the wild-type sequence in pME18s. Similar strategies were used to generate the ΔSH2 and K390R mutants. The

resulting ΔSH3 and ΔSH2 mutants encode proteins devoid of residues 178–226 and 239–337, respectively (19).

The SH2 domain point mutant R265K and SH3 point mutant W208K of Itk were created by site-directed mutagenesis of H902-tagged wild-type Itk in the pME18s vector using the QuikChange Site-Directed Mutagenesis Kit following the manufacturer's instructions (Stratagene, La Jolla, CA). The sense primer 5'-GGAGCTTTCATGGTCAAAGATTCCAGG-3' and the antisense primer 5'-CCTGGAATCTTTGACCATGAAAGCTCC-3' (mutated codon underlined) were used for the construction of the R265K mutant, whereas the sense primer 5'-CCGAGATCCACAAGTGGAGGGTTC-3' and antisense primer 5'-GAACCCTCCACTTGTGGATCTCGG-3' (mutated codon underlined) were used for the construction of the W208K mutant. Both of the mutant constructs were confirmed by sequencing.

GFP was added to the C terminus of Itk by removing the stop codon and creating an in-frame KpnI site by PCR amplification of H902-tagged pME18s-WT-Itk. The following primers were used: sense primer 5'-TTGAATTCCGTTGGAAGCCATGGCCCGTAT-3' (containing an EcoRI site and a.a. 1–4 of the H902 epitope tag), antisense primer 5'-TTTCTAGAGGTACCCAAGCCCAGCTTCTGCGATTT-3' (containing a.a. 613–619 of Itk, KpnI, and XbaI sites). The PCR products were purified as described above and subcloned into the EcoRI-KpnI sites of pEGFP-N2 expression vector (Clontech, Palo Alto, CA).

2.3.3 Stimulation and fluorescence labeling

For stimulation, 20 X 10^6 Jurkat large-T antigen (JTAg) cells (39) were incubated (30 min on ice) in RPMI 1640 culture medium containing 20 μg/ml concentrations of the anti-CD3ε monoclonal antibody OKT3 or an isotype (IgG2a) control antibody UPC-10 (Bionetics, Charleston, SC). Cells were washed, resuspended in culture medium containing 20 μg/ml of goat anti-mouse IgG (cross-linking antibody, Jackson Immunoresearch, West Grove, PA), and incubated at 37°C for the indicated time period. After incubation, the cells were placed on ice until further analysis. For fluorescence microscopy, 1 X 10^6 cells were incubated in 250 μl of PBS containing 10 μg/ml antibody OKT3 for 30 minutes on ice. After washing with PBS, the cells were incubated for 30 minutes on ice in PBS containing 10 μg/ml Texas Red-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). The cells were then exposed to 37°C for 7 minutes to induce capping and were immediately cooled with 1 ml of ice-cold PBS containing 0.1% sodium azide (Sigma-Aldrich) to block TCR internalization. After two washes in azide-containing PBS, the cells were placed on poly-L-lysine-coated glass slides and fixed with 2% paraformaldehyde (Sigma-Aldrich) in PBS. The slides were mounted with Prolong Anti-fade (Molecular Probes) according to the manufacturer's instructions (40).

2.3.4 Immunoprecipitation and Western blotting

Twenty million JTAg cells were lysed with 1 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris (pH 7.3), 0.4 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 μM sodium o-vanadate, and 1 μM PMSF) for 1 hour at 4°C. Lysed cells

were spun at 14,000 rpm in a microcentrifuge for 20 minutes at 4ºC to remove cellular debris. Cell lysates were incubated for 2 hours at 4°C with 5 μg monoclonal anti-H902 (IgG1) antibody, followed by overnight incubation with 20 μl protein G-Sepharose at 4°C. Immune complexes were washed three times with lysis buffer, resolved by 6% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (PVDF, Gelman Sciences, Ann Arbor, MI). For Western blotting, the membrane was blocked with 5% BSA in TBST for two hours, probed with primary antibodies titrated in 2.5% BSA in TBST for 1 hour, washed three times for five minutes with TBST, probed with secondary antibodies titrated in 2.5% BSA in TBST for 30 minutes, and washed three times for 10 minutes with TBST. The membrane was then exposed to an enhanced chemiluminescence substrate reaction (West Pico SuperSignal, Pearce, Rockford, IL). The light reaction was then captured with film at various exposure times (X-OMAT LS film, Kodak, Rochester, NY).

2.3.5 Autophosphorylation assay

Itk immune complexes, prepared as described above, were washed once with kinase reaction buffer (50 mM HEPES (pH 7.4), 2 mM $MnCl_2$, 200 μM ATP, 10 mM $MgCl_2$, and 10 mM DTT) and then resuspended in 100 μl of the same buffer. The immune complexes were incubated for 1 hour at 37°C, resolved by SDS-PAGE (6%), and analyzed by Western blotting with anti-phosphotyrosine antibodies.

2.3.6 Bead endocytosis

One million Jurkat cells in 0.5 ml of RPMI 1640, expressing Itk-or LAT-green fluorescent protein (GFP) transgenes or GFP alone were incubated with 2 X 10^6 latex beads (6-μm diameter, Polysciences Inc., Warrington, PA) precoated either with anti-human CD3ε antibody (OKT3) or isotype control antibody (UPC10) (10 μg/10^7 beads). Cells and beads were centrifuged (50*g*) and incubated for 7 minutes at 37°C (optimal as assessed by time kinetics) to allow for engagement of the cells and beads. The cell/bead mixtures were then fixed on slides with 3.7% paraformaldehyde at room temperature for 30 minutes and analyzed under a Zeiss epifluorescence microscope (Zeiss, Oberkochen, Germany). Positive cells were those GFP (transgene)-expressing cells extending protrusions around the beads as to endocytose them (for example, see Figure 2.8). For each transfected cell line, multiple fields were visualized from which several (30–100 depending on the experimental group) cell-bead conjugates were assessed.

2.3.7 Actin polymerization index

Jurkat cells transfected with Itk-GFP were treated similarly as above with except for the cell/bead incubation was performed for 5 minutes at 37°C to minimize extension of cytoplasmic protrusions around the beads, but still allow for optimal actin polarization at the cell-bead interface. The cells were then permeabilized in PBS 1% BSA containing 0.05% saponin for 30 minutes at 4°C. Phalloidin-tetramethylrhodamine isothiocyanate (Phalloidin-TRITC, 100 ng/ml; Sigma-Aldrich, St. Louis, MO) was added and cells were incubated for 30 minutes at 4°C to visualize filamentous actin. After washing, samples

were fixed on slides as described above and analyzed on a laser scanning confocal microscope (Leica TCS SP2; Leica, Heidelberg, Germany). Images were acquired and analyzed with the Leica confocal software. To determine the actin polymerization index, a transect was drawn across each cell-bead conjugate that included the cell-bead contact site and the portion of the cell membrane diametrically opposite to the contact site.

Actin polymerization indices were also assessed and compared between splenocytes from $Itk^{-/-}$ and strain-, sex-, and age-matched control mice in a manner similar to that described above for Jurkat cells. However, beads were coated with the anti-murine CD3ε antibody 2C11 (BD PharMingen, San Diego, CA), incubated with cells for 10 minutes at 37°C (determined to be optimal by time kinetic analysis), and visualized with phalloidin-TRITC concentration at 50 ng/ml.

2.3.8 Quantification of actin polymerization by flow cytometry

Splenic cells from $Itk^{-/-}$ or strain-, sex-, and age-matched control mice were stimulated at 37°C with anti-mouse-CD3ε (2C11) followed by cross-linking with FITC-conjugated goat anti-Armenian hamster secondary antibody (Jackson ImmunoResearch, West Grove, PA) for various periods. The cells were permeabilized and stained with phalloidin-TRITC (50 ng/ml) as described above and then analyzed on a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). Using appropriate controls, signal intensity was compensated to minimize spectral overlap and then FITC-positive cells (antibody reactive), defined by an electronic gate, were analyzed for red fluorescence (produced by cells stained with phalloidin-TRITC). The data are expressed

as fold increase in actin polymerization calculated by the ratio of median fluorescence intensity at each time point to that of the non-stimulated zero point.

2.3.9 Laser scanning confocal microscopy

Confocal imaging was performed using the Leica TCS SP2 (Leica, Heidelberg, Germany). GFP and Texas Red were imaged sequentially to avoid channel bleed-through. GFP was excited at 488 nm and analyzed through the 522–535 nm channel photon multiplier tube (PMT), whereas Texas Red was excited at 568 nm and detected by the 598–640 nm channel PMT. Images were acquired with the Leica software, converted with GraphicConverter (Lemke Software, Peine, Germany), and pseudocolored and overlaid with Adobe Photoshop (Adobe, San Jose, CA).

2.3.10 Statistics

Graphing and statistical analyses were done using either GraphPad Prism (GraphPad Software, San Diego, CA), Excel (Microsoft, Seattle, WA), or Cricket Graph (Cricket Software, Cupertino, CA). Student t-tests were evaluated through these programs.

2.4 Results

2.4.1 Itk is organized into discrete modular domains

Itk is organized in discrete modular domains, which includes a Pleckstrin homology (PH) domain, a Tec homology (TH) domain, a Src homology 3 domain (SH3), a Src homology 2 domain (SH2), and a kinase domain (SH1). Figure 2.1 is a cartoon diagram of the domains of Itk and the representations of the mutations created within Itk for use in the following studies. At top is the full-length, wild-type Itk with the amino acid positions of each of the domains. Deletion mutants of both the SH2 and the SH3 domains are represented. Point mutants of Itk generated are also shown in this figure. The SH2 domain point mutant (R265K) was generated based on the disruption of the phosphorylated tyrosine binding pocket similarly seen in PLCγ1 (41, 42) and Btk (43, 44). The SH3 domain point mutant (W208K) was generated based on *in vitro* GST-fusion protein work done in the Berg lab (45). The SH1 domain kinase inactive mutant (K390R) was generated according to a known mutation in Btk's kinase domain which is thought to disrupt the ability of this domain to bind ATP (46).

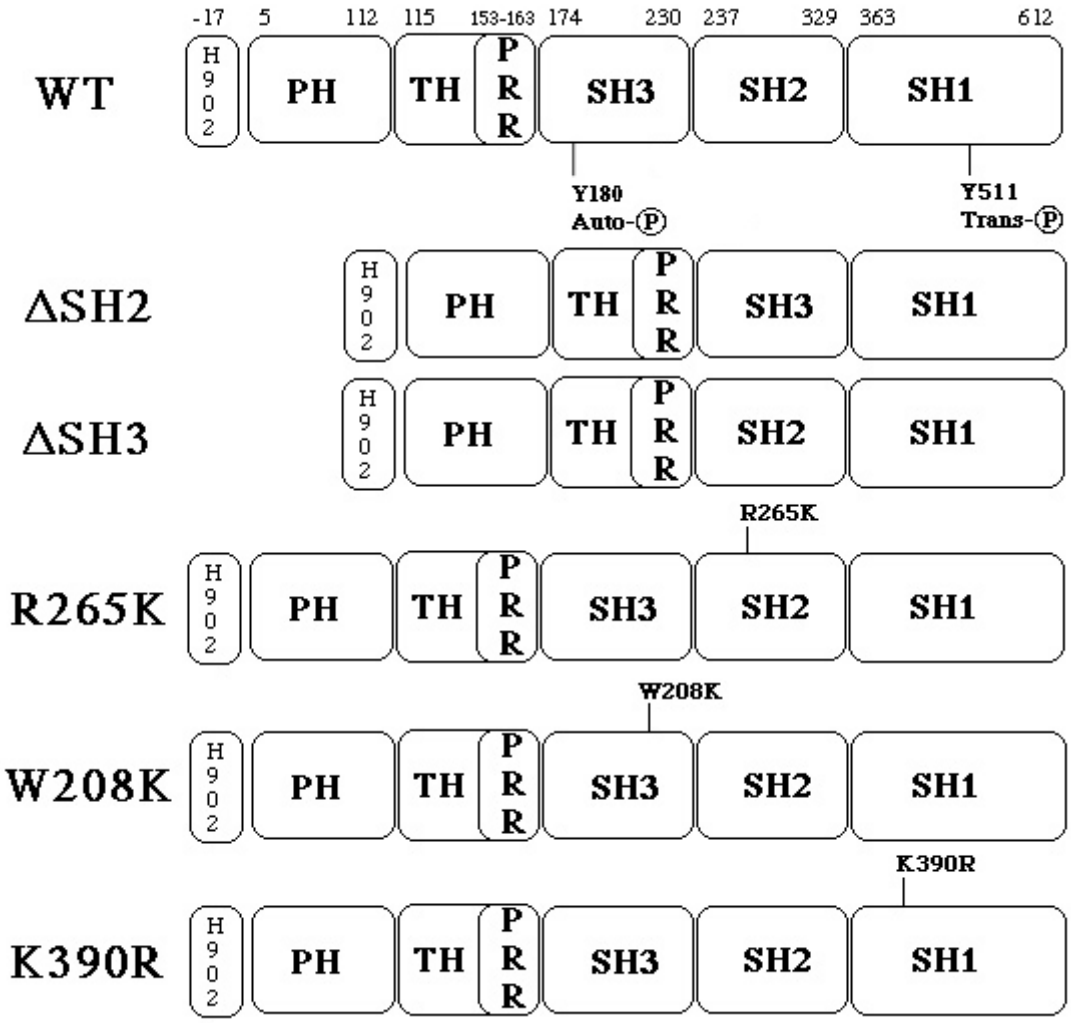

Figure 2.1. *Schematic representation of Itk structure.*

H902-tag at the N-terminus followed by the Pleckstrin Homology (PH) domain, Tec Homology (TH) domain, a Proline Rich Region (PRR), Src Homology 3 (SH3) domain, Src Homology 2 (SH2) domain, and the Src Homology 1 (SH1) or kinase domain. Deletion and point mutants used in the experiments are also displayed.

2.4.2 The Itk SH2 domain is required for the TCR/CD3-induced activation of Itk

To investigate which domain(s) of Itk play a role in its TCR/CD3-induced activation, JTAg T cells were transfected with H902-tagged WT-Itk or various mutants. These cells were stimulated by cross-linking with anti-CD3ε antibodies (OKT3) or isotype control antibodies, and the phosphorylation of the transgene products was analyzed. Wild-type Itk becomes highly tyrosine phosphorylated, as assessed by immunoprecipitation with anti-H902 antibodies and Western blotting with anti-phosphotyrosine antibodies (Figure 2.2A and Figure 2.2B, top panels). Similarly, SH3 domain mutants of Itk, either lacking the SH3 domain (ΔSH3) or containing a point mutation (W208K), are also highly tyrosine phosphorylated upon anti-CD3ε cross-linking (Figure 2.2A and Figure 2.2B, top panels). In sharp contrast, mutants lacking the SH2 domain (ΔSH2) or mutants with an inactivating SH2 domain point mutation (R265K) display deficient phosphorylation under the same experimental conditions (Figure 2.2A, top panels). The differences in transphosphorylation are not due to unequal loading, as is shown by the anti-Itk blotting (Figure 2.2A, bottom panels). It is interesting that a mutation in the SH2 domain of the related kinase Btk, analogous to the R265K mutation in Itk used here, has also been shown to disrupt Btk function (44). Similar to WT-Itk, mutation of the SH1 domain of Itk (K390R), with theoretically deficient enzymatic activity, also displayed intact tyrosine phosphorylation upon stimulation (Figure 2.22).

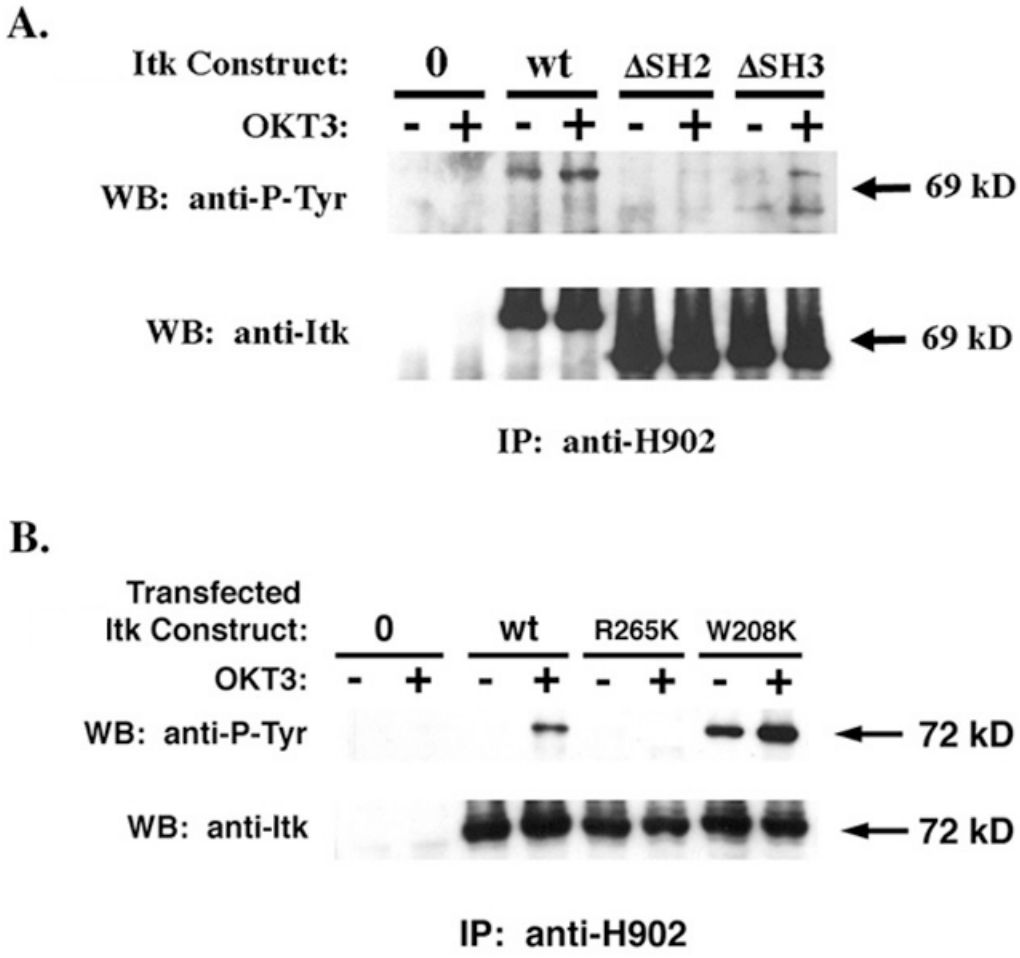

Figure 2.2. *Inhibition of Transphosphorylation in SH2 Domain Mutants of Itk.*

Jurkat T cells transfected with the indicated Itk constructs were stimulated by incubating with OKT3 (anti-CD3ε) antibodies (+) or with isotype control antibodies (-). Transfected Itk was immunoprecipitated by using anti-H902-tag antibodies, then analyzed by Western blotting with anti-phospho-tyrosine (top panels) or anti-Itk (bottom panels) antibodies. Panel A shows effects of deleted domains on transphosphorylation, while panel B displays effects of point mutations on transphosphorylation. Wild-type Itk and SH3 mutants display increased tyrosine phosphorylation when incubated with anti-CD3ε antibodies, while Itk SH2 mutants do not become tyrosine phosphorylated.

The transphosphorylation of Itk correlates with an increase in its catalytic activity (47). Therefore, it was tested whether the lack of transphosphorylation seen with the SH2 domain mutants correlated with lower catalytic activity. To assess this relationship, Itk immune complexes isolated from JTAg T cells transfected with different Itk constructs were subjected to an *in vitro* kinase assay after stimulation with anti-CD3ε antibodies or with isotype control antibodies. Consistent with the transphosphorylation data in Figure 2.2B, the ΔSH2 domain deletion mutant and the R265K point mutant both display a severe deficiency in enzymatic activity (Figure 2.3A and Figure 2.3B, top panels). This is in contrast to WT-Itk and to the SH3 domain mutants (ΔSH3 and W208K), all of which display CD3ε-inducible kinase activity (Figure 2.3A and Figure 2.3B, top panels). It should be noted that the conversion of tryptophan to lysine at amino acid position 208 has been shown to be an inactivating mutation at the predicted binding pocket of the SH3 domain (45). In further experiments, this SH3 domain mutant displayed some autophosphorylation even in the absence of stimulation (Figure 2.22). This non-induced and spontaneous kinase activity may be due to partial release of autoinhibition by the SH3 domain. As expected, the kinase domain mutant K390R does not display any enzymatic activity (Figure 2.4, top panels). The results with the K390R mutant exclude the possibility that the kinase activity detected with WT- or W208K-Itk might be due to a fortuitously co-precipitating kinase, if this were the case, one would observe phosphorylation of the mutant kinase. Western blotting with anti-Itk antibodies shows that the data cannot be explained simply because of unequal loading of samples (Figure 2.3A and Figure 2.3B, bottom panels), rather it must due to the kinetic activity (or lack thereof) of the mutant Itk. Furthermore, lack of detection of enzymatic activity cannot be

due to insufficient expression of the R265K transgene because its expression is comparable to that of W208K, which displays detectable kinase activity (Figure 2.3A and Figure 2.3B, bottom panels).

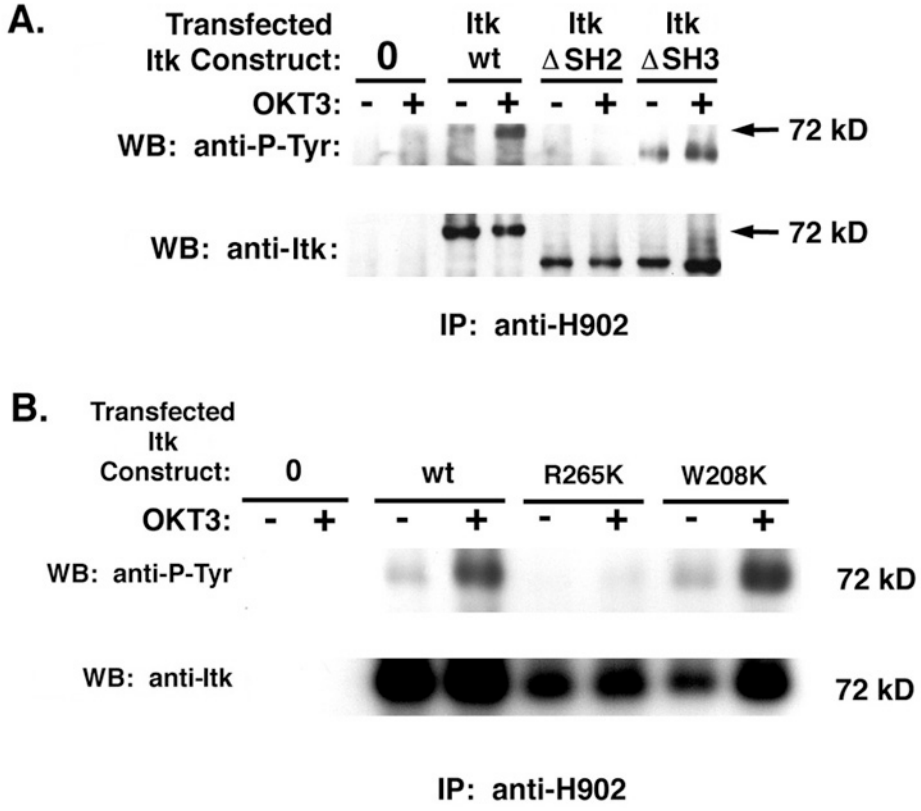

Figure 2.3. *Itk SH2 Domain Mutation Abolishes Activation Induced Auto-Phosphorylation.*

Experiments were performed as in Figure 2.2 with the exception that Itk immune complexes (anti-H902) were analyzed for enzymatic activity in an *in vitro* autokinase assay. Immunoprecipitates were incubated in a kinase buffer for 1 hour at 37°C. Deletion mutants are shown in panel A and point mutants are shown in panel B. The stimulation-induced kinase activity of the mutant SH2 Itk is reduced compared to that of wild-type or mutant SH3 Itk.

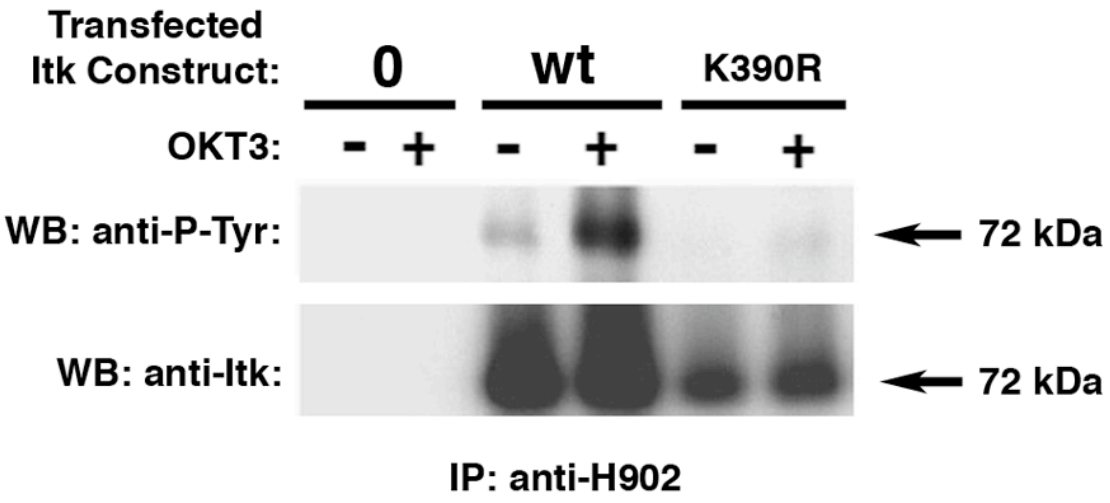

Figure 2.4. *Itk SH1 Domain Mutant Is Catalytically Inactive.*
Jurkat T cells, non-transfected, or transfected with either WT-Itk or K390R-Itk, were stimulated for 3 minutes at 37°C with anti-CD3ε OKT3 (+) or with isotype control (-) antibodies. Cells were lysed, immunoprecipitated with H902, and subjugated to the autophosphorylation assay described in the Materials and Methods. The Itk SH1 domain mutant displays no catalytic activity, nor are there any co-precipitating enzymes that phosphorylate the mutant protein.

2.4.3 SH2 domain mutants of Itk interfere with its co-localization with the TCR/CD3 complex and affects TCR/CD3 capping

TCR/CD3 complex polarization as a result of cross-linking with antibodies has been visualized since the anti-CD3 antibodies themselves were first characterized (48, 49). This phenomenon became known as TCR "capping", a precursor to the structured accumulation of signaling molecules later termed the immunological synapse. In an attempt to visualize Itk's localization within a T cell, wild-type Itk was genetically fused with green fluorescent protein (Itk-GFP) and transfected into Jurkat T cells to monitor its localization from non-stimulated to stimulated conditions. Labeling of the TCR/CD3 complex with anti-CD3ε (OKT3) antibodies and secondary labeling with Texas-Red

conjugated antibodies shows that the TCR/CD3 complex is roughly evenly distributed on the surface of the T cell prior to stimulation (Figure 2.5F). When the cells are stimulated (warmed at 37°C for 7 minutes), TCR/CD3 capping occurs (Figure 2.5C) where the complexes coalesce and polarize to one end of the cell. In these transfected Jurkat cells, Itk-GFP is localized primarily at the cell membrane, and fairly well distributed when the cell has not been stimulated (Figure 2.5D). Please note, however, that this membrane localization pattern is most likely due to the lack of a regulatory phosphatase in Jurkat cells, phosphatase and tensin homolog (PTEN), which allows Itk to be constitutively localized at the membrane, while in wild-type non-immortalized cells it would normally be located in the cytoplasm (50). Upon stimulation, Itk aggregates into a cap (Figure 2.5A), and co-localizes with the TCR/CD3 complex (Figure 2.5B).

While further exploring the effects of SH2 domain mutants of Itk, it was discovered that these mutants, when fused to GFP, were found not to co-localize with the TCR/CD3 complex upon stimulation (Figure 2.6, panels A-F). Even more remarkable, T cells transfected with these mutant molecules failed to fully organize a cap, or a polarized aggregation of TCR/CD3 complexes. Other mutants of Itk when similarly fused to GFP, including the SH3 domain deletion (ΔSH3) and point mutants (W208K), as well as the catalytically inactive mutant (K390R), all seemed to co-localize with the TCR/CD3 complex competently, as well as having little to no effect on the size and number of the T cell caps that took place upon stimulation (Figure 2.6, panels G-O).

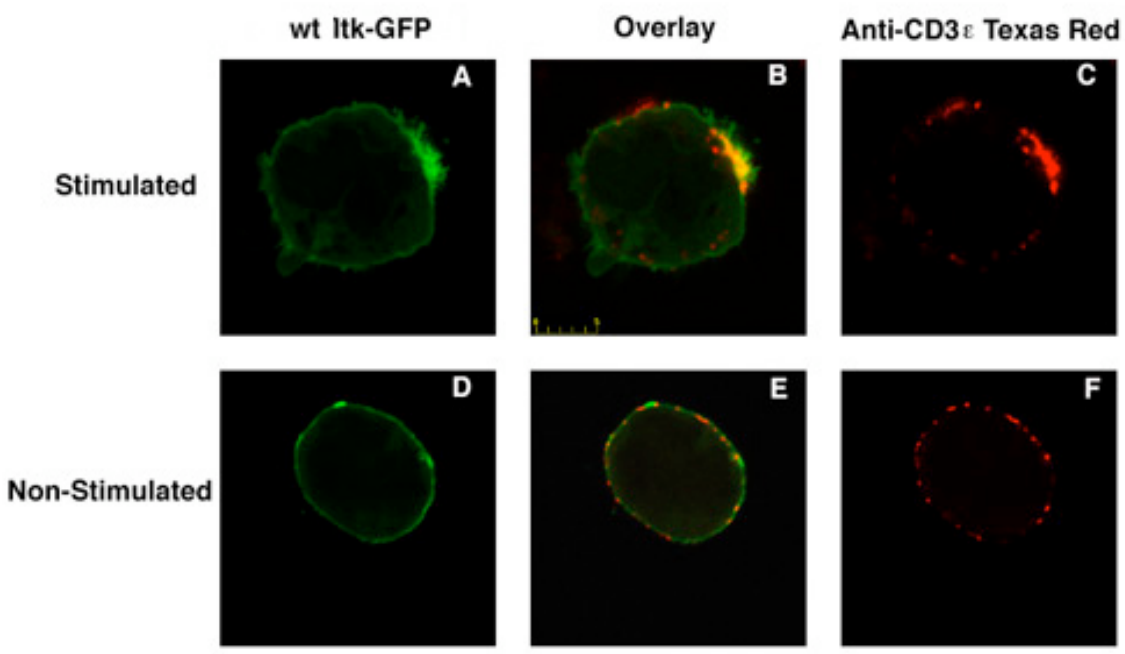

Figure 2.5. *Stimulation Induced Co-localization of Itk and TCR/CD3.*

Jurkat T cells were transfected with wild-type Itk-GFP (green), then incubated with OKT3 antibodies, followed by Texas-Red conjugated secondary antibodies (red) to cross-link. Samples were incubated at 37°C (Stimulated, panels A-C) or kept at 4°C (Non-Stimulated, panels D-F) for 7 minutes to induce capping. Confocal microscopic analysis indicates that stimulation induces the formation of Itk clusters (panel A) and a distinct TCR/CD3 "cap" (panel C). Overlay of Itk cluster and TCR/CD3 cap show a co-localization event (panel B).

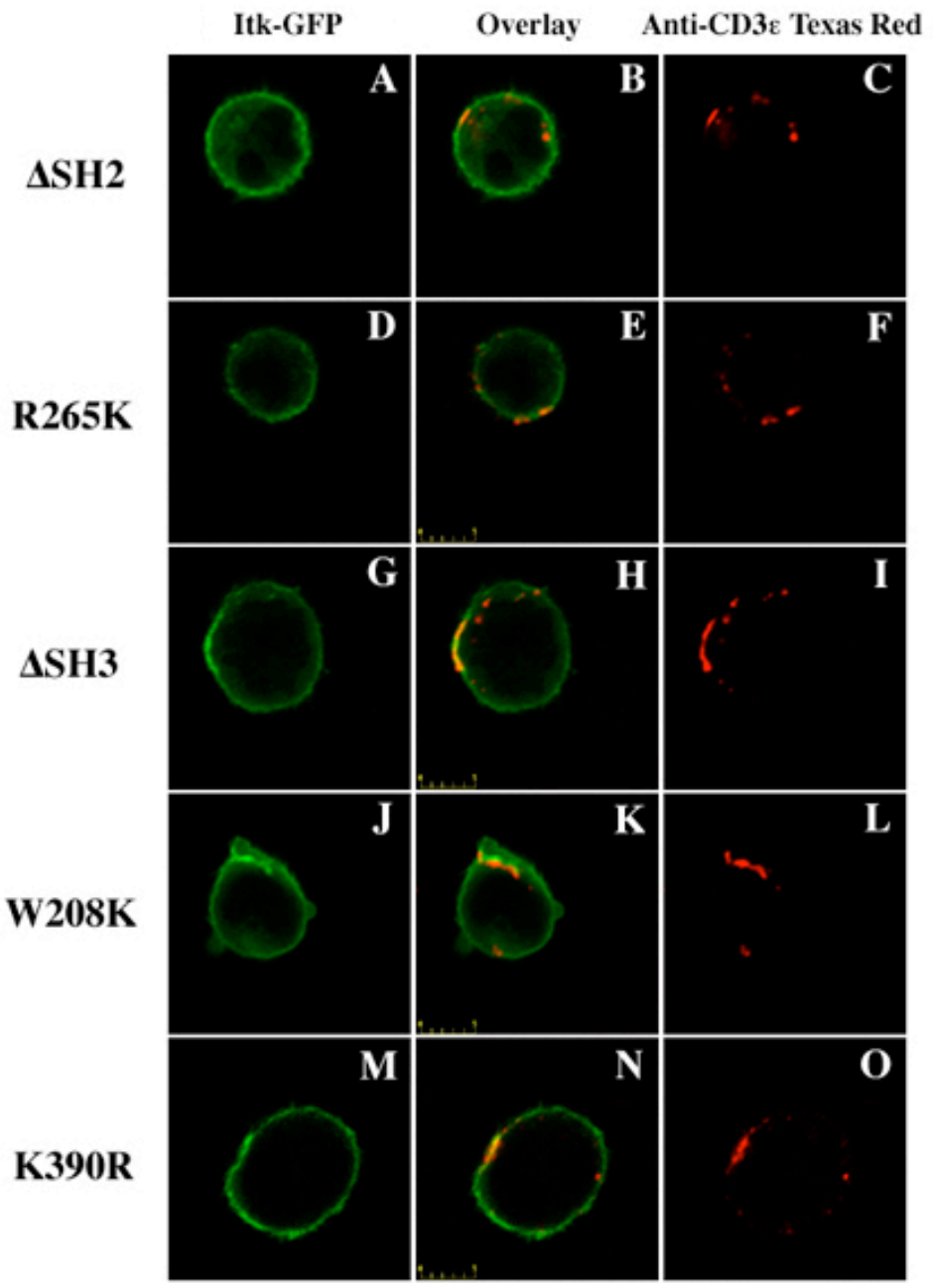

Figure 2.6. *Antigen Receptor Clustering is Inhibited by SH2 Mutants of Itk.*

Similar experiments were conducted with cells transfected with Itk-GFP constructs bearing mutations in the SH2 domain (ΔSH2, panels A-C; R265K, panels D-F). Upon stimulation, SH2 domain mutants showed a reduction in the percentage of cells forming TCR/CD3 "caps". In contrast, SH3 domain mutants (ΔSH3, panels G-I; W208K, panels J-L) or a kinase-inactive mutant (K390R, panels M-O) display no disruption in "cap" formation or co-localization of Itk with TCR/CD3.

Of note, the presence of GFP remarkably does little to affect the activity of a protein to which it is attached. This inert feature is astonishing in light of GFP's large molecular size (27 kDa). However, very little has been reported in the scientific literature as to any adverse biological effect due to its presence outside of some slow fluorescent maturation times and some oligomerization issues with certain isoforms of the protein (51). As such, it was tested whether the GFP fusion onto Itk affected its ability to become trans-phosphorylated upon stimulation. As shown in Figure 2.7, the presence of GFP on Itk did not alter the ability of Itk to become trans-phosphorylated upon stimulation, nor did it differ in its kinetics or amplitude of phosphorylation when compared to non-GFP tagged Itk constructs.

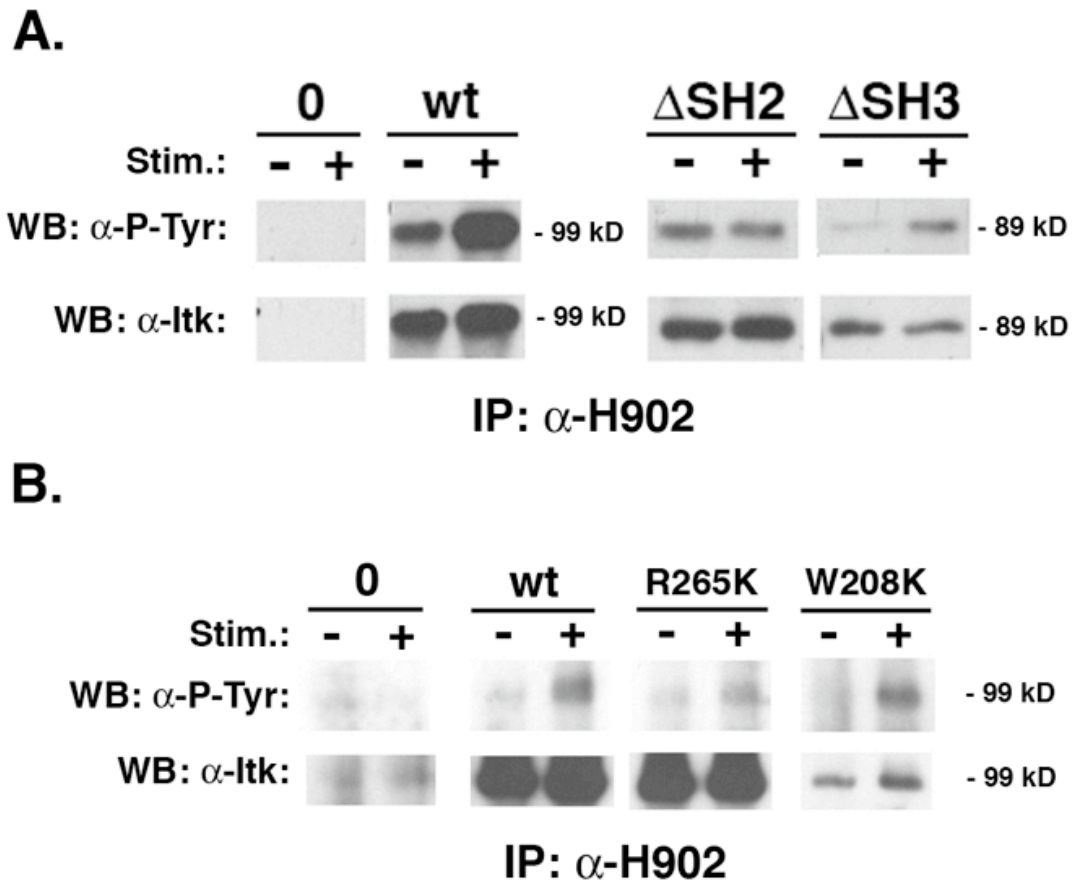

Figure 2.7. *GFP Does Not Interfere With Itk Trans-Activation.*
Jurkat T cells transfected with the indicated Itk-GFP constructs were stimulated by incubating with OKT3 (anti-CD3ε) antibodies (+) or with isotype control antibodies (-). Transfected Itk was immunoprecipitated with anti-H902-tag antibodies, then analyzed by western blotting with anti-phospho-tyrosine (top panels) or anti-Itk (bottom panels) antibodies. Panel A shows deletion mutants effect on transphosphorylation, while panel B displays point mutants effect on transphosphorylation.

2.4.4 SH2 mutant Itk interferes with TCR/CD3-induced cytoskeletal events

The overexpression of the SH2 domain Itk mutants into Jurkat T cells interferes with the capping of the TCR-CD3 complex upon cross-linking with anti-CD3ε antibodies. Since capping of the TCR depends on the polymerization of the actin cytoskeleton (9, 52-54), the role of Itk in this phenomenon was investigated through the

use of a conjugate formation assay previously reported by Lowin-Kropf et al. (55). In this assay, incubation of Jurkat cells with anti-TCR/CD3ε antibody-coated polystyrene beads induces cytoskeletal changes at the cell-bead attachment site such as reorientation of the microtubule-organizing center (MTOC) and localized actin polymerization. When Jurkat cells are incubated with anti-CD3ε-coated polystyrene beads, they extend cytoplasmic processes around the beads as to endocytose them (see (25) and Figure 2.8A). This cellular action is dependent on the reorganization and polymerization of the actin cytoskeleton (25). In sharp contrast, Jurkat cells incubated with isotype control antibody-coated beads do not extend cytoplasmic protrusions around the beads (Figure 2.8B). It should be noted that in these experiments Jurkat cells have been transfected with WT-Itk-GFP.

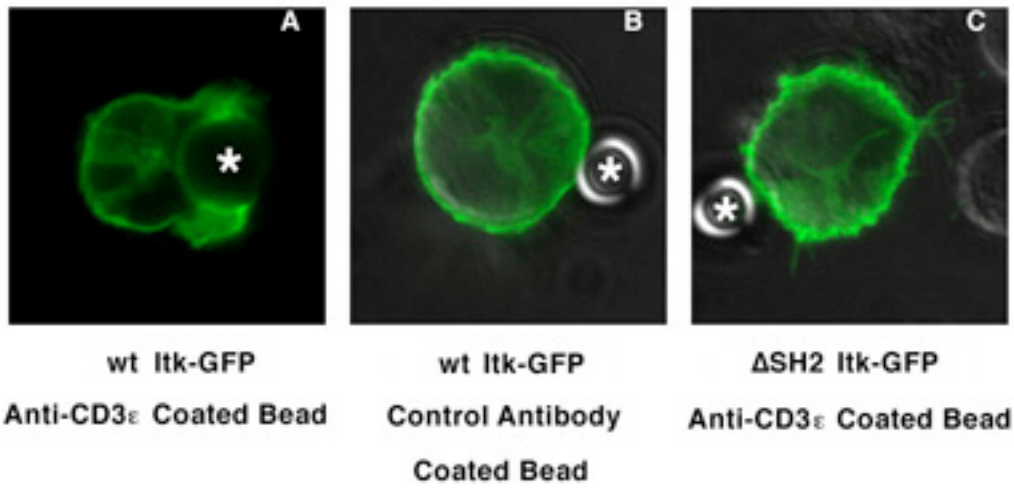

Figure 2.8. *Endocytosis of Anti-CD3ε Coated Beads by Itk-GFP Transfected Jurkat T Cells.*

Representative examples of Jurkat T cells transfected with Itk-GFP and incubated with antibody-coated polystyrene beads as indicated. Asterisks designate the location of coated beads.

Using this bead endocytosis assay, analysis of the effects that expression of the R265K SH2 mutant of Itk has on TCR/CD3-induced cytoskeletal events were quantified. Jurkat cells expressing equal amounts of transfected GFP-chimeric constructs of either WT- or R265K-Itk were incubated with polystyrene beads coated either with anti-CD3ε or isotype control antibodies (Figure 2.9B). After an optimal incubation period (7 minutes), determined by kinetic analysis, the cells were fixed on slides and analyzed by fluorescence microscopy for GFP-positive cells that extended protrusions around the beads. Eighty-six percent of Jurkat cells transfected with WT-Itk extended protrusions around the beads as to endocytose them (Figure 2.9C). In contrast, 29% of Jurkat-expressing R265K-Itk displayed bead endocytosis (Figure 2.9C), a statistically significant reduction ($p \leq 0.05$, Student's t test) in comparison to WT-Itk transfected cells. The percentage of bead endocytosis displayed by WT-Itk transfectants was similar to that seen with non-transfected Jurkat (81±8%, Figure 2.10) when visualized under differential interference contrast (DIC) microscopy, or with Jurkat transfected with GFP alone (84±9%, Figure 2.10). Thus, the reduction in bead endocytosis is specific to R265K-Itk expression and not due to non-specific overexpression of the protein. This defect cannot be explained by delayed time kinetics because the percentage of bead endocytosis displayed by the SH2 mutant transfectants is not altered at longer incubation times lasting up to 30 minutes. Furthermore, Jurkat cells transfected with an SH2 deletion (ΔSH2) mutant of Itk-GFP display a similarly reduced ability in bead endocytosis (39±7%, Figure 2.10).

SH3 domain mutants of Itk-GFP transfected T cells displayed a slight reduction in their ability to endocytose stimulating microbeads (ΔSH3 68±4%, W208K 74±6%,

Figure 2.10). When K390R-Itk-GFP is expressed in Jurkat cells at levels equivalent to those of R265K-Itk-GFP and WT-Itk-GFP (Figure 2.9B), there is no effect on TCR/CD3-mediated cytoskeletal events (Figure 2.9C). This is interesting, since the K390R protein carries a point mutation in the SH1 domain that inactivates the enzymatic activity of Itk (Figure 2.4 and (56)). This was the first indication that Itk could have a kinase-independent role in T cell regulation.

Using the same bead conjugate assay, Lowin-Kropf et al. found that the Src tyrosine kinase, Lck, is important for the TCR/CD3-induced cytoskeletal events, as Jurkat mutants lacking expression of Lck (JCaM1.6) were deficient in both MTOC reorientation and localized actin polymerization (55). Comparison of the behavior of JCaM1.6 to R265K transfectants in the bead endocytosis assay reveals that both cell types are similarly deficient in TCR/CD3-induced actin events (Figure 2.9C).

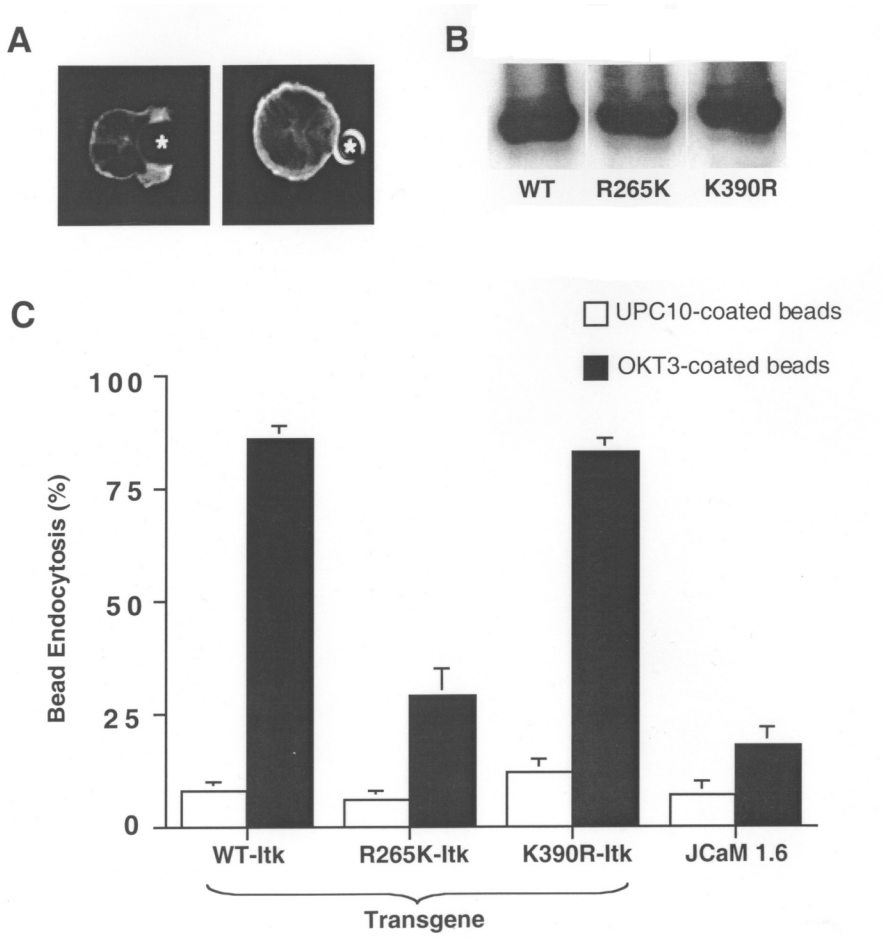

Figure 2.9. *Mutant Itk SH2 Domain Significantly Reduces the Ability of Jurkat T Cells to Endocytose Anti-CD3ε Microbeads.*

A.) Jurkat cells transiently transfected with murine WT-Itk-GFP were incubated (7 minutes, 37°C) with polystyrene beads coated with either anti-CD3ε OKT3 (left) or isotype control UPC10 (right) antibodies. After fixation on slides, GFP-positive cells were visualized for extension of cytoplasmic processes around the beads using an epifluorescence microscope. Results are from one of seven replicate experiments and they display representative GFP-positive cells out of 100 cell-bead conjugates assessed per condition. B.) Jurkat cells transiently transfected with murine Itk-GFP (WT, R265K, K390R) constructs were lysed, Itk transgenes were isolated by immunoprecipitation with H902 antibodies, and immune complexes were analyzed by Western blotting with anti-murine Itk antibodies. C.) Jurkat cells transiently transfected with murine Itk-GFP transgenes as indicated and JCaM1.6 cells were incubated with OKT3 or UPC10 control antibody-coated beads as indicated and analyzed as described in A. Results are displayed as percentage of cells extending cytoplasmic processes around the beads (bead endocytosis). Results are averages (±SEM) of at least four replicate experiments.

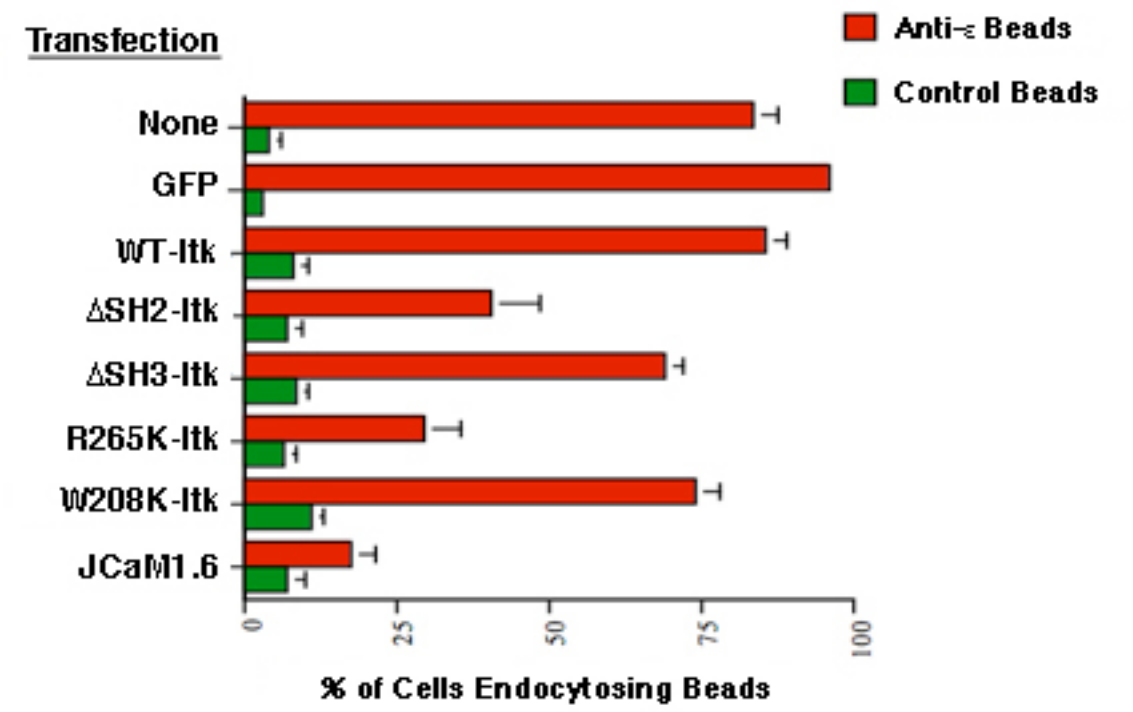

Figure 2.10. *Effects of Itk mutants on TCR/CD3-induced cytoskeletal events.*
Jurkat T cells were transfected with either wild-type or mutant Itk-GFP and then incubated with microbeads coated with anti-CD3ε or isotype control antibodies. Percentage endocytosis was assessed by a combination of bright field and fluorescent microscopy where the number of transfected cells (green) that conjugate 1 or 2 beads was quantified. Typical examples are shown in Figure 2.8. Data were collected from four replicate experiments with 100 conjugates scored per condition. Data are shown as percent endocytosis (±SEM) when compared to wild-type Itk-GFP. The Lck-deficient mutant cell line JCaM1.6, which is known to display deficiencies in bead endocytosis and Itk activation, was used as a reference control.

2.4.5 Itk co-localizes with actin

Since this process of protrusion around an surrogate APC has been reported to be dependent on actin polymerization (25, 55), the decision was made to stain for polymerized actin using the fungal toxin phalloidin (which binds filamentous actin, preventing actin depolymerization (57)). When a T cell engages an APC, it polymerizes and polarizes actin at the site of interaction. Staining T cells engaging artificial APCs

with fluorophore conjugated phalloidin allows one to observe the effects of Itk on directed actin polarization at the site of T cell interaction with the artificial APC. Figure 2.11 shows this directed actin polymerization within the T cell when engaging a stimulating anti-CD3ε antibody coated microbead (Figure 2.11B). Further, when Itk-GFP is transfected into these Jurkat T cells, Itk transgene molecules localize to the site of actin polymerization (Figure 2.11, A and C). When T cells engage a non-stimulating isotype control antibody coated microbead, they no longer direct actin polymerization towards the cell/bead engagement site, instead actin polymerization appears uniform amongst the intracellular periphery (Figure 2.11F). Although Itk similarly appears to co-localize with the actin stain in transfected cells, these molecules do not localize towards the site of interaction with the non-stimulating microbead (Figure 2.11, F and G). Please note that spectral overlap between the two fluorophores (GFP and TRITC) are eliminated by first exciting either a non-transfected GFP cell or a non-TRITC-stained cell and visualizing through the other respective channel, GFP fluorescence on the TRITC channel in a non-transfected cell or TRITC fluorescence on a non-stained cell. Further, the images were collected sequentially for each of the fluorophore channels so that no other excitation factor could illuminate the fluorophore. The elimination of spectral overlap provides confidence in the analysis of co-localization without the possibility of artifact due to the fluorescence of random fluorophores.

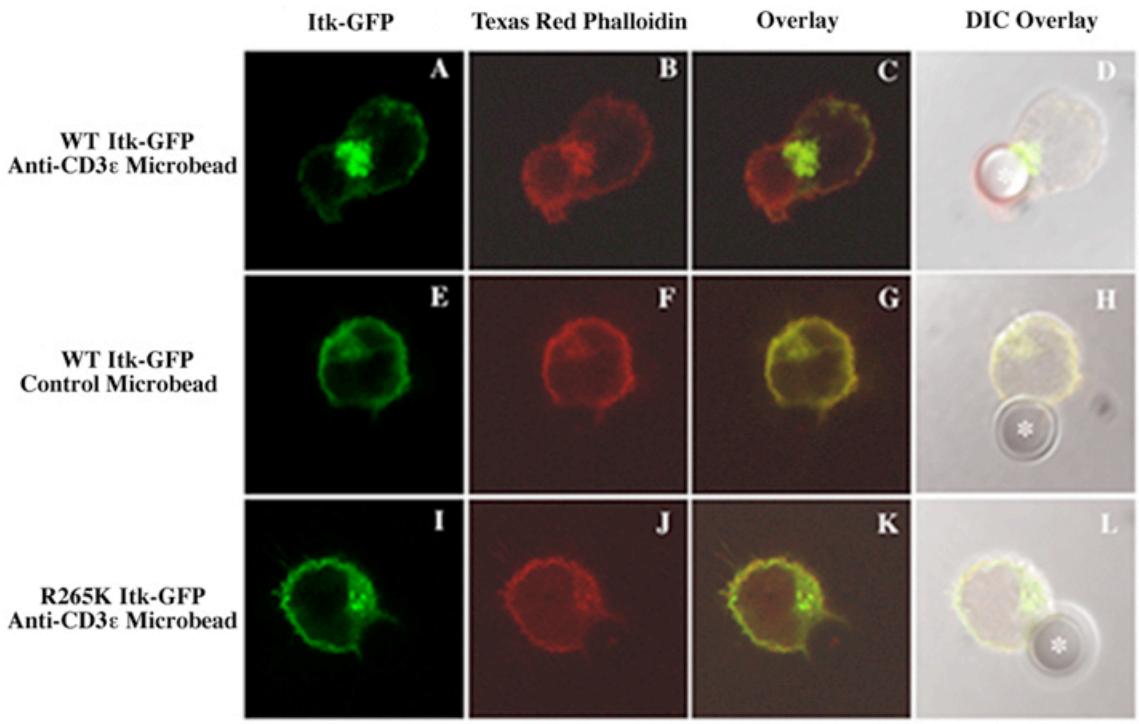

Figure 2.11. *Itk Co-localizes with Actin.*

Jurkat cells transfected with Itk-GFP constructs were incubated with beads coated with anti-CD3ε antibody, then stained with Texas-Red phalloidin to visualize polymerized actin, and analyzed by confocal microscopy. Itk (green) aggregates at the cell-bead interface (panel A). An actin collar (red) has similar polarity and orientation (panel B). The two signals show co-localization (panel C). Panel D is a DIC overlay. Control antibody-coated beads fail to induce aggregation of Itk or actin at the cell-bead interface (panels E-H). Interestingly, the R265K SH2 mutant of Itk does not aggregate to the cell-bead interface and, in the same cell, neither does actin (panels I-L). This finding suggests that the R265K mutant acts in a trans-dominant negative fashion.

2.4.6 SH2 mutant Itk interferes with TCR/CD3-induced actin polymerization

The above data suggests that Itk has both an effect on actin-mediated events and that it co-localizes with actin. Therefore, it was tested whether transfected mutants of Itk could affect actin polymerization in Jurkat T cells. In these experiments, Jurkat cells were transfected with either wild-type, R265K SH2 domain mutant, or W208K SH3 domain mutant Itk constructs, all possessing the GFP tag. The cells were then stimulated with anti-CD3ε (OKT3) or isotype control (UPC10) antibodies for 7 minutes at 37°C, permeabilized, and stained with TRITC-phalloidin. The cells were analyzed by flow cytometry by gating on the GFP-positive cells and measuring the increase in TRITC-phalloidin intensity. Data from stimulated cells were compared to non-stimulated cells for each condition and presented as a percent increase in actin polymerization induced under stimulation. As seen in Figure 2.12, wild-type transfected Jurkat cells displayed an inducible increase in actin polymerization (38±4%) upon stimulation, while SH2 domain point mutant transfected cells displayed significantly less ability to polymerize actin (16±5%). Additionally, SH3 domain mutant transfected cells displayed slightly less ability to polymerize actin (31±7%), but was not significant in comparison to wild-type transfected cells (Figure 2.12). Unfortunately, Jurkat cells displayed a large amount of actin polymerization even without stimulation (data not shown), which made the evaluation of actin polymerization in Jurkat cells through use of flow cytometry difficult. Therefore, to better resolve and evaluate Itk effects on actin polymerization, laser scanning confocal microscopy was used.

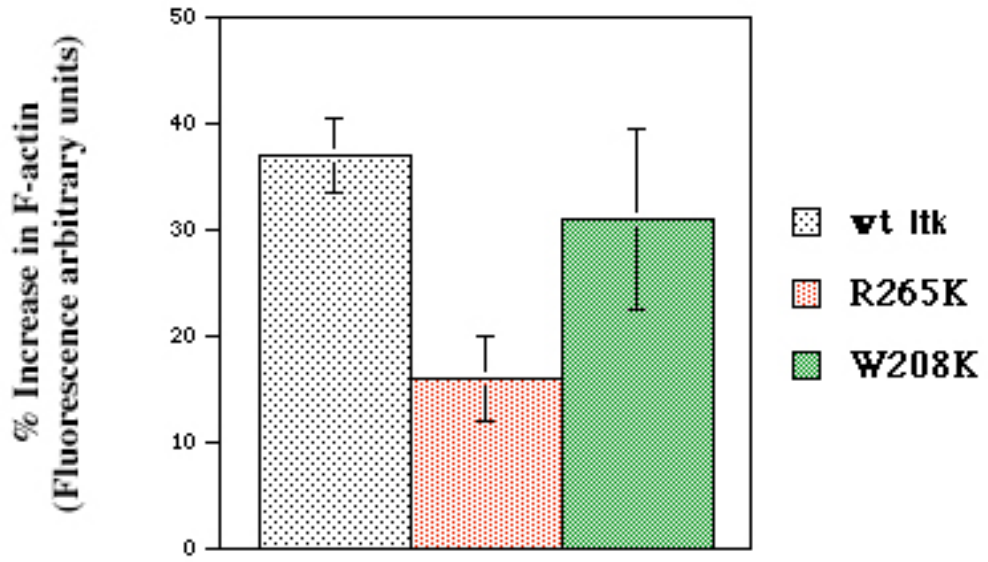

Figure 2.12. *Actin Polymerization in T Lymphocytes Is Diminished With Itk SH2 Domain Point Mutant.*

Jurkat T cells were transfected with wild-type or mutant Itk-GFP, then incubated with anti-CD3ε or isotype control antibodies for 7 minutes at 37°C, permeabilized, and then stained with TRITC-phalloidin. The cells were analyzed by Flow Cytometry. GFP-positive transfected cells were gated and analyzed for mean TRITC fluorescence, which measures F-actin content in Itk transfected cells. Results are presented as the average mean fluorescence intensity increase (±SEM) of stimulated transfectants compared to that of non-stimulated transfectants. The data show that the SH2 point mutant (R265K) of Itk inhibits actin polymerization in T lymphocytes upon stimulation, thus behaving in a dominant-negative fashion.

The extension of cytoplasmic protrusions around the anti-CD3ε-coated beads depends on localized actin polymerization at the bead-cell attachment site. To further explore the effects of SH2 mutant Itk on TCR/CD3-induced cytoskeletal events, the bead-conjugate assay was used to quantify the localized actin polymerization at the contact sites between Jurkat cells and polystyrene beads. To this end, Jurkat cells expressing equal amounts of R265K-Itk-GFP or WT-Itk-GFP (Figure 2.13B) were incubated with polystyrene beads coated with either anti-CD3ε or isotype control

antibodies. Incubation was conducted for 5 minutes, to minimize extension of cytoplasmic protrusions around the beads, but still allow for optimal actin polarization at the cell-bead interface. Following incubation, the cells were permeabilized, stained with TRITC-phalloidin to visualize polymerized actin, and then analyzed by confocal microscopy. Actin polymerization at the cell-bead contact sites was visualized by pseudocoloring (Figure 2.13A). The degree of polymerization relates to the color scale shown on top of Figure 2.13A, with blue representing the highest and red the lowest intensities, respectively. Pixel intensities at the GFP (transgene)-positive cell-bead contact sites were quantified and expressed as actin polymerization index. An actin polymerization index was determined by measuring the intensity of fluorophore actin signal at the site of interaction versus at the polar opposite cellular end from the interaction site. T cells transfected with wild-type Itk-GFP displayed an actin polymerization index of more than 2 when engaging a stimulating condition microbead. This indicates that twice as much actin was polymerized at the site of T cell interaction with the artificial APC than actin polymerized at an equal area opposite of the interaction site. Jurkat cells expressing WT-Itk transgene display about a 2-fold increase in the actin polymerization index when stimulated with anti-CD3ε (OKT3)-coated beads as compared to non-stimulating conditions with isotype control (UPC10) antibody-coated beads (Figure 2.13C). This difference is statistically significant ($p \leq 0.05$, Student's t test). Similar results were obtained with non-transfected Jurkat cells (data not shown). Importantly, when cells expressing the R265K mutant are stimulated with anti-CD3ε (OKT3)-coated beads, they display a very small increase in the actin polymerization index (Figure 2.13C) as compared with stimulation with isotype control antibody-coated

beads. This small increase is barely significant ($p = 0.04$, Student's t test). Consistent with the data shown in Figure 2.9C, expression of the kinase-dead K390R-Itk mutant has no effect on the actin polymerization index when compared to wild-type Itk (Figure 2.13C).

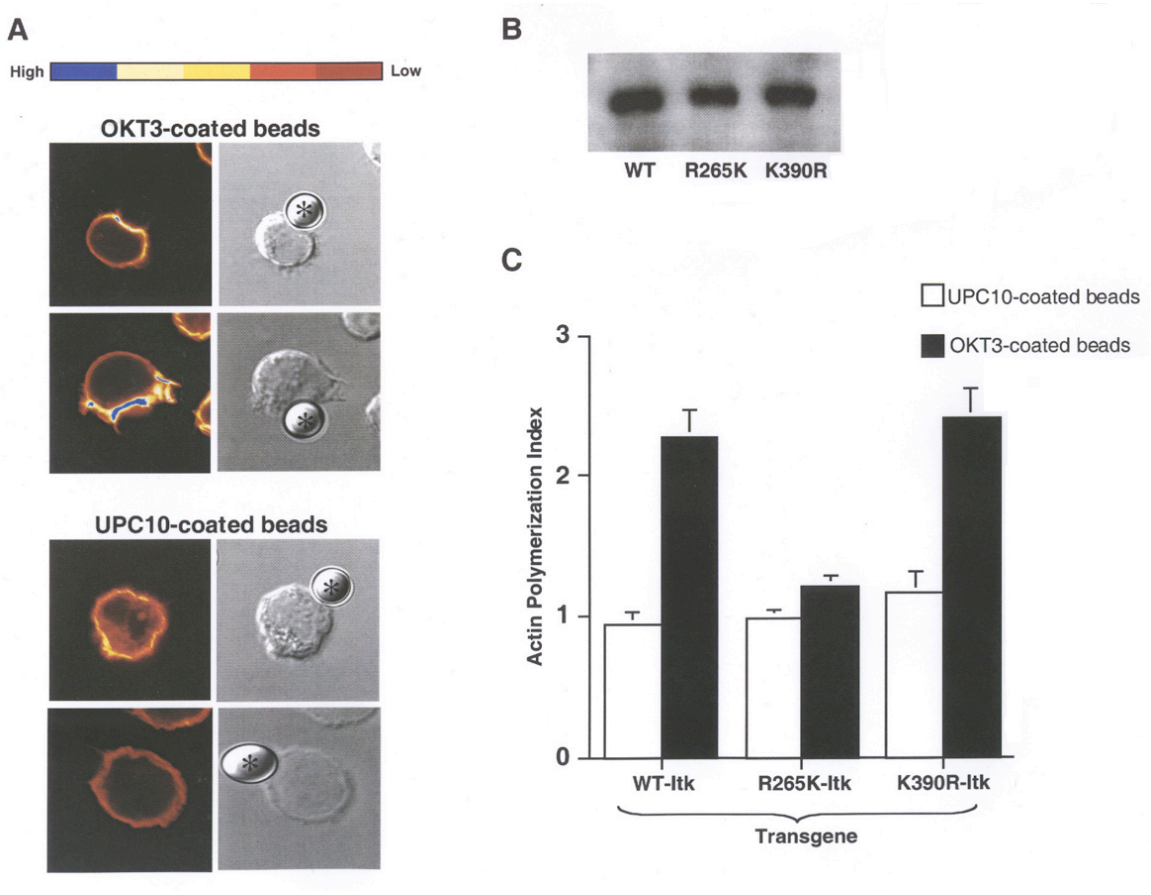

Figure 2.13. *Directed Actin Polymerization is Inhibited by Mutant Itk SH2 Domain.*

A.) Jurkat cells transiently transfected with murine WT-Itk-GFP were incubated (5 minutes, 37°C) with polystyrene beads coated with either OKT3 (*top panels*) or UPC10 control (*bottom panels*) antibodies. Cells were then permeabilized, stained with phalloidin-TRITC, and analyzed by laser scanning confocal microscopy. Results are from one of seven replicate experiments. Two representative cell-bead conjugates are shown out of at least 30 analyzed per condition. Results are displayed as phalloidin fluorescence intensity visualized by pseudocoloring (*left panels*) and differential interference contrast images (*right panels*). Intensity correlates to the color scale on the *top*. Beads are designated by asterisks. B.) Jurkat cells transiently transfected with Itk-GFP transgenes as indicated were analyzed as described in Figure 2.9B. C.) Jurkat cells transfected with Itk-GFP transgenes were incubated with anti-CD3ε (OKT3) or isotype control (UPC10) antibody-coated beads as indicated and analyzed as described in *A*. Results are displayed as the actin polymerization index calculated as described in *Materials and Methods*. Results are averages (±SEM) of seven replicate experiments per condition.

2.4.7 Itk-deficient mice are defective in TCR/CD3-mediated actin polymerization

Since this work was done in the immortalized T cell line Jurkat and the molecular defects within the cell can complicate interpretation (50, 58), similar experiments were performed in primary cells to determine if the previous observations were reproducible in mice lacking Itk. Splenocytes collected from $Itk^{-/-}$ mice and from mice of strain, age, and sex matched wild-type mice were analyzed. Cells were stimulated with anti-murine CD3ε antibodies, cross-linked with Texas Red secondary antibodies, incubated at 37°C over time, then a sample of cells were taken at various time-points to be fixed, placed on a slide and analyzed for TCR/CD3 capping. As seen in Figure 2.14, capping between wild-type mouse splenocytes and $Itk^{-/-}$ mouse splenocytes are nearly identical up to 5 minutes of stimulation, despite the low percentage of cells showing TCR capping for each cell type. After 7 minutes of stimulation, however, a large percentage of wild-type splenocytes continued to display a capping phenotype over time. This was not the case with $Itk^{-/-}$ cells, as they displayed a complete divergence from the wild-type cells, and did not show any further increase in the percentage of TCR capping cells.

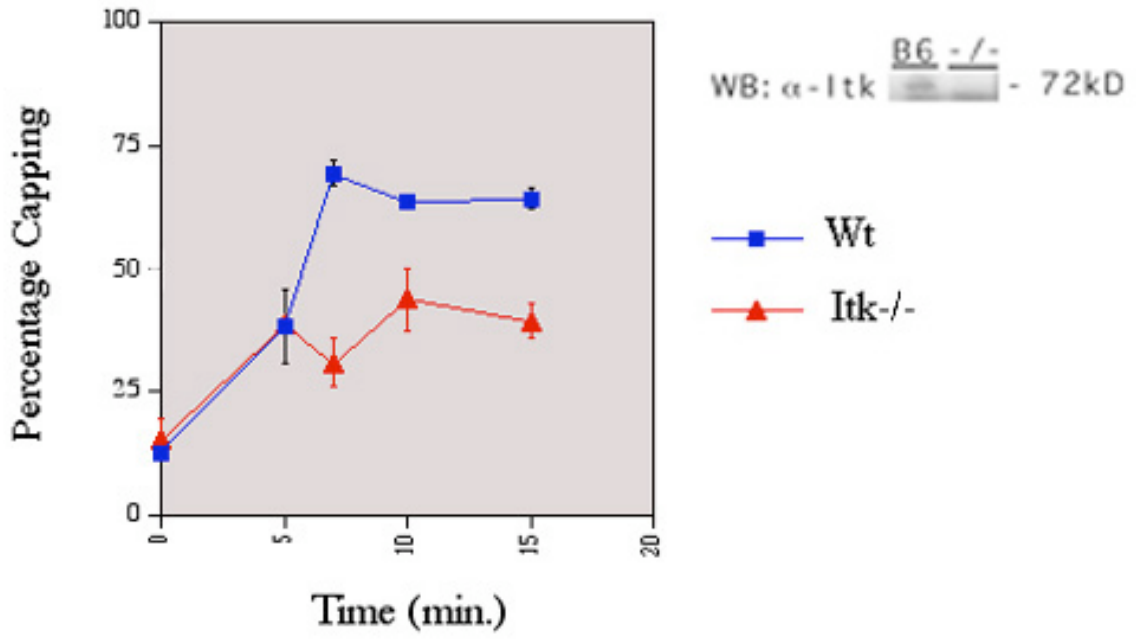

Figure 2.14. *T cells from Itk$^{-/-}$ Mice Display Deficiency in TCR-induced "cap" Formation.*

Splenic T cells were isolated from Itk$^{-/-}$ mice and from normal, strain and age matched controls. TCR/CD3 receptors were cross-linked, then stimulated at 37°C for various time points, and the percentage of cells forming caps were evaluated. Data are presented as percentage of cells displaying TCR caps observed over time (±SEM). At 7 minutes, a divergence between the two groups in their ability to form caps was seen, a phenomenon sustained with time. *Inset*, expression of Itk in both the wild-type (C57Bl/6) and Itk$^{-/-}$ splenocytes as shown with Western blotting. These data provide additional *in vivo* evidence of the involvement of Itk in cytoskeletal events and they further confirm a putative role of this kinase in the formation and continued maintenance of the immunological synapse.

It must be noted that the evaluation of capping is both tedious and subjective, since the delineation between a mature and immature cap formation is difficult to make. Thus, a more quantifiable assay was employed to determine the role of Itk in actin polymerization in primary cells. To do this, isolated splenic T cells from Itk-deficient ($Itk^{-/-}$) mice were compared to strain, age, and sex matched controls (WT). Cells were stimulated with anti-murine CD3ε antibody (2C11) followed by cross-linking with FITC-conjugated secondary antibody for various periods, then stained with phalloidin-TRITC, and were analyzed by flow cytometry. This assay quantitatively measures the total amount of actin polymerization within the cells analyzed. Within 5 minutes of stimulation, significant differences ($p \leq 0.05$, Student's t test) in TCR/CD3-induced actin polymerization were seen between T cells of $Itk^{-/-}$ and control mice (Figure 2.15). Splenic T cells from Itk-deficient animals displayed statistically significant 30-40% lower TCR/CD3-induced actin polymerization through the 5-to 30-minute time points (Figure 2.15, $p \leq 0.05$, Student t-test).

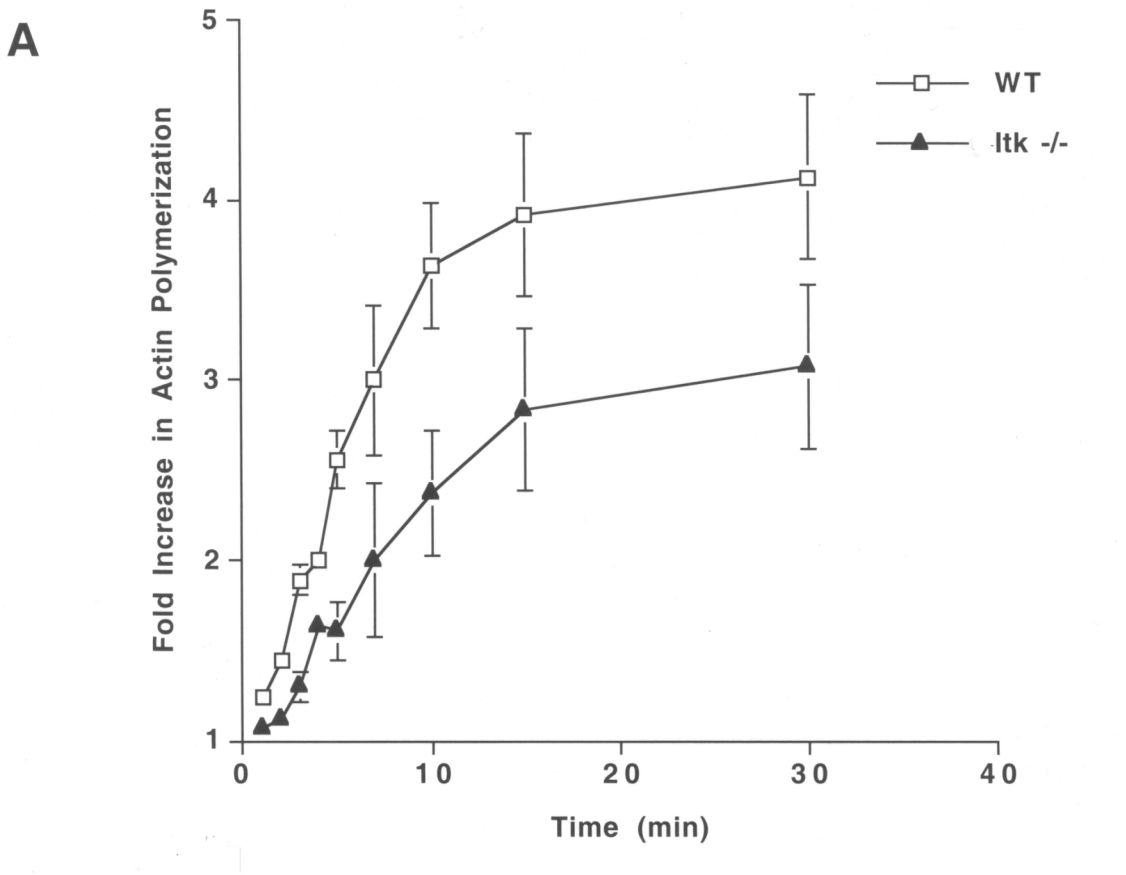

Figure 2.15. *Inducible Tyrosine Kinase Deficient Mouse Splenocytes Display Defects in Actin Polymerization.*

Preliminary evidence using cell culture Jurkat T cells indicated that Itk affected TCR-induced, actin-mediated cytoskeletal events. To confirm these results, T cells from mice lacking expression of Itk were tested for deficiencies in actin polymerization. Spleen cells from either $Itk^{-/-}$ (Δ) or control WT mice (□) were stimulated with anti-TCR antibodies followed by cross-linking with FITC-conjugated secondary antibodies for various periods as indicated. The cells were then fixed, permeabilized, and stained for polymerized actin with phalloidin-TRITC. The cells then were analyzed by flow cytometry. The results are displayed as the fold increase in actin polymerization calculated by the ratio of median fluorescence intensity at each time point as compared to the non-stimulated zero point. The results are averages (± SEM) of five replicate experiments using different mice.

Even though splenic T cells from $Itk^{-/-}$ mice display significantly lower TCR/CD3-induced actin polymerization, they are not totally devoid of it. This is most likely due to the method of measurement of total actin polymerization employed in the experiments in Figure 2.15. To further extend these experiments, the bead conjugate assay previously used was utilized again to quantify localized actin polymerization at the cell-bead interface in primary cells. In contrast to splenic T cells from WT mice that display a significant increase ($p \leq 0.05$, Student's t test) in the actin polymerization index, T cells from $Itk^{-/-}$ mice displayed no significant ($p \leq 0.05$) increase in directed actin polymerization (Figure 2.16). The increase in actin polymerization seen with T cells from WT mice is specific to TCR/CD3-induced stimulation, since incubation with beads coated with antibodies to MHC ($H\text{-}2K^{b}$) did not cause a significant increase in the actin polymerization index (data not shown). These data confirm that splenic T cells from $Itk^{-/-}$ mice have defective TCR/CD3-induced actin polymerization. The actin polymerization index increases seen with splenocytes were smaller than those observed with Jurkat cells (see Figure 2.13), probably due to the smaller cell morphology and smaller areas of contact between splenic T cells and beads.

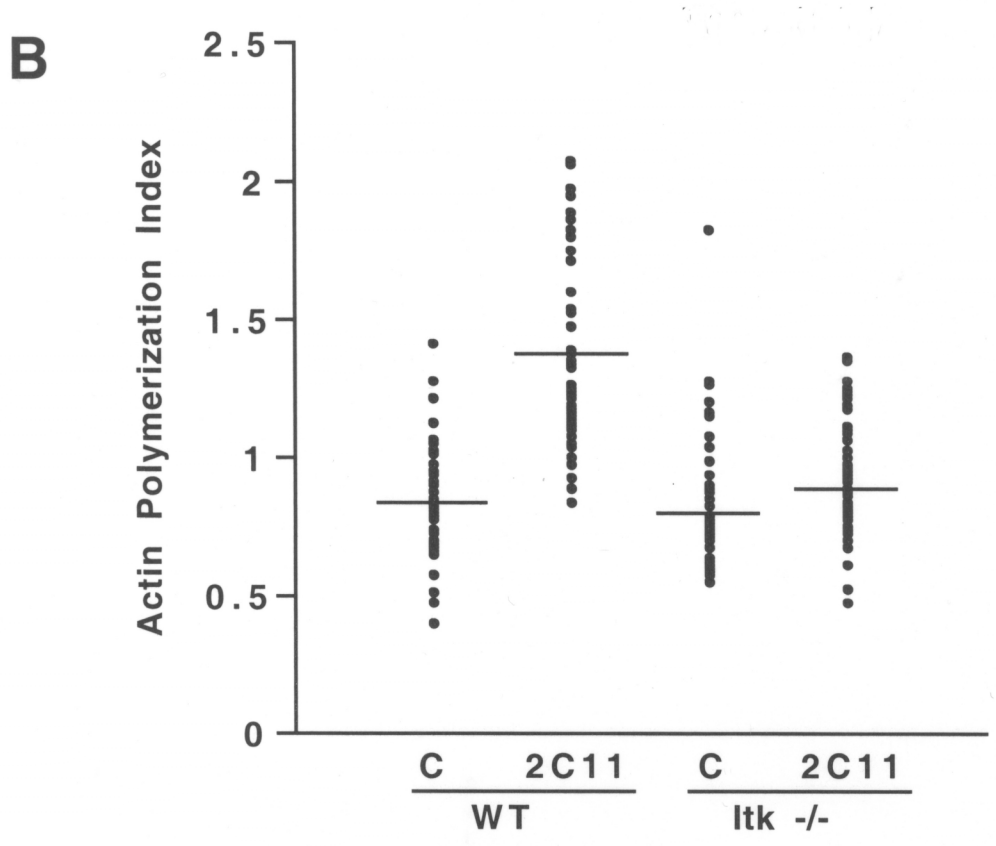

Figure 2.16. *Directed Actin Polymerization is Inhibited Within Itk$^{-/-}$ Mouse Splenocytes.*

The results displayed in Figure 2.15 indicate significantly reduced TCR-induced actin polymerization, but not an absence of actin polymerization. This is due to the measuring of total actin polymerization in the cell, whereas directed actin polymerization at the site of cellular interaction is important for the function of the T cell. Therefore, we sought to determine if Itk deficient T cells have a directed actin defect. Spleen cells from either Itk$^{-/-}$ or WT control mice were incubated with beads coated either with anti-CD3ε (2C11) or isotype control (C) antibodies. Cells were then permeabilized, stained with phalloidin-TRITC, and analyzed by laser scanning confocal microscopy. Results are cumulative of three experiments using different mice. The actin polymerization index represents the ratio of pixel intensity at the contact site to the pixel intensity of an equal area on the opposite side. The horizontal lines represent the average actin polymerization index for each group. The decrease in stimulated actin polymerization index in Itk$^{-/-}$ cells is significant in relation to the actin polymerization index in WT cells (student t-test, $p < 0.05$).

2.4.8 Itk reconstitutes TCR/CD3-induced actin-dependent events in LAT-negative cells

It has been previously demonstrated that Itk fails to become activated upon TCR/CD3 engagement of Jurkat cells deficient in the expression of LAT (56, 59). Therefore, it was reasoned that these cells could be deficient in actin-mediated events. This turned out to be the case, as LAT-deficient JCaM2.5 cells fail to extend cytoplasmic protrusions around anti-CD3ε-coated latex beads (GFP-transfected group in Figure 2.17). This defect in LAT-negative cells has been also independently demonstrated by Bunnell et al. (7), who used an anti-TCR-coated coverslip assay to assess antigen receptor-induced actin-dependent cytoskeletal events. Evidence that the lack of LAT expression is responsible for this deficiency displayed by JCaM2.5 cells is that the reconstitution with the LAT transgene renders JCaM2.5 cells capable of mediating a significant ($p \leq 0.05$, Student's t test) degree of endocytosis of anti-CD3ε-coated beads as compared with control antibody-coated beads (Figure 2.17).

To determine whether Itk could reconstitute this deficiency in LAT-negative cells, either WT-Itk or an SH3 domain mutant (W208K-Itk) were transfected into JCaM2.5 cells. The W208K mutation does not interfere with the TCR/CD3-induced transphosphorylation and enzymatic activation of Itk (Figure 2.2, Figure 2.3 and (56)), nor does it affect the inducible colocalization of this mutant with TCR/CD3 (Figure 2.6). Expression of WT-Itk partially reconstitutes the ability of JCaM2.5 cells to endocytose anti-CD3ε-coated beads (Figure 2.17). Albeit small, this increase is statistically significant ($p \leq 0.05$, Student's t test). Interestingly, expression of W208K-Itk results in significant reconstitution ($p \leq 0.05$, Student's t test) of bead endocytosis at levels

equivalent to those seen with the LAT-reconstituted cells (Figure 2.17). The increase in bead endocytosis occurs only when the cells encounter anti-CD3ε-coated beads and not when encountering isotype control antibody coated beads indicating that this increase is a specific phenomenon, requiring events induced by the engagement of the TCR/CD3 complex. The specificity of this phenomenon is indicated by the lack of bead endocytosis in JCaM2.5 cells transfected with the non-related protein GFP (Figure 2.17).

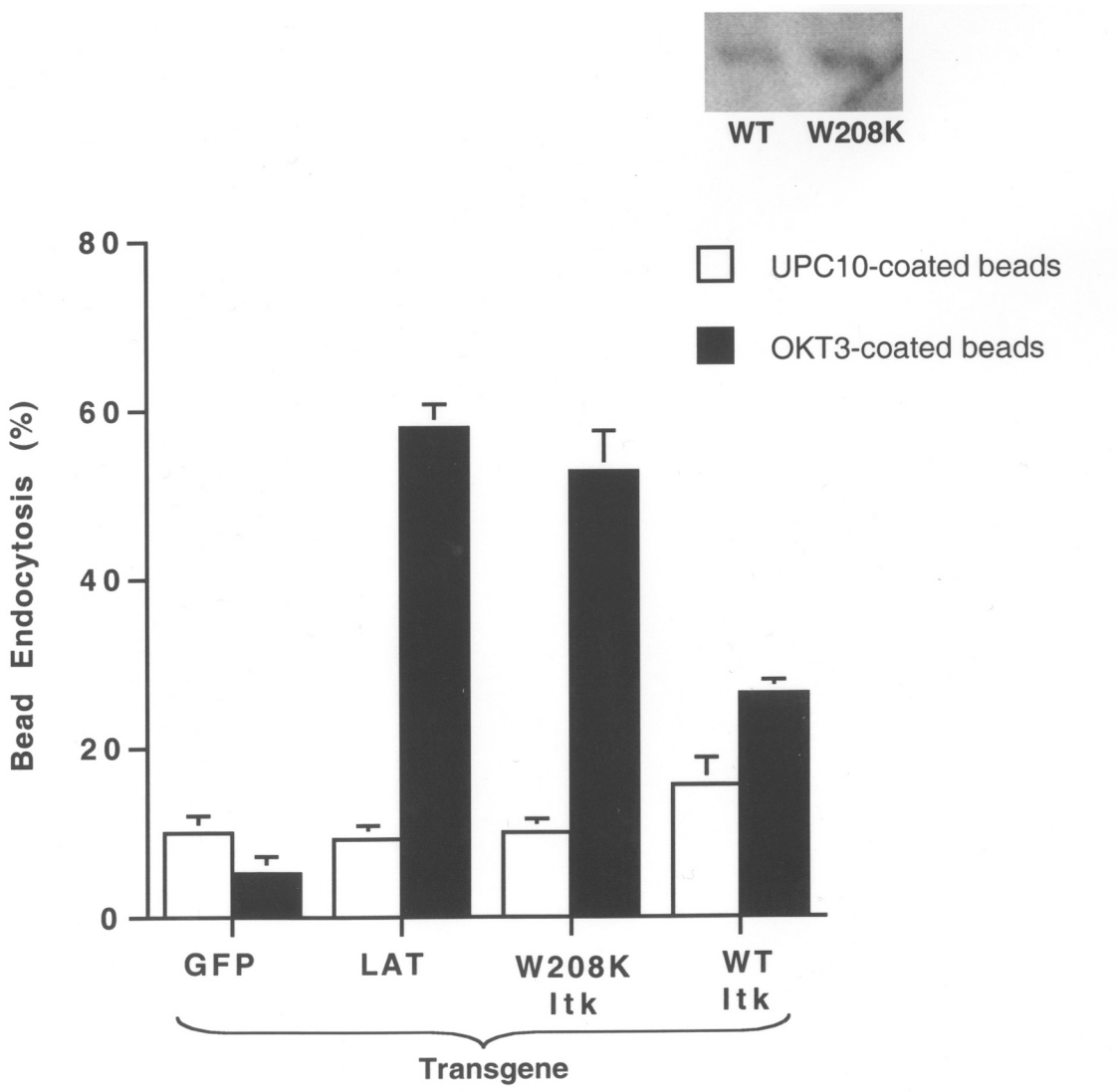

Figure 2.17. *SH3 mutants of Itk reconstitute actin polymerization-deficient Jurkat cells.*
LAT-deficient JCaM2.5 cells were transiently transfected with the indicated GFP chimeric constructs. Cells were then stimulated with either UPC10-or OKT3-coated beads as indicated and the percentages of cells extending cytoplasmic protrusions around the beads were assessed as described in the legend of Figure 2.9. Results are averages (±SEM) of four replicate experiments. *Inset,* Itk transgene expression as assessed by Itk specific immunoprecipitation and Western blotting analyses.

2.4.9 Association of Itk with Molecules Involved in Actin Polymerization

These sets of data shown thus far unequivocally support Itk involvement in the regulation of actin polymerization in the T cell upon engagement of the T cell antigen receptor. To help elucidate the mechanism behind this phenomenon, the physical interaction between Itk and that of a protein known to be involved in the regulation of actin polymerization was sought. Two such proteins reported to be intimately involved in TCR-induced actin regulation and localize to the LAT signalosome, as is similar with Itk, is the actin-nucleating factor, Wiskott-Aldrich Syndrome protein, or WASp (60-62) and the GTP exchange factor Vav1 (63-66). Both proteins have been reported to be deficient in receptor capping and both proteins are activated through TCR/CD3 stimulation.

Co-immunoprecipitation assays to determine whether Itk associates with either of these proteins were conducted, and through this method, it was found that Itk physically associates with both proteins (Figure 2.18 and Figure 2.19). In the Vav1 co-immunoprecipitation experiments, Jurkat T cells were transfected with WT-Itk, stimulated at 37°C for the times indicated (up to 30 minutes) with anti-CD3ε (OKT3) antibodies, lysed, immunoprecipitated with anti-Vav1 antibodies, then Western blotted with anti-Itk to determine co-association and with anti-Vav1 to determine loading. The observed association of Itk with Vav1 (Figure 2.18) is a non-inducible, constitutive association, which was later confirmed by the Schwartzberg lab (67). Further experiments were conducted to determine endogenous co-association between these two proteins, but were unsuccessful (data not shown).

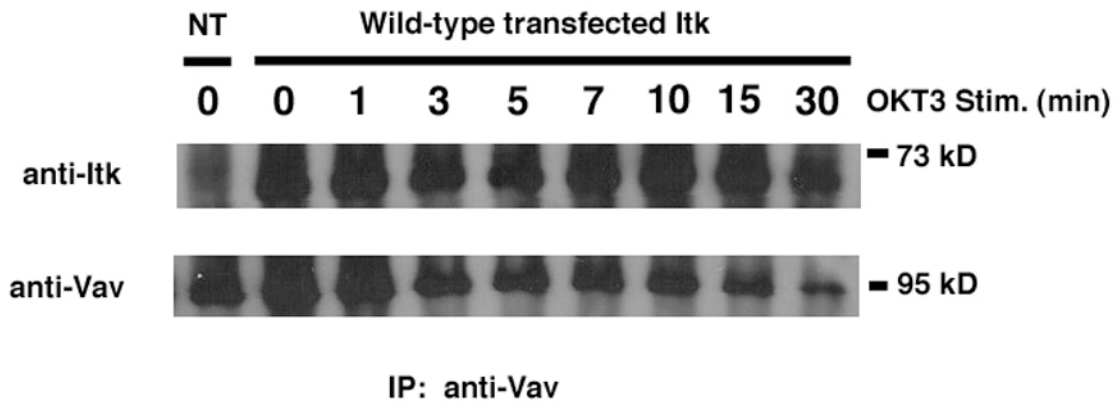

Figure 2.18. *Itk constitutively associates with Vav.*
Wild-type Itk transfected Jurkat T cells were stimulated for up to 30 minutes with anti-CD3ε, lysed, and immunoprecipitated with anti-Vav antibodies. Immunocomplexes were run on SDS-PAGE, transferred to PVDF membrane, and Western blotted with anti-Itk antibodies specific for the transfected Itk. Non-transfected Jurkat T cells were used as a control (NT).

The association of Itk with WASp, however, displayed a disparate interaction (Figure 2.19). Non-transfected Jurkat cells were stimulated with anti-CD3ε (OKT3) antibodies for various time-points (up to 30 minutes), lysed, immunoprecipitated with anti-WASp (Figure 2.19, left side) or with anti-Itk (Figure 2.19, right side) antibodies, then Western blotted with anti-Itk (Figure 2.19, top) or with anti-WASp (Figure 2.19, bottom) antibodies to determine co-immunoprecipitation. The two proteins inducibly associate early upon TCR/CD3 engagement, and then gradually decline in their association over time.

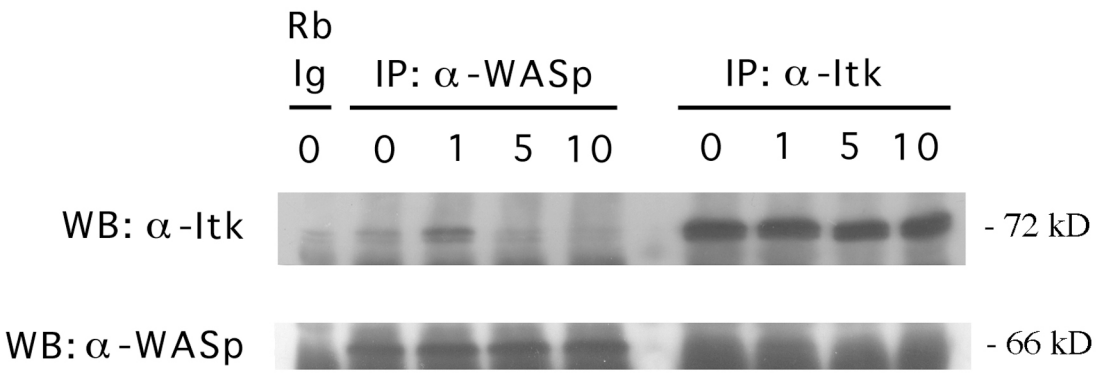

Figure 2.19. *Inducible association of endogenous WASp and Itk proteins in Jurkat T cells.*

Jurkat T cells were stimulated with anti-CD3ε for upwards of 10 minutes at 37°C, lysed, pre-cleared with normal rabbit serum, and immunoprecipitated with either anti-WASp or anti-Itk antibodies. Samples were then run on SDS-PAGE, transferred to PVDF membrane, and sequentially Western blotted with anti-Itk antibodies, then anti-WASp antibodies.

Time course kinetic stimulation studies were extended through use of wild-type transfected Jurkat T cells (Figure 2.20). These experiments revealed that Itk and WASp inducibly associate early and gradually dissociate over time (Figure 2.20, second panel). This dissociation is dependent on the tyrosine phosphorylation of both molecules (Figure 2.20, top panel and third panel), and consequently, their full activation (47, 68-70). The co-association and activation of these two molecules were controlled through the simultaneous evaluation of CD3ε phosphorylation to show that these cells were stimulated (Figure 2.20, bottom panels). Since this association supported earlier work using GST fusion proteins of Itk, the following experiments focused on the interaction between Itk and WASp (45).

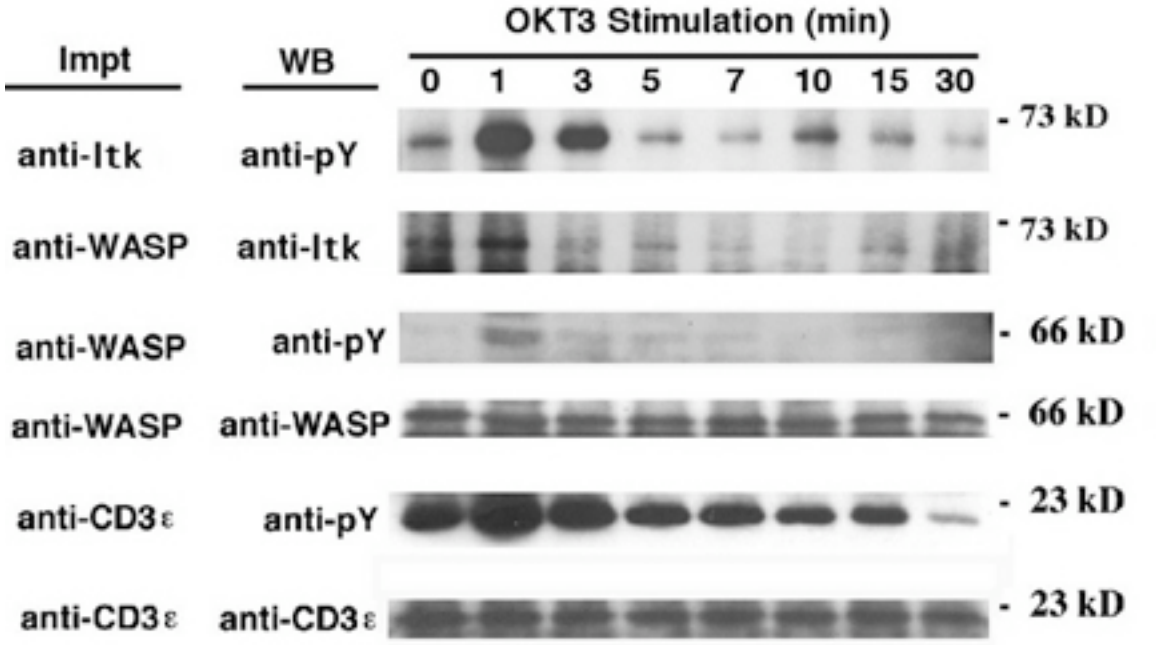

Figure 2.20. *Inducible Tyrosine Kinase Association with Wiskott-Aldrich Syndrome Protein.*

Since Itk is involved in actin reorganization, experiments were performed to determine whether Itk physically interacts with other proteins known to be important in actin polymerization events. One such protein is the actin-organizing molecule Wiskott-Aldrich Syndrome protein (WASp). To this end, Jurkat T cells were stimulated with anti-TCR antibodies for various periods as indicated (up to 30 minutes). Lysates were immunoprecipitated with anti-WASp, anti-Itk, or anti-CD3ε antibodies then analyzed by Western blotting with anti-Itk (2nd panel), anti-phospho-tyrosine (1st, 3rd, and 5th panels), anti-WASp (4th panel), or anti-CD3ε (last panel) antibodies. Results show that inducible association of Itk with WASp occurs within one minute of stimulation. Following this event, the molecules become tyrosine phosphorylated, and Itk dissociates from WASp, only to re-associate with it later in a non-phosphorylated state. The stimulation control of CD3ε is displayed in the bottom two panels. The results are representative of five individual experiments.

2.4.10 Association of WASp with mutant Itk molecules

Given the inducible association between transfected WT-Itk and WASp, it was decided to pursue which domain of Itk is responsible for this interaction and whether this correlates with the defect seen in actin polymerization. Jurkat T cells were transfected

with wild-type Itk constructs, R265K SH2 domain point mutant constructs (Figure 2.21), W208K SH3 domain point mutant or K390R SH1 domain point mutant constructs (Figure 2.22). Cells were stimulated up to 10 minutes with anti-CD3ε antibodies (OKT3), lysed, and immunoprecipitated with anti-WASp antibodies. A secondary immunoprecipitation for transfected Itk was used with anti-Itk-tag (H902) antibodies to determine equal expression levels between the different transfections. As seen in Figure 2.21, Itk association with WASp increases upon stimulation, and decreases over time, coincident with Itk phosphorylation and activation. By contrast, the SH2 domain mutant (R265K) constitutively associates with WASp, neither increasing its association with WASp when the cells become activated, nor decreasing its association at later time points. This may be due to the lack of phosphorylation and consequent activation of R265K-Itk. By comparison, the SH3 domain mutant of Itk (W208K) failed to associate with WASp, while the SH1 domain mutant (K390R) displayed an association with WASp much like that of wild-type (Figure 2.22). These latter results were expected since the Itk W208K mutation was shown to disrupt association with WASp *in vitro* (45), and the K390R Itk mutation had not shown any previous adverse effects on actin polymerization (Figure 2.9 and Figure 2.13, and (71)).

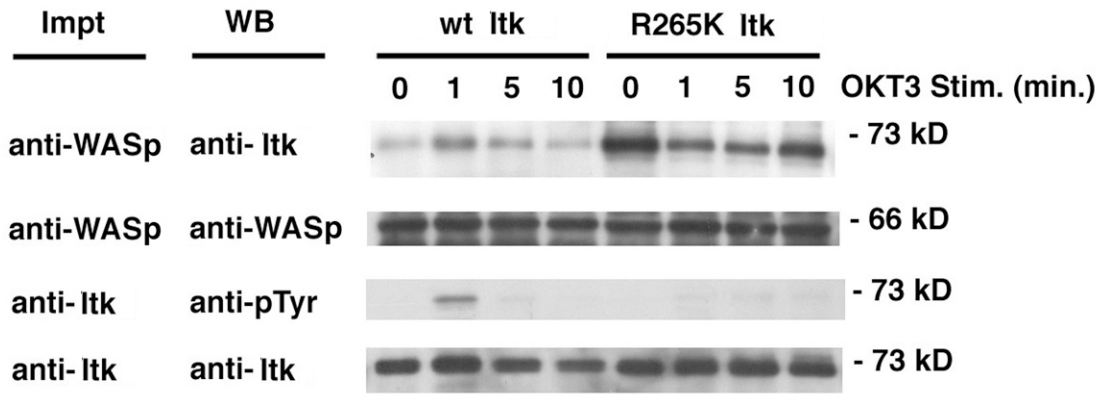

Figure 2.21. *Constitutive Association of Itk SH2 Domain Mutant with WASp.*

Jurkat T cells were transfected with either wild-type Itk or with SH2 domain mutant Itk (R265K) and stimulated with anti-CD3ε antibody for up to 10 minutes. Lysates were split into two samples, immunoprecipitated with anti-WASp (top two panels) or with anti-Itk antibodies (lower two panels) respectively. The anti-WASp immunoprecipitates were analyzed by Western blotting sequentially with anti-Itk and then with anti-WASp antibodies, while the anti-Itk immunoprecipitates were analyzed by Western blotting sequentially with anti-phospho-tyrosine and then with anti-Itk antibodies. The data show that while the wild-type Itk has a decrease in association with WASp over time, the SH2 domain mutant does not show any dissociation from WASp even after 10 minutes of cellular stimulation. This lack of dissociation between WASp and the SH2 domain mutant can be correlated to the lack of tyrosine phosphorylation of this mutant when compared to the wild-type Itk. This data suggests that Itk needs to become activated before it can dissociate from WASp.

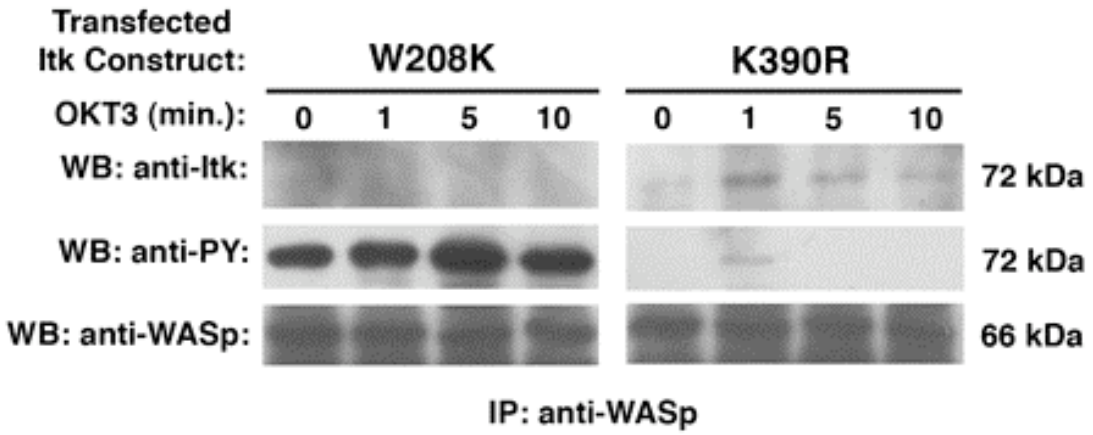

Figure 2.22. *WASp association with other Itk mutants.*
Jurkat T cells were transfected either with W208K SH3 domain mutant of Itk or with K390R SH1 domain mutant of Itk. The cells were stimulated with anti-CD3ε (OKT3) antibodies for upwards of 10 minutes. The cells were lysed, immunoprecipitated with anti-WASp antibodies, run on SDS-PAGE, transferred to PVDF, and Western blotted with anti-Itk antibodies, anti-phospho-tyrosine antibodies, and anti-WASp antibodies.

2.4.11 Itk fails to dissociate from WASp in cells deficient in LAT expression

This requirement of complete activation of both Itk and WASp being necessary for the dissociation between these two molecules is confirmed in experiments identifying the interaction between the two proteins in cells where Itk does not become activated (56, 59). LAT-deficient (JCaM2.5) Jurkat T cells were stimulated through the TCR/CD3 complex for up to 10 minutes of time. The cells were lysed, immunoprecipitated with anti-WASp antibodies, run on SDS-PAGE, transferred to PVDF membrane and Western blotted for anti-Itk. The data show no inducible association between the two molecules, nor is there any gradual dissociation between the two molecules over time (Figure 2.23). Rather, Itk and WASp seem to stay associated to the same degree in these LAT deficient cells. This supports the idea that at least Itk needs to become fully activated for the two

molecules to dissociate and go about their normal processes. It may also partially explain the deficiencies in actin dependent events in cells lacking LAT, since WASp itself may not be activated in these cells, as may remain sequestered by Itk from its role in actin reorganization.

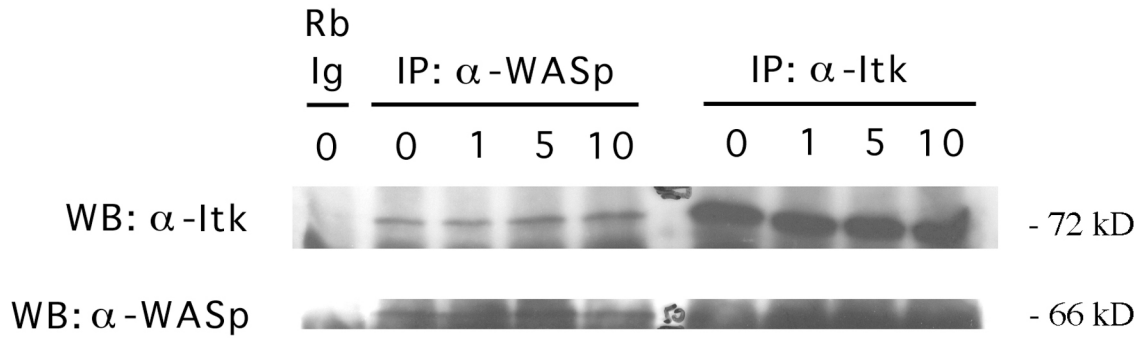

Figure 2.23. *Itk and WASp Constitutively Associate in the Absence of LAT.*
Jurkat cells deficient in LAT (JCaM2.5) were stimulated with anti-CD3ε antibodies for up to 10 minutes at 37°C. Cells were lysed, precleared with rabbit IgG, immunoprecipitated with anti-WASp or anti-Itk antibodies and Western blotted with either anti-Itk or anti-WASp antibodies. Immunoprecipitation with rabbit IgG is used as an immunoprecipitation control.

2.4.12 Using a WASp biosensor to determine activity

In a paper by Labno, et al., which confirms the finding that Itk is involved in the regulation of actin polymerization in T cells (72), the researchers extended this observation by determining that activated WASp and activated Cdc42 were localized at the immunological synapse. Conversely, they showed that these two activated molecules were not located at the IS in $Itk^{-/-}$ cells. This work was done using antibody staining for activated WASp and using a GST-GBD domain of WASp to probe for GTP bound Cdc42 in fixed cells.

In an effort to determine the localization of active WASp, the use of Förster Resonance Energy Transfer (FRET) can also be used to determine the location of these activated molecules. This method would not only allow one to confirm the Labno et al. work in fixed cells, but would also extend the work into live cells. The FRET based WASp molecule is taken from two papers using N-WASp, the ubiquitously expressed familial homolog to WASp (73, 74). Briefly, this method takes advantage of the closed conformation of N-WASp while in the autoinhibited state, and the open conformation state it takes while active. These N-WASp biosensor constructs contain a cyan fluorescent protein (CFP) at one end of the protein and yellow fluorescent protein (YFP) at the other end of the protein (one research group had CFP on the amino terminus, the other research group had YFP on the amino terminus). While in the closed-conformational state, the amino and carboxyl termini are in proximity allowing for this N-WASp biosensor to undergo FRET with the emission of CFP providing the excitation energy for the YFP. When activated, the N-WASp biosensor undergoes a change to a open conformation which separates the amino and carboxyl termini such that the molecule no longer FRETs. The amount of activated WASp at the IS can be measured by the ratiometric analysis of the amount of non-FRET fluorescent signal at the interaction site versus that at a site distal to the site of interaction. To accomplish this goal, the WASp gene was sub-cloned into the N-WASp FRET construct obtained from the Lorenz research group. This construct was transfected into COS-7 cells, to determine CFP and YFP expression and to similarly reproduce the FRET work done with N-WASp constructs in the previously reported research (Figure 2.24, panels B and C). FRET, as exhibited by the transfer of light from CFP molecules excited at 433 nm and light emitted

at 527 nm by YFP molecules, is displayed in Figure 2.24 panel D, indicating a plausible transfer of light photons from one CFP molecule to a juxtaposed YFP molecule. This transfer of light can be quantitatively evaluated by calculating efficiency FRET (E-FRET) using the amount of light emitted from each channel (CFP_{ex}-CFP_{em}, YFP_{ex}-YFP_{em}, CFP_{ex}-YFP_{em}) and calculating the efficiency of photons transferred. As seen in Figure 2.24 panel E, FRET efficiency can be visualized by pseudocoloring the areas of high levels of E-FRET with violet, to areas of blues and yellows where E-FRET is less intense. The areas of higher E-FRET indicate the higher presence of WASp molecules in the closed conformational state. Overall, these cells present a large amount of WASp molecules in the FRET conformation, indicating that most of the WASp molecules are in some autoinhibited state.

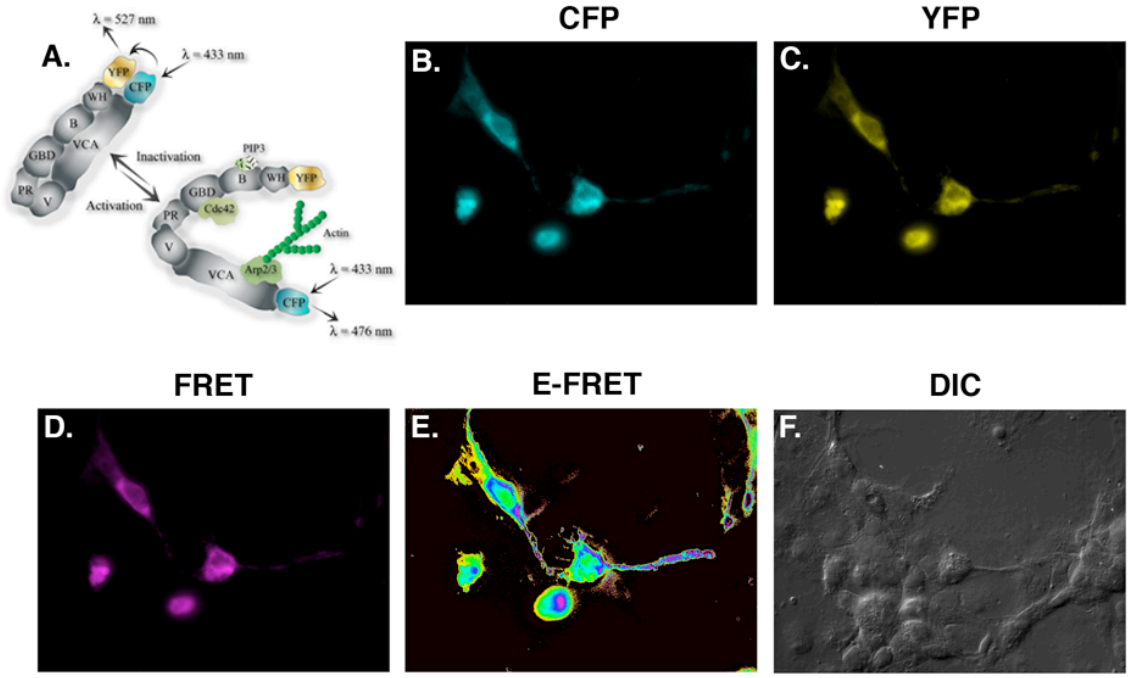

Figure 2.24. *Inactive Wiskott-Aldrich Syndrome Protein Biosensor.*

To determine the functional significance of Itk's association with WASp, a WASp biosensor was generated to determine the activity of this protein. Using a Förster Resonance Energy Transfer (FRET) based reporter, one can determine the activation state of WASp. A.) Diagram of the WASp FRET protein. In its inactive state, WASp folding allows for the juxtaposition of the N-terminal YFP and the C-terminal CFP, leading to FRET fluorescence (YFP emission at 527 nm when CFP is excited at 433 nm). B.) WASp biosensor transfected COS-7 cells as visualized with CFP excitation and CFP emission. C.) WASp biosensor transfected COS-7 cells as visualized with YFP excitation and YFP emission. D.) WASp biosensor transfected COS-7 cells as visualized with CFP excitation and YFP emission. E.) FRET efficiency index as represented by intensity coloring. Black background displays zero FRET efficiency, while a scale from yellow to blue to violet displays an increase in FRET efficiency. F.) Differential interference contrast of COS-7 cells. Panels B-E display representative example of WASp biosensor FRET occurrence indicating the position of inactive WASp molecules within the cell.

To determine if the WASp FRET molecule could respond to stimulus, COS-7 cells were again transfected with the WASp FRET construct and cells were stimulated with epidermal growth factor (EGF) for 1 minute prior to fixation as reported (74). EGF is a growth factor known to activate many signal transduction pathways, including the activation of GEF proteins leading to WASp activity (75). As shown in Figure 2.25, one again can observe the expression of CFP (panel B), YFP (panel C), and FRET (panel D). With the addition of EGF as a stimulus, E-FRET, by comparison to non-stimulated cells, shows a striking difference for molecules within the cells (panel E). The very little E-FRET generated upon the addition of EGF stimulus indicated that the WASp FRET molecules within these cells are not effectively transferring energy, leading one to conclude that the molecule is in an open conformation, active state. This indicates that this FRET biosensor construct is responsive to stimulus and can be used in further more quantitative studies.

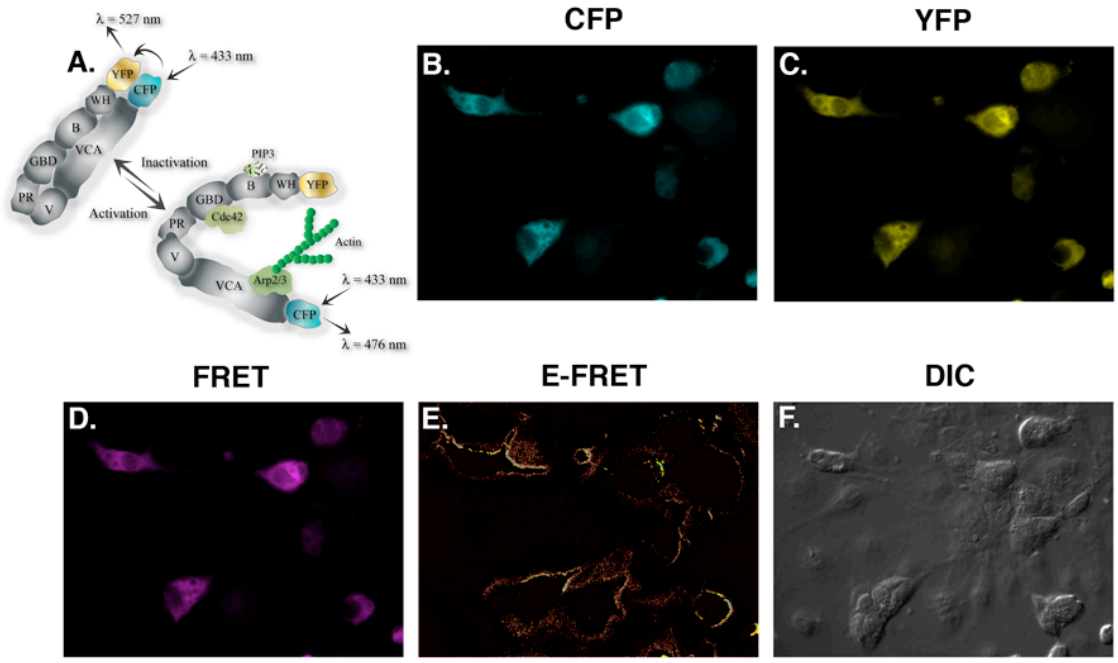

Figure 2.25. *Active Wiskott-Aldrich Syndrome Protein Biosensor.*

Visualization of regulated WASp activity *in vivo*. A.) Diagram of the WASp FRET protein. Upon activation of the WASp biosensor, the unfolding of WASp leads to an increase in the distance between the N-terminal YFP and the C-terminal CFP, which in turns leads to low levels of FRET (emission at 476 nm when excited at 433 nm). B.) WASp biosensor transfected COS-7 cells as visualized with CFP excitation and CFP emission. C.) WASp biosensor transfected COS-7 cells as visualized with YFP excitation and YFP emission. D.) WASp biosensor transfected COS-7 cells as visualized with CFP excitation and YFP emission. E.) FRET efficiency index as represented by intensity coloring. Red background displays zero FRET efficiency, while a scale from yellow to blue to violet displays an increase in FRET efficiency. F.) Differential interference contrast of COS-7 cells. Panels B-E display representative example of lack of WASp biosensor FRET occurrence upon stimulation of the cells with EGF for 1 minute.

After determining that the WASp FRET construct could respond to stimulus, it was stably transfected into Jurkat cells. Stable WASp FRET transfectants were selected for the cells expressing the Neo^r gene through growth in the presence of neomycin antibiotics. After selection, the cells expressing the FRET construct were further selected by Fluorescently Activated Cell Sorting (FACS) (Figure 2.26A). This method allowed for the sequential selection of YFP-positive expressing cells through primary and secondary sorts, to not only obtain a higher percentage of cells expressing YFP (Figure 2.26B), but to also obtain cells that were expressing the construct at higher expression levels (Figure 2.26A, right panel, and Figure 2.26C). Western blotting for WASp also showed an increase in WASp expression, particularly at the WASp FRET size of 117 kDa (Figure 2.26C), which also indicates that the molecule is being fully expressed. Finally, confirmation of WASp biosensor protein expression by confocal microscopy shows YFP expression in this selected cell line appears to be ubiquitous (Figure 2.26D). The multiple WASp FRET positive cell lines generated through this (extensive) selection method were maintained and used by other lab research members to further explore Itk involvement in the activation of WASp. In turn, other projects on Itk protein associations and role in T cell activation were pursued.

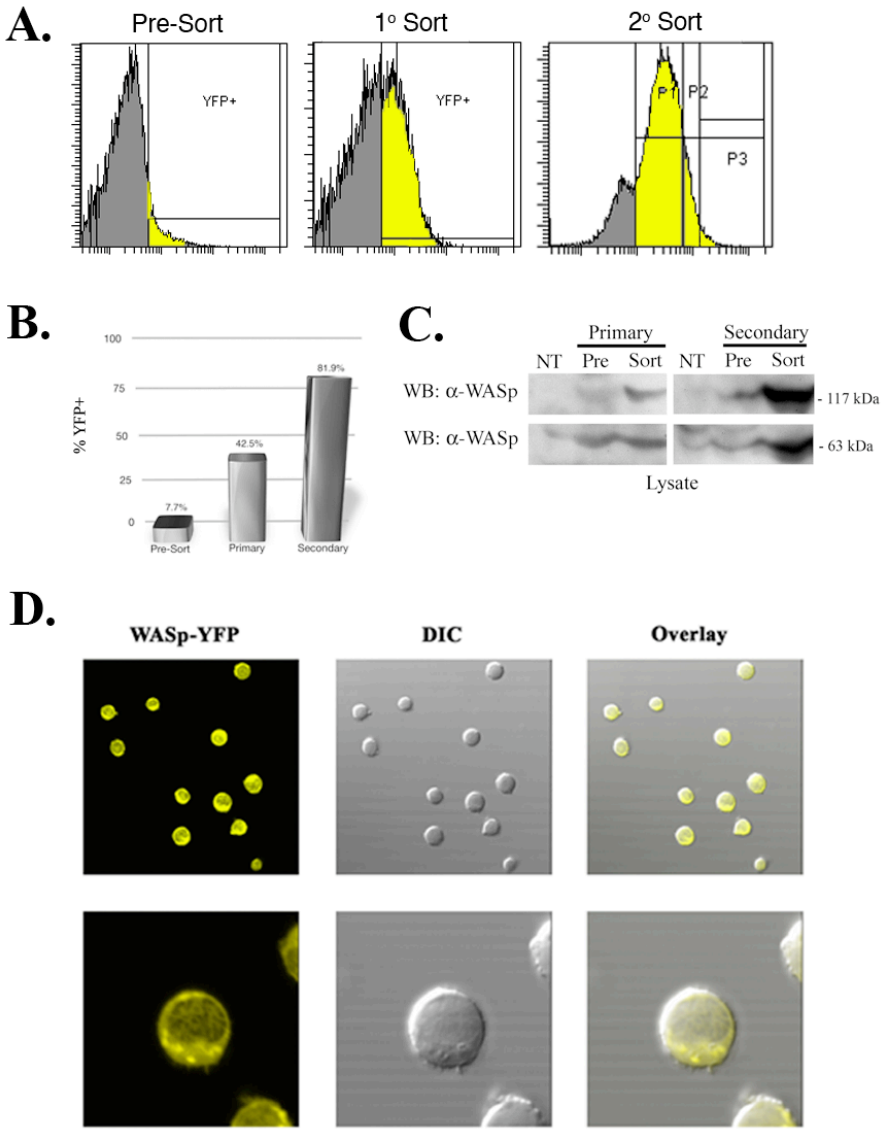

Figure 2.26. *Selecting for a high expressing WASp FRET Jurkat cell line.*

A.) Cell sorting and selection of high YFP expression in WASp FRET positive Jurkat transfectants by Fluorescently Activated Cell Sorting. B.) Graph representing the percentage of YFP positive cells obtained at each sorting activity. C.) WASp FRET molecule expression from each sort as represented by Western blotting. D.) Confocal microscope images of stable, sorted WASp FRET molecules as visualized through the YFP emission channel.

2.4.13 Itk fails to associate with WASp in the absence of SLP-76

In a continuing effort to determine how Itk and WASp associate, and to clarify conflicting or confusing evidence in the literature as to whether Itk SH3 domain binds to WASp or SLP-76 preferentially (45, 76), Itk association with WASp in the absence of SLP-76 was investigated. The previously reported work on Itk association with these molecules used exogenous GST-fusion proteins of Itk SH3 domain using *in vitro* settings. In contrast, the method employed here uses whole, endogenous molecules in stimulated cells. Briefly, JTAg T cells or Jurkat T cells deficient in the expression of SLP-76 (J14) were stimulated with anti-CD3ε antibodies for up to 10 minutes prior to lysis. Lysates were immunoprecipitated for WASp or Itk, resolved on SDS-PAGE, transferred to PVDF membrane, then probed (Western blotted) for Itk or WASp. As shown in Figure 2.27, Itk initially associates with WASp, with only a minimal increase upon stimulation (right side). This near constitutive association between Itk and WASp in JTAg T cells is unique to this clone of T cells, as another Jurkat T cell clonal line (E6.1) did not display nearly as much co-immunoprecipitation prior to stimulation (Figure 2.19). By stark contrast, cells lacking the cytoplasmic adaptor protein SLP-76 (J14 T cells) yielded no assocaiton between Itk and WASp at all, despite the presence of both Itk and WASp (Figure 2.27, left side). This was not due to a delay in kinetics of the protein-protein association between these two molecules, since cellular stimulation up to 10 minutes still displayed no association. From this, one can conclude that the association between Itk and WASp is indirect, presumably occurring through SLP-76.

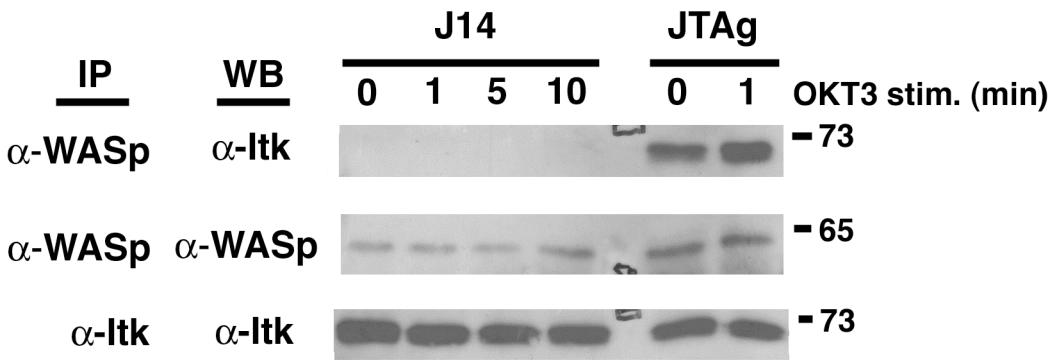

Figure 2.27. *Itk and WASp Association is Absent in Jurkat Cells Deficient in SLP-76.* Jurkat cells, either JTAg containing SLP-76 or J14 deficient for SLP-76, were stimulated with anti-CD3ε (OKT3) antibodies for the times indicated. Cells were lysed, immunoprecipitated with either anti-WASp or anti-Itk antibodies, run on SDS-PAGE, transferred to PVDF membrane, and Western blotted with either anti-WASp or anti-Itk antibodies. Results are representative of four individual experiments.

To confirm this finding, reconstitution of J14 cells with SLP-76 should restore the interaction between Itk and WASp, as SLP-76 would now serve as the bridge linking these two molecules in proximity. Using SLP-76 deficient Jurkat T cells reconstituted with SLP-76-GFP, and selected for GFP expression, cells were stimulated for up to 10 minutes with anti-CD3ε antibodies, immunoprecipitated with anti-WASp antibodies, run on SDS-PAGE, transferred to PVDF, and probed for Itk or WASp through Western blotting. Upon stimulation of the SLP-76-GFP J14 reconstituted cells, Itk and WASp resumed their transient association (Figure 2.28, right side), while this association is absent within the same time frame in stimulated non-reconstituted SLP-76-deficient cells (Figure 2.28, left side). This provides confirmative evidence that Itk and WASp need SLP-76 present to indirectly associate.

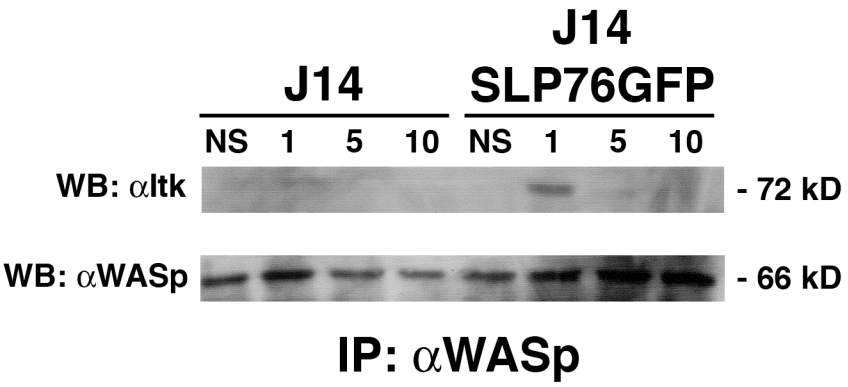

Figure 2.28. *Itk/WASp Association is Restored When SLP-76 is Reconstituted into SLP-76-Deficient Cells.*

SLP-76-deficient Jurkat T cells J14 and a stable cell line of SLP-76-GFP reconstituted J14 generated by the laboratory were stimulated with anti-CD3ε (OKT3) antibodies for the times indicated or with isotype control (UPC10) antibodies (NS). Cells were lysed, immunoprecipitated with anti-WASp antibodies, samples were resolved on SDS-PAGE, transferred to PVDF membrane, and Western blotted with either anti-WASp or anti-Itk antibodies. Results are representative of four individual experiments.

Table 2.1. *Structural defects of Itk leading to functional T cell outcomes.*

Cumulative table of experiments performed using Itk mutants or the effects on Itk in mutant T cell lines. The comparative functions are listed in the first column, followed by how wild-type Itk functions for each of these processes. Differences observed with the mutant Itk constructs or with the mutant T cell lines are highlighted in red. C.A. indicates "constitutively active", while n.d. indicates "not determined".

Structure-Function Chart

	Wild-Type Itk	PH* Itk (ΔPH)	TH* Itk (ΔPR)	SH3* Itk (W208K)	SH2* Itk (R265K)	SH1* Itk (K390R)	Endo-Itk (Lck$^{-/-}$)	Endo-Itk (LAT$^{-/-}$)	Endo-Itk (SLP-76$^{-/-}$)
Trans - PY	Y	Y	n.d.	Y	N	Y	N	N	N
Auto - PY	Y	C.A.	n.d.	C.A.	N	N	N	N	N
Co-Local w/TCR	Y	N	n.d.	Y	N	Y	N	N	N
Bead Endocyt.	Y	n.d.	n.d.	Y	N	Y	N	N	N
Actin Polym.	Y	n.d.	n.d.	Y	N	Y	N	N	N
Assoc. w/ WASp	Y	n.d.	n.d.	N	Y	Y	n.d.	Y	N

2.5 Discussion

The series of investigations presented here aimed to elucidate the requirements necessary for the activation of the T cell receptor proximal kinase, Itk. In particular, an effort was made to identify the domain(s) within Itk necessary for its activation, its localization to the TCR/CD3 complex, and determine whether any of these domains play a role in downstream events in which Itk may be involved. Characterization of these domains required mutating these protein regions either through deletion or critical point mutation. Through these mutational studies, it was identified that Itk plays a pivotal role in actin polymerization initiated through the T cell receptor. The mechanism for this role was of great importance, though the direct involvement was not elucidated.

Mutational analysis of the SH2 and SH3 domains of Itk led to some interesting results that affect Itk activation. Itk SH2 domain mutations affecting its ability to bind phosphorylated tyrosines (either through complete ablation of the domain or through pinpoint disruption of a critical residue) consequently led to the protein's inability to become tyrosine phosphorylated and fully activated. Further, SH2 domain mutation led to very real defects in Itk localization to the TCR/CD3 complex, complicating this observation more by preventing the coalescing of these complexes. Conversely, Itk SH3 domain mutations affecting its ability to bind polyproline rich regions (either within its own Tec Homology domain or with other proteins) led the protein to become phosphorylated and activated. Additionally, these SH3 domain mutations did little to affect Itk localization to TCR/CD3 complex. Initially, this would indicate that the SH2

domain of Itk is of vital importance in the activation of this molecule, while the SH3 domain would be considered dispensable.

Moreover, the above data suggests that Itk plays an important role in TCR/CD3-induced, actin-dependent cytoskeletal events. This has been demonstrated using both the immortalized Jurkat cell system and primary T cells from $Itk^{-/-}$ mice. Using a previously published conjugate formation assay (55) where Jurkat cells are stimulated with polystyrene beads coated with anti-CD3ε antibodies, actin-mediated events are measured either by the extension of cytoplasmic protrusions around the beads or by quantification of actin polymerization at the cell-bead interface (Figure 2.8 and Figure 2.11). The overexpression of a SH2 domain Itk mutant (R265K) fails to become activated upon antigen receptor engagement and inhibits actin-mediated events (Figure 2.9, Figure 2.10, Figure 2.12, and Figure 2.13; and (56)). The analogous mutation at the SH2 domain of another Tec family kinase, Bruton's tyrosine kinase (Btk), disrupts the ability of this kinase to interact with its cellular targets (44). Furthermore, mutations in this position of Btk have been found in patients with X-linked agammaglobulinaemia, suggesting the functional importance of this residue and domain (77, 78). The lack of TCR/CD3-induced activation of R265K-Itk is probably attributable to its inability to interact with LAT, as this adaptor protein is known to be critical for the activation of Itk (56, 59).

For its activation, Itk undergoes two tyrosine phosphorylation events; transphosphorylation by the Src family kinase Lck followed by autophosphorylation (47, 70). Furthermore, Itk needs to localize to the inducible LAT signalosome, which places Itk in proximity to Lck for its activation. This localization plays an additional function, as the signaling complex formed on the LAT platform contains proteins that play critical

roles in the reorganization and polymerization of the actin cytoskeleton. Among these are SLP-76, Nck, Wiskott-Aldrich Syndrome protein (WASp), Vav1 and ADAP, among others (25, 32). Most notably, WASp, through its interaction with the Arp2/3 complex, plays a very important role in actin polymerization and patients carrying mutations in the WASp coding gene display severe immunodeficiency characterized by recurrent infections and hematopoietic malignancies (61, 79-81). Previously published data indicate that Itk, WASp, and Arp2/3 colocalize with TCR/CD3 upon stimulation (72). Furthermore, Bunnell et al. have provided *in vitro* evidence that Itk interacts with several of the members of the actin polymerization machinery including WASp (45) and a cooperative association with SLP-76 through Itk's SH2 and SH3 domains (76). In the above studies, an inducible association between Itk and WASP is shown (Figure 2.19 and Figure 2.20). This association indicated a dependency of both the SH2 and the SH3 domains of Itk, as R265K-Itk displayed a constitutive association with WASp, while W208K-Itk failed to associate with WASp. This would support the cooperative binding scenario seen with Itk and SLP-76, with the SH3 domain initially being pivotal for the interaction, while the SH2 domain being of secondary importance. If the SH3 domain of Itk is rendered non-functional, it can no longer associate with WASp, and if the SH2 domain is non-functional, it could initially associate with WASp through its SH3 domain, but could not release WASp (as seen with wild-type Itk or with kinase-inactive SH1 domain mutant Itk) once it has associated with its phosphorylated target. Of course, this scenario would also confirm the *in vitro* data seen for a cooperative association between Itk and SLP-76 (76). More likely, this is the scenario of interaction between Itk and

WASp, through the intermediary adaptor molecule SLP-76 (as evidenced in Figure 2.27 and Figure 2.28).

Even if the association between Itk and WASp is shown to be indirect, Itk can still have a role in WASp's activation. Intriguingly, Labno et al. found no WASp activation in T cells from $Itk^{-/-}$ mice upon stimulation through their antigen receptors when assessed with an antibody that can distinguish between active and inactive WASp (72). These observations are consistent with the data presented here in which T cells from $Itk^{-/-}$ mice display significantly reduced TCR/CD3-mediated actin polymerization (Figure 2.14-Figure 2.16). The construction and characterization of a WASp FRET construct was intended to be utilized as a biosensor that could identify areas of WASp activity within the cell. Initially, fixed cells were used to reproduce the observation seen by Labno, et al., in large part because of the ease in observing static cellular images versus that of live and motile T cell conjugates. The subsequent work using live cells extends the work already published, with the construct shown to be responsive to stimulus in non-hematopoietic cells (Figure 2.24 and Figure 2.25), and was stably selected for high expressing T cells using FACS (Figure 2.26). This was done with the intention of using the FRET based WASp biosensor to observe how WASp becomes activated in the presence of various mutants of Itk. This would extend the biochemistry of protein-protein interactions between Itk and WASp into the realm of real-time *in vivo* interactions. After determining the biochemistry of the interaction between these two molecules, one can observe the localization of activated WASp in the context of mutant Itk and make the connection of how the structure of Itk affects the function of WASp. Unfortunately, this was not the case. After transferring the selected stable cell lines to

other lab members, it was discovered that the cell lines lacked CFP expression the longer they were kept in culture. This was probably due to homologous recombination between the very similar genetic structures of CFP and YFP. Since FACS selection had focused on YFP expression, and although the size of the full length molecule was revealed by Western blotting (Figure 2.26), recombination of the genes under restriction *in vivo* probably led to the demise of CFP expression, rendering the cell lines irrelevant.

In view of the above observations, a dominant-negative mechanism could account for the inhibitory properties of R265K-Itk. This SH2 domain mutant could compete with endogenous WT-Itk for binding to a signaling complex, such as the one assembled by phosphorylated LAT or phosphorylated SLP-76. Thus, the R265K-Itk mutant could inhibit the formation of this complex or subsequent signaling events critical to TCR/CD3-induced actin polymerization in a dominant-negative fashion.

The Lck-mediated transphosphorylation of Itk as well as its subsequent autophosphorylation play key roles in the activation of this kinase (47, 70). However, additional observations have suggested that through its various domains Itk may interact with other signal transducers and become conformationally altered and activated. In particular, the analysis of a fragment of Itk by multidimensional nuclear magnetic resonance reveals an intramolecular association between the SH3 domain and a proline-rich sequence located within the TH domain of Itk (82). The authors of this study suggested that this association might regulate the interaction of Itk with its targets/substrates and its subsequent activation. Thus, the ability of the SH3 domain mutants of Itk to reconstitute TCR/CD3-induced actin-dependent events in LAT-deficient cells might be through the disruption of the SH3-polyproline interaction and the

conformational alteration of Itk into an active state. However, in this scenario the participation of the SH3 mutant Itk in actin-mediated events still requires the engagement of the TCR/CD3 for its recruitment to relevant sites (Figure 2.17). It is interesting that comparison of GFP-tagged W208K-and R265K-Itk mutants by confocal microscopy reveals the ability of the former, but not the latter mutant to co-localize with the antigen receptor complex (Figure 2.6). Furthermore, unlike R265K, the W208K mutant can be inducibly transphosphorylated and enzymatically activated (Figure 2.2, Figure 2.3 and (56)).

Since Itk contains an SH1 domain, its involvement in TCR/CD3-induced actin-dependent events would primarily focus on its enzymatic activity. Lack of catalytic activity could be important in the mechanism through which the SH2 mutant of Itk, R265K, inhibits actin-dependent events since this mutant is enzymatically inactive (Figure 2.3). Thus, if enzymatic activity were important, it would be reasonable to expect that overexpression of the kinase-dead mutant of Itk (K390R) would behave similar to the R265K, namely, K390R-Itk would inhibit actin-dependent events. However, this does not appear to be the case (Figure 2.9, Figure 2.10, Figure 2.12, and Figure 2.13). The inability of the kinase-dead mutant to inhibit actin-dependent events is in agreement with the observations of Donnadieu et al. who used a similar SH1 mutant of Itk and found it to have no effect on antigen receptor-induced cytoskeletal events (71). Thus, it is possible that either the kinase activity of Itk is not important for the regulation of actin-dependent events or inactivation by itself may not be sufficient to affect actin polymerization. Several investigators have recently reported the effects mediated by different protein kinases in a manner independent of their enzymatic activity. Examples

include the IFN-inducible protein kinase PKR that can induce NF-κB activation through a mechanism independent of its kinase activity (83), and the p210 BCR-ABL tyrosine kinase that can modulate cell-extracellular matrix interactions independent of its catalytic activity (84). Interestingly, however, Woods et al. have reported that a similar kinase-dead mutant of Itk can inhibit TCR/CD3-induced β1-integrin-mediated adhesion to the extracellular matrix when transfected in Jurkat cells (85). This interesting finding raises the possibility that Itk may behave differently in "inside-out" signaling pathways.

In conclusion, the evidence presented here strongly supports an important role for Itk in TCR/CD3-induced cytoskeletal events, perhaps in a kinase-independent fashion. Since Itk is known to regulate TCR/CD3-induced intracellular Ca^{2+} mobilization through its ability to phosphorylate phospholipase Cγ1 (86-89), the present data support the hypothesis that Itk is an important link between TCR/CD3-mediated Ca^{2+} mobilization through its enzymatic activity and cytoskeletal reorganization through its interaction with WASp via SLP-76 protein (90).

Portions of Chapter 2 were published as: Grasis, J.A., C. D. Browne, and C. D. Tsoukas. (2003) Inducible T cell tyrosine kinase regulates actin-dependent cytoskeletal events induced by the T cell antigen receptor. J. *Immunol.* 170:3971-3976.

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3 IP_4 Guides the Localization of Itk to the Immunological Synapse

3.1 Abstract

Production of the second messengers inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG) by PLCγ1 plays a paramount role in signaling from the T cell receptor. The phosphorylation of IP_3 to inositol (1,3,4,5)-tetrakisphosphate (IP_4) by inositol (1,4,5)-trisphosphate 3-kinase B (IP 3-kinase B, ItpkB) generates another inositol-polyphosphate with a novel signaling function. Previous studies have shown that IP_4 acts as a soluble ligand for the pleckstrin homology (PH) domain of the Tec kinase Itk. The following studies show that ItpkB regulates Itk recruitment, thereby enabling its full activation and that of its target PLCγ1 and subsequent downstream effectors, including the activation of Erk.

3.2 Introduction

T lymphocytes are critical components of the adaptive immune system, protecting us from such maladies as infections and cancer. However, the mechanisms necessary for this response to take place are very well balanced to not only take on the myriad of potential disease states the immune system may encounter, but to also prevent an unnecessary response against self. As such, T cells undergo an exhaustive instructive period within the thymus to emerge as diligent caretakers and protectors of the body.

T cells recognize foreign antigen via interactions between the T cell receptor (TCR) and antigenic peptides presented by MHC proteins (pMHC) on an antigen presenting cell (APC). Variations in the context, strength and duration of TCR signals direct distinct outcomes during peripheral T cell activation and T cell development in the thymus. During development, thymocytes progress through a series of stages distinguishable by CD4, CD8, CD25, and CD44 surface expression (1). The different developmental stages encountered by developing T cells can be distinguished based on the expression of cell surface marker proteins that are dynamically up- or down-regulated as development proceeds. From early precursors, thymocytes first develop through several substages (DN1-DN4) of the $CD4^-CD8^-$ double-negative (DN) stage, distinguished via surface expression of CD25 and CD44. At the DN3 stage, cells express a pre-TCR composed of a pre-TCRα and a mature TCRβ subunit. Pre-TCR surface expression triggers upregulation of CD4 and CD8 on the cell surface and progression into the $CD4^+CD8^+$ double positive (DP) stage. DP thymocytes express a mature TCR composed of a TCRα and a TCRβ subunit. At the DP stage, selection processes

eliminate non-functional or potentially auto-reactive cells through death by neglect or by negative selection. This is lightheartedly referred to as the Goldilocks theory of thymic selection. When a DP T cell undergoes TCR engagement with pMHC presented by an APC from the thymic epithelia that creates a weak (or no) signal, the T cell undergoes death by neglect. If it produces too strong a response, or strongly auto-reactive to the self-epitope presented by the thymic APC, the T cell is negatively selected. Only T cells whose TCR generates appropriately strong signals are positively selected to survive. This leads to the down-regulation of one of the two marker proteins and maturation into either $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells. The former are key regulators of the immune system, and the latter are involved in the direct killing of pathogen-infected cells. Thymic selection warrants that only T cells with desired functions populate the body. Thus, thymic selection at the DP stage is critical for the development of a normal T cell repertoire. Defects in thymic selection yield malfunctioning T cells or T cells with undesired functions to emerge. This can lead to a variety of severe immune disorders.

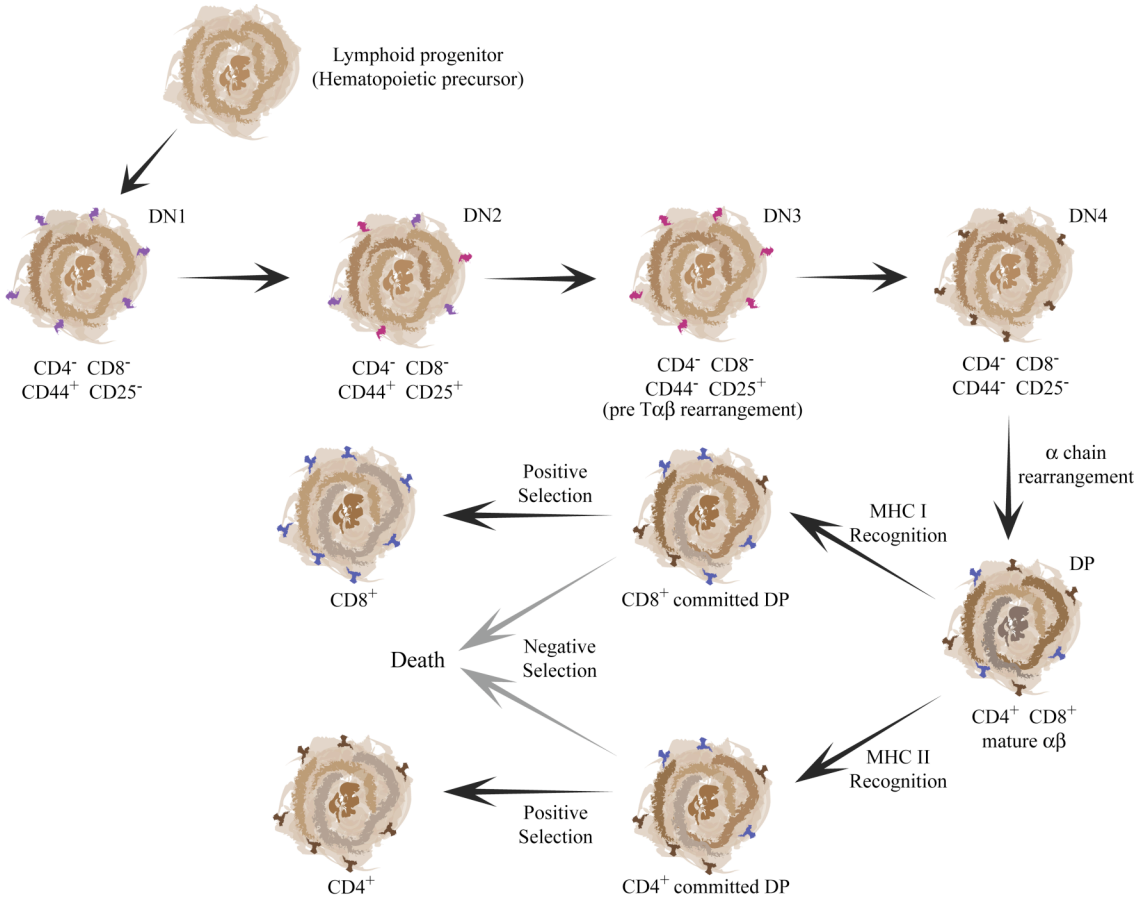

Figure 3.1. *Thymic T cell development.*

Cartoon diagram of the development of a T cell from a bone marrow derived hematopoietic progenitor to that of a mature $CD4^+$ or $CD8^+$ T cell capable of mounting an immune response in the periphery.

TCR engagement triggers the activation of intracellular kinases including the lipid kinase phosphatidylinositol 3-kinase (PI_3K) and the Tec family protein kinase, interleukin-2 inducible kinase (Itk). Itk activation is dependent on the activity of PI_3K, since the non-covalent tethering of Itk to the plasma membrane is via the binding of its pleckstrin homology (PH) domain to PI_3K generated membrane phosphatidyl inositol (3,4,5) triphosphate (PIP_3). Itk activation leads to the phosphorylation of phospholipase C-gamma-1 (PLCγ1), which mediates the catabolism of phosphatidyl inositol (4,5)

bisphosphate (PIP_2) into equimolar amounts of secondary messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binding to IP_3 receptors (IP_3R) triggers sustained (capacitative) Ca^{2+} mobilization leading to NFAT-dependent transcription (2, 3). DAG mediates protein kinase C-theta (PKCθ) and RasGRP membrane recruitment and activation. Deficiencies in either IP_3-mediated Ca^{2+} mobilization (4) or in DAG-initiated RasGRP activation (5) leads to a block in thymocyte development, particularly at the $CD4^+CD8^+$ (DP) stage, during which T cells undergo selection by signaling through the TCRs.

Karsten Sauer's research group, in collaboration with Michael Cooke's group (both at the Genomics Institute of the Novartis Research Foundation, or GNF), utilized an ENU-induced forward genetic screen in mice to find genes that affect T cell selection and development. In doing so, they identified that inositol 1,4,5-triphosphate kinase B (ItpkB), which phosphorylates IP_3 to IP_4 (inositol 1,3,4,5-tetrakisphosphate), is involved in T cell positive selection (6). Deficiency of ItpkB in T cells results in a block in positive selection, causing a profound accumulation of DP T cells in the thymus. Through much work, they embarked upon a series of studies to determine where the defect manifests itself, and specifically, to determine upon what IP_4 acts. They found that IP_4 acts within an intermediate window between that of TCR-inducible proximal and distal signals.

The forward genetic screen identified a nonsense mutation within the ItpkB gene that causes a premature STOP codon within position 199 in the amino acid sequence. As a result, no ItpkB protein is expressed and is phenotypically indistinguishable from mice genetically targeted to be deficient in ItpkB (7). Thymocyte development in these

ItpkB$^{-/-}$ mice displayed a profound block at the DP stage with very little production of single positive (SP) CD4^{+} or CD8^{+} cells indicating a problem with positive selection. The amount of expression of this gene seems to dictate this defect in positive selection. Haplotypic (ItpkB$^{+/-}$) mice displayed only a 50% reduction in single positive mature CD4^{+} or CD8^{+} T cells, and transgenic overexpression of ItpkB showed an overwhelming recurrence of single positive T cells. Therefore, the expression level of ItpkB determines the efficiency of positive selection.

As stated above in the Goldilocks theory of thymic selection, very weak or very strong TCR-initiated signals result in the death of the T cell during thymic development. As such, using transgenic mice that have weak or strong TCR affinities can help determine which signals are necessary for progression through thymic development. Sauer's group crossed the ItpkB$^{-/-}$ mice onto five different transgenic strong TCR mouse backgrounds, including the 2C, AND, HY, OT-1 and OT-2 mice. Positive selection was still blocked on all of these backgrounds, indicating how profoundly important IP_4 is for positive selection and thymic development (8).

To evaluate the effects of ItpkB on negative selection, the Sauer group utilized a transgenic TCR strategy that involved the use of HY^{+} transgenic TCRs. In this experimental system, male mice normally undergo negative selection to the male-specific HY antigen during the DN to DP transition, because of the strong reaction induced against this antigen. The female mice would positively select the HY^{+} TCR thymocytes and these cells would become CD8 single positive cells that could emigrate into the periphery. In ItpkB$^{-/-}$ mice, thymocytes were similarly selected, as male mice negatively selected the HY^{+} cells and the female mice positively selected the HY^{+} cells to become

$CD8^+$ single positives. Additionally, $ItpkB^{-/-}$ DP thymocytes were compared to WT DP thymocytes in their ability to undergo apoptosis in response to strong *in vitro* stimulation with plate-bound anti-CD3 and anti-CD28 antibodies. The cells from each group displayed similar dose-responsive death profiles indicating that negative selection is independent of ItpkB expression. Finally, distinct levels of Ca^{2+} signaling have been proposed to bifurcate the TCR signals driving positive versus negative selection (2, 9). In this way, TCR-induced low sustained Ca^{2+} signals induce calcineurin activity, which is required for positive selection (4), while TCR-induced high Ca^{2+} signals up-regulate expression of the pro-apoptotic protein Bim, which is required for negative selection (10). *In vitro* stimulated Ca^{2+} signaling was also compared in $ItpkB^{-/-}$ mice. The Ca^{2+} flux was indistinguishable and calcineurin activity was likewise unaffected in thymocytes from ItpkB-deficient mice when compared to WT mice. Similarly, the expression of Bim was consistent between the thymocytes from the two strains of mice. Therefore, negative selection of thymocytes is not affected in mice lacking ItpkB.

To determine the molecular mechanisms behind the effect on positive selection, the Sauer group identified that Erk activation was reduced in ItpkB-deficient thymocytes. They also found that the upstream activators of Erk, Raf and MEK, were also impaired in their activation. However, TCR-proximal enzymes such as Lck and ZAP-70 had activities that were intact. Their findings indicated that the molecular defect had to exist somewhere in-between. Since activation of Raf and MEK is Ras-dependent, and a similar DP block is seen in the Ras activator RasGRP, Sauer's group determined to address the activation of RasGRP through its upstream effector DAG, which is a product of PLCγ1 activation. Interestingly, and in contrast to the normal levels of Ca^{2+} seen,

ItpkB$^{-/-}$ thymocytes displayed a significant reduction in DAG production indicating a dichotomy between normal IP_3 production leading to Ca^{2+} flux and abnormal DAG production leading to RasGRP activation. They confirmed the DAG effect on positive selection by complementing ItpkB$^{-/-}$ defects with use of the DAG chemical analog, phorbol myristate acetate (PMA). Using fetal thymic organ cultures, PMA-stimulated ItpkB$^{-/-}$ thymocytes were rescued to produce CD4+ and $CD8^+$ T cells indicating that DAG is part of the defect seen in ItpkB-deficient positive selection. Therefore, mechanistically ItpkB acts as a bimodal regulator of PLCγ1 function by affecting only one of its products, the conversion of IP_3 to IP_4.

Since the delineation between IP_3 and DAG is seen at PLCγ1 activation, it was then sought how Itk, a known activator of PLCγ1, is involved in this process. Since Itk contains a pleckstrin homology domain that binds to membrane PIP_3 and soluble IP_4 is structurally similar to PIP_3, it was decided to find out whether Itk binds IP_4 *in vitro*. Precipitation of Itk with PIP_3-coated beads from WT DP thymocytes was reduced in a dose-dependent manner with soluble IP_4. Using IP_4-coated beads, IP_4 could precipitate Itk transiently in the same kinetics as Itk phosphorylation. To determine whether Itk activity was duly affected by IP_4, Itk was immunoprecipitated from ItpkB$^{-/-}$ DP thymocytes and compared to WT DP thymocytes. Specific Itk phosphorylation on tyrosine 511 was severely reduced in cells lacking ItpkB, indicating that IP_4 is necessary for Itk activation. The purpose of the following studies was to determine how IP_4 affects Itk activity, and whether IP_4 has any effect on Itk localization that could affect its activity. This was done in collaboration with the Sauer group to add to the biochemical and developmental evidence they had generated.

3.3 Materials and Methods

3.3.1 Mice and cell purification

C57Bl/6 Ms. T-less (ItpkB$^{-/-}$) mice were generated as described (6). The animals were analyzed between the ages of 5-12 weeks. Thymocytes were purified by staining excised thymus with anti-CD53 antibodies followed by anti-IgM-biotin on ice for 15 minutes in MACS staining buffer (PBS, 5% BSA, 2 mM EDTA). Cells were incubated with anti-biotin microbeads on ice for 15 minutes followed by separation via MACS depletion. Flow-through depleted thymocytes were found to be > 95% CD4^{+}CD8^{+} by flow cytometry.

3.3.2 Analysis of Itk and LAT localization

Wild-type-, Pleckstrin Homology deletion mutant (ΔPH), or myristoylated PH deletion mutant (myrΔPH) constructs of Itk-GFP, or LAT-GFP constructs were introduced into thymocytes from C57Bl/6 (WT), from Major Histocompatibility Complex deficient thymocytes (MHC$^{-/-}$), or from Inositol 1,4,5-triphosphate kinase B deficient (ItpkB$^{-/-}$) thymocytes using nucleofection. Nucleofection was achieved by using the Amaxa primary mouse T cell nucleofector kit and Amaxa setting X-001 (Amaxa, Köln, Deutschland). 24 hours after nucleofection, 2 X 10^{6} sorted GFP^{+} cells were incubated with polystyrene beads (3 μm diameter, Polysciences Inc., Warrington, PA) precoated with murine anti-CD3ε, CD3ε/CD4, or isotype control antibodies (10 μg/10^{7} beads). The cells were mixed with the antibody-coated beads, centrifuged at 50*g*, and

incubated for 2-5 minutes at 37°C. The cells were fixed on slides (3.7% paraformaldehyde in PBS) at room temperature for 30 minutes. Images were acquired on a laser scanning confocal microscope (Leica TCS SP2, Leica, Heidelberg, Deutschland) and were analyzed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD). Equal area regions of interest were drawn at the cell membrane/bead interface and at a membrane site not undergoing bead contact. A localization index was calculated as the ratio of pixel intensity at the contact site over pixel intensity of an equal area at the control site. Indices were plotted and calculated for statistics by using GraphPad Prism software (GraphPad Software, San Diego, CA).

3.4 Results

3.4.1 Itk localizes to the site of interaction between a T cell and an APC

When Itk becomes targeted to the membrane through the binding of its PH domain to PI_3K-generated PIP_3, Itk is brought into proximity of its activator, Lck. To identify the localization of Itk, an assay utilizing GFP-tagged Itk (Itk-GFP) transfected cells and stimulatory microbead engagement was employed. When Itk-GFP transfected mouse thymocytes engage a microbead coated with anti-TCR antibodies, Itk-GFP becomes localized to the site of interaction between the bead and the cell (Figure 3.2, Anti-CD3). Alternatively, when an Itk-GFP transfected thymocyte engages a microbead coated with antibodies that are of similar isotype, but cannot stimulate the cell, Itk-GFP does not localize to the contact site between the cell and the microbead (Figure 3.2, Isotype). Using this system, a index of localization can be created by evaluating the pixel intensity of an area at the site of engagement and comparing that to the pixel intensity of an equal area at a membrane site directly opposite of the site of engagement. A localization index near one indicates little redistribution of the GFP-tagged molecule to the contact site. The yellow boxes in Figure 3.2 indicate areas of pixel intensity used to determine the index of localization.

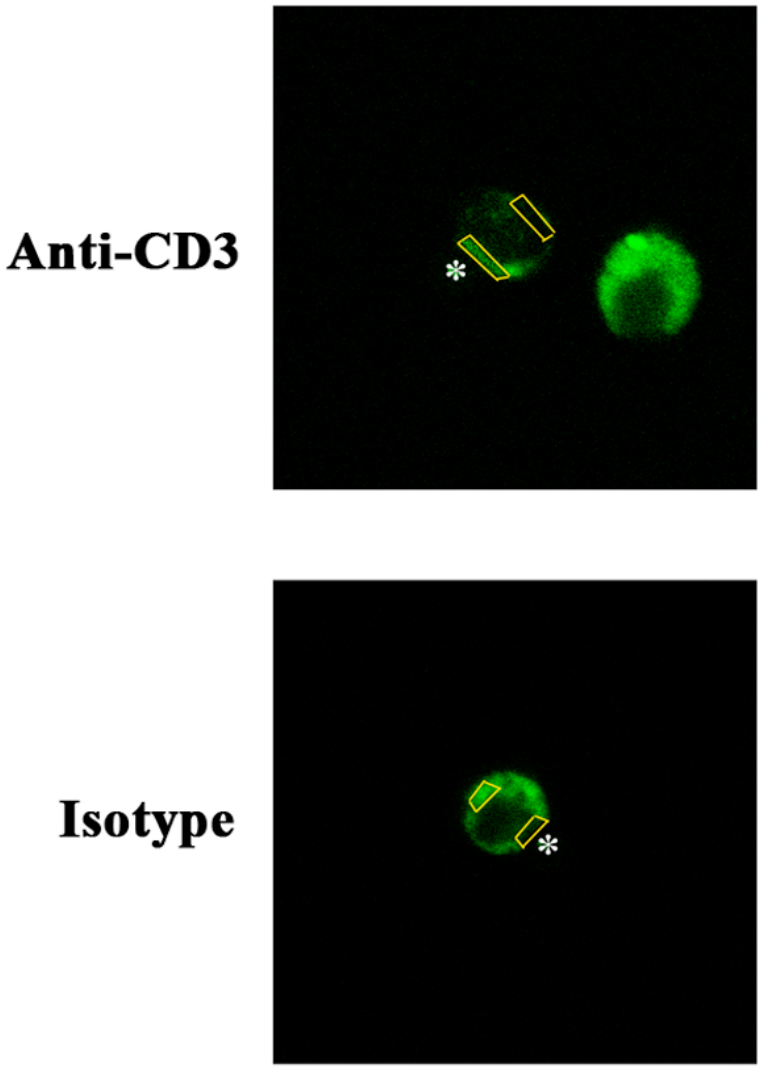

Figure 3.2. *Itk localizes the site of interaction between a T cell and a surrogate APC.*
Wild-type thymocytes nucleofected with Itk-GFP are mixed with anti-CD3ε coated microbeads (top panel) or mixed with isotype control coated microbeads (bottom panel). The pixel intensity of an area at the site of interaction is compared to the pixel intensity of an equal area at a site opposite to create a localization index (yellow boxes). The asterisk denotes the position of the antibody-coated microbead engaged by the Itk-GFP expressing cell.

3.4.2 IP_4 affects Itk localization to the T cell/surrogate APC contact site

Upon TCR/CD3 engagement, Itk localizes to the activated TCR complex and to the LAT signalosome in particular. This association is dependent on the presence of the PH domain of Itk. To determine whether IP_4 had any effect on Itk localization, it was decided to analyze the effect of ItpkB deficiency on Itk redistribution in DP thymocytes upon stimulation with antibody-coated beads. These cells were compared to MHC-deficient cells, as they have a similar (though not identical) block in thymic development, at the DP stage. As shown in Figure 3.3, Itk-GFP nucleofected cells from both strains of mice were subjected to engagement with stimulating or non-stimulating antibody-coated microbeads. Please note that Itk failed to accumulate to the site of interaction between the cell and the isotype control beads in both strains. Additionally, Itk accumulation at the site of interaction with stimulating antibody microbeads is seen in the cells from $MHC^{-/-}$, but not in cells from the $ItpkB^{-/-}$ mice. Figure 3.4 displays more examples of cells transfected with Itk-GFP engaging beads that are coated with stimulating antibodies to further discriminate the differences in Itk localization between the two strains of mice.

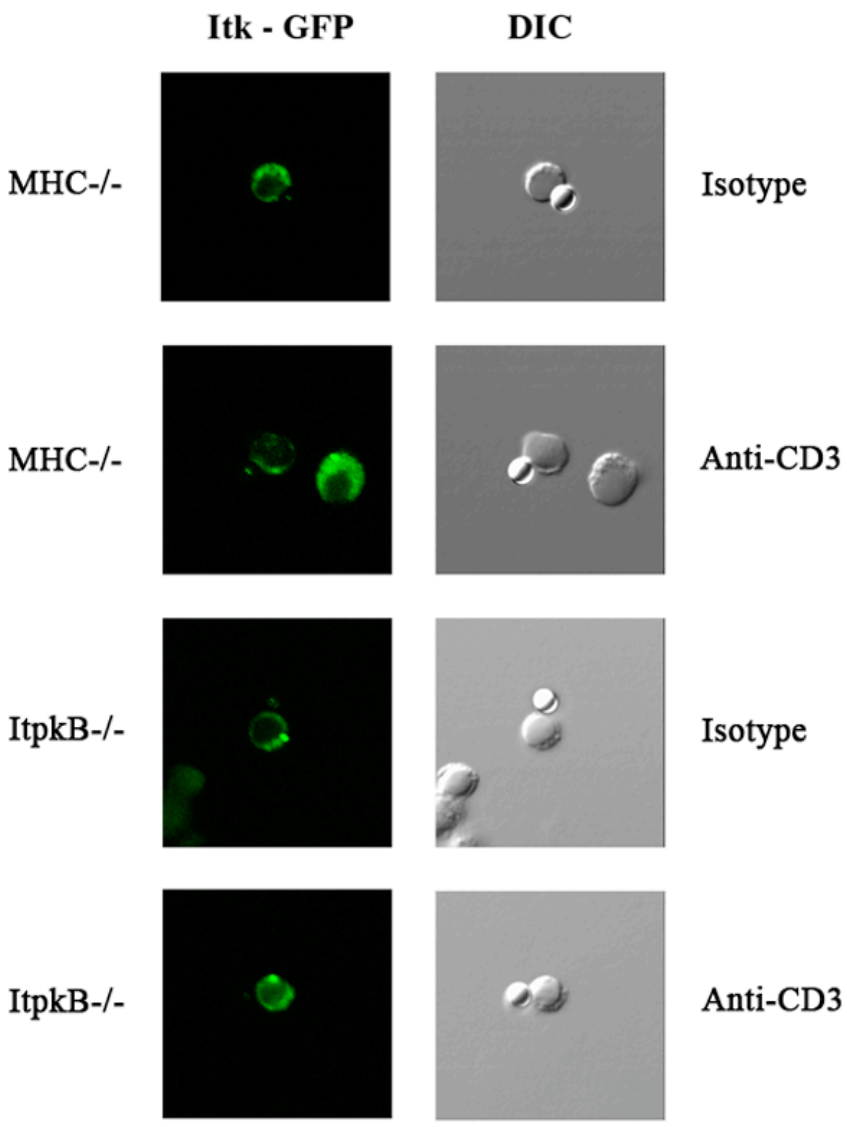

Figure 3.3. *Localization of Itk-GFP in DP thymocytes.*

Itk-GFP nucleofected thymocytes either from $MHC^{-/-}$ mice or from $ItpkB^{-/-}$ mice were mixed with either anti-CD3ε or with isotype control antibody-coated microbeads. Cells were allowed to engage for either 2 or 5 minutes at 37°C and evaluated for Itk localization at the cell/bead contact site. Left panel images were captured using laser scanning confocal microscopy while right panel images were captured using differential interference contrast microscopy to indicate the bead location relative to the engaged cell.

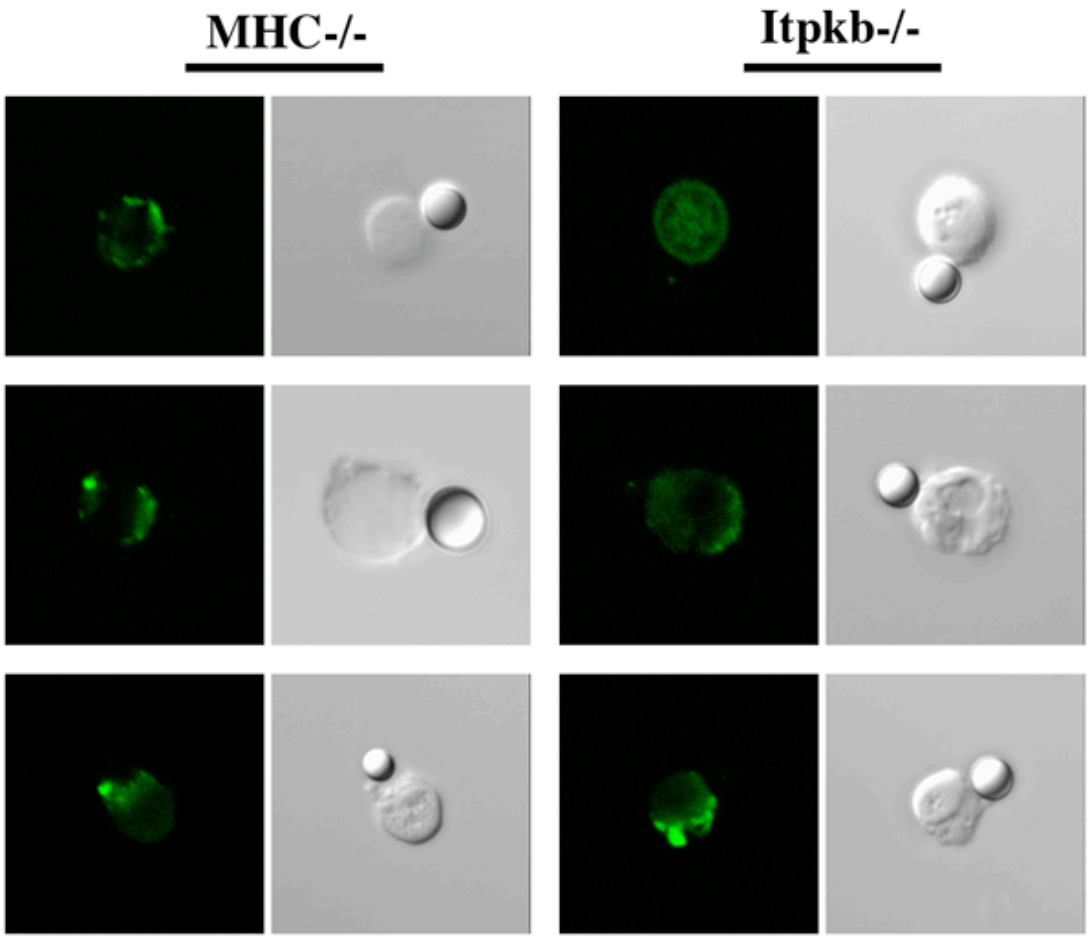

Figure 3.4. *Localization of Itk-GFP in stimulated DP thymocytes.*

Itk-GFP nucleofected thymocytes either from MHC$^{-/-}$ or ItpkB$^{-/-}$ mice were mixed with anti-CD3ε antibody-coated microbeads. Cells were allowed to engage for either 2 or 5 minutes at 37°C and evaluated for Itk localization at the cell/bead contact site. Confocal microscopy images (left panels) show Itk-GFP localization in nucleofected thymocytes of each strain. DIC microscopy images (right panels) show the anti-CD3ε antibody coated bead location relative to the location of the cell.

Quantitative analysis of cells transfected with Itk-GFP showed that the relative Itk-GFP abundance at the bead contact site increased at 2 minutes post CD3 or CD3/CD4 stimulation of $MHC^{-/-}$ DP thymocytes, but not in $ItpkB^{-/-}$ DP cells (Figure 3.5). Upon encountering anti-CD3 antibody stimulating microbead, Itk-GFP localized to the contact site in $MHC^{-/-}$ cells (localization index of 1.57 ± 0.10, Figure 3.5, blue filled squares), while Itk-GFP failed to localize in cells lacking IP_4 under similar stimulating conditions (1.06 ± 0.08, Figure 3.5, blue filled circles). A similar trend is observed when the cells from each strain of mouse were incubated with microbeads coated with anti-CD3 and anti-CD4 antibodies. The location index for $MHC^{-/-}$ thymocytes is 1.60 ± 0.12 (Figure 3.5, filled red squares), while the location index for $ItpkB^{-/-}$ thymocytes is 1.08 ± 0.08 (Figure 3.5, filled red circles). At 5 minutes of engagement with stimulating microbeads (Figure 3.6), the localization index of Itk-GFP showed similar increases both in wild-type (C57Bl/6) DP thymocytes (anti-CD3, 1.60 ± 0.19; anti-CD3/4, 1.73 ± 0.19, filled squares) and in $MHC^{-/-}$ DP thymocytes (anti-CD3, 1.69 ± 0.10; anti-CD3/4, 1.33 ± 0.05, filled circles). Similar to the observation with 2 minutes of stimulation, prolonged incubation to 5 minutes displayed no statistically significant increase in Itk-GFP localization in cells lacking ItpkB with 5 minutes of stimulation (anti-CD3, 1.16 ± 0.10; anti-CD3/4, 1.05 ± 0.06, filled diamonds). These results show a reproducible increase in Itk-GFP localization in DP thymocytes from two different strains of mice (C57Bl/6 and $MHC^{-/-}$) at two different time-points relevant for signaling in T cells. Furthermore, DP thymocytes lacking IP_4, through the deficiency of the kinase that converts IP_3 to IP_4, are unable to localize Itk-GFP to the engaging bead contact sites observed in cells from the

other strains of mice. This correlates the biochemical data collected in the Sauer lab to a functional outcome affecting the molecule within the cell.

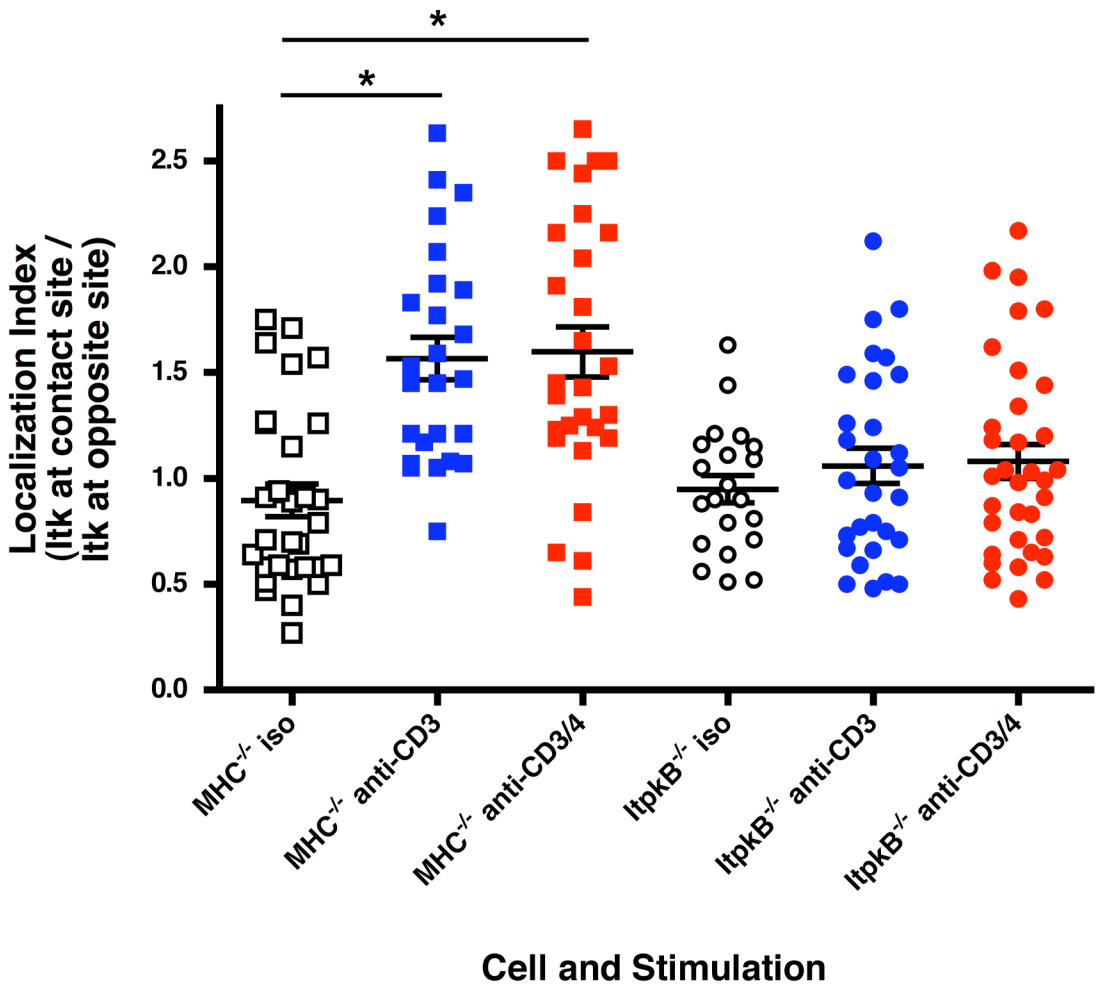

Figure 3.5. *Itk fails to inducibly localize to the cell/bead interface in the absence of IP_4 after 2 minutes of stimulation.*

Itk-GFP transfected DP thymocytes from either $MHC^{-/-}$ or $ItpkB^{-/-}$ mice were mixed with antibody-coated microbeads as indicated and allowed to engage at 37°C for 2 minutes. Cell/bead conjugates were analyzed for Itk localization as described in Figure 3.2. Each square ($MHC^{-/-}$) or circle ($ItpkB^{-/-}$) represents one cell/bead conjugate analyzed for Itk localization. The lines within each group are the mean ± SEM. The bars across with asterisks indicate a statistically significant change between compared groups (student t-test, $p < 0.05$).

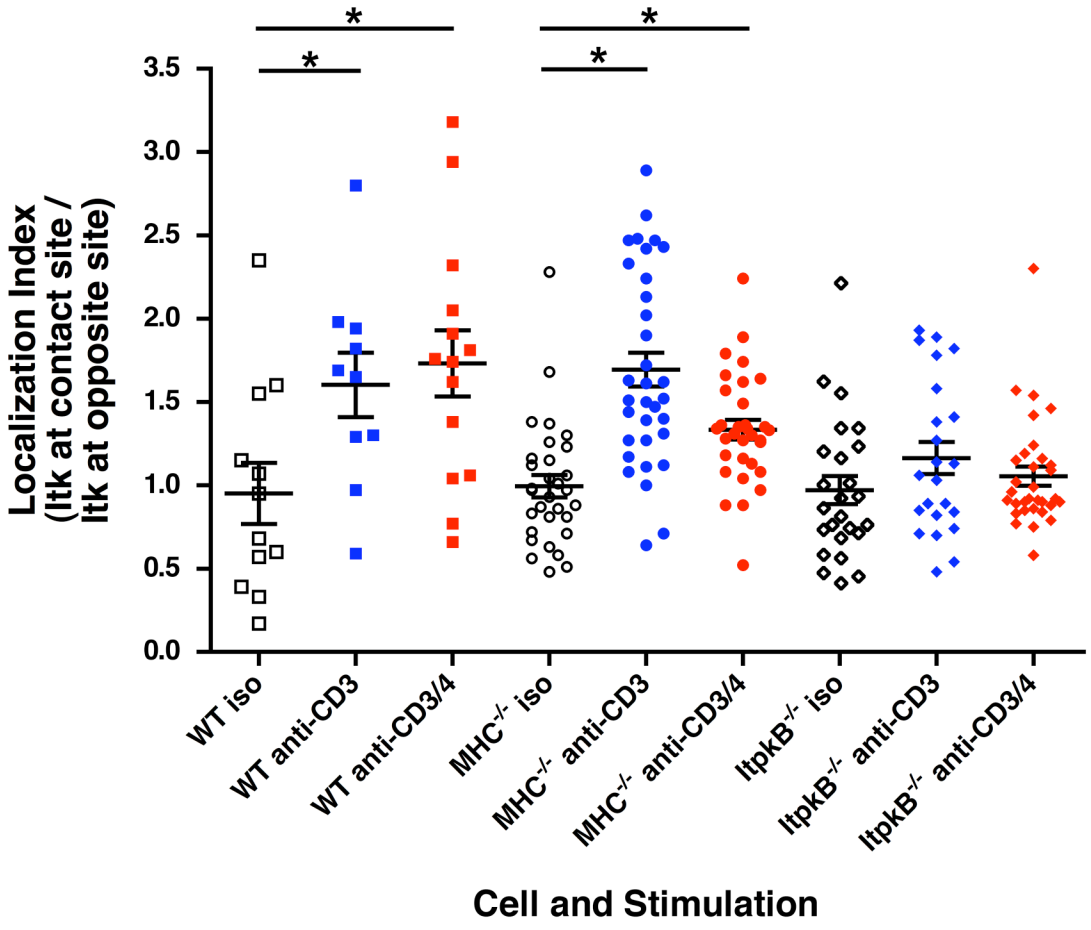

Figure 3.6. *Itk fails to localize to the bead/cell contact site in the absence of IP_4 after 5 minutes of stimulation.*

DP thymocytes from WT, $MHC^{-/-}$, and from $ItpkB^{-/-}$ were treated as in Figure 3.5. Briefly, the cells were incubated with antibody-coated microbeads as indicated for a period of 5 minutes. Localization of Itk was analyzed as described in Figure 3.2. Each symbol represents one cell/bead conjugate analyzed and the lines within each group are the Mean ± SEM. The bars across with asterisks indicate a statistically significant change between compared groups (student t-test, $p < 0.05$).

The adaptor protein LAT is also known to functionally localize to the immunological synapse upon stimulation of the T cell. To compare the localization of Itk to that of another protein that also co-localizes to the T cell/APC interface, a LAT-GFP tagged construct was transfected into DP thymocytes either from $MHC^{-/-}$ mice or from $ItpkB^{-/-}$ mice. Figure 3.7 shows LAT-GFP molecules in $ItpkB^{-/-}$ DP thymocytes localize to the plasma membrane and coalesce at the APC contact site upon engaging a stimulatory microbead for a period of 5 minutes. Evaluation of many cell/bead conjugates shows that LAT enrichment was indistinguishable between $MHC^{-/-}$ and $ItpkB^{-/-}$ DP thymocytes. Localization indices of LAT-GFP in $MHC^{-/-}$ thymocytes were 1.32 ± 0.08 when engaging anti-CD3 antibody coated microbeads (Figure 3.8, blue filled squares) and 1.48 ± 0.10 when engaging anti-CD3/4 antibody coated microbeads (Figure 3.8, red filled squares). The indices of localization for LAT-GFP in ItpkB-/- thymocytes were 1.34 ± 0.11 when engaging anti-CD3 antibody coated microbeads (Figure 3.8, blue filled circles) and 1.42 ± 0.11 when engaging anti-CD3/4 antibody coated microbeads (Figure 3.8, red filled circles). The inducible increase in LAT-GFP localization at the contact site in both strains of mice is statistically significant (student t-test $p < 0.05$). Therefore, the absence of IP_4 does not affect LAT-GFP localization to the T cell/bead interface upon stimulation.

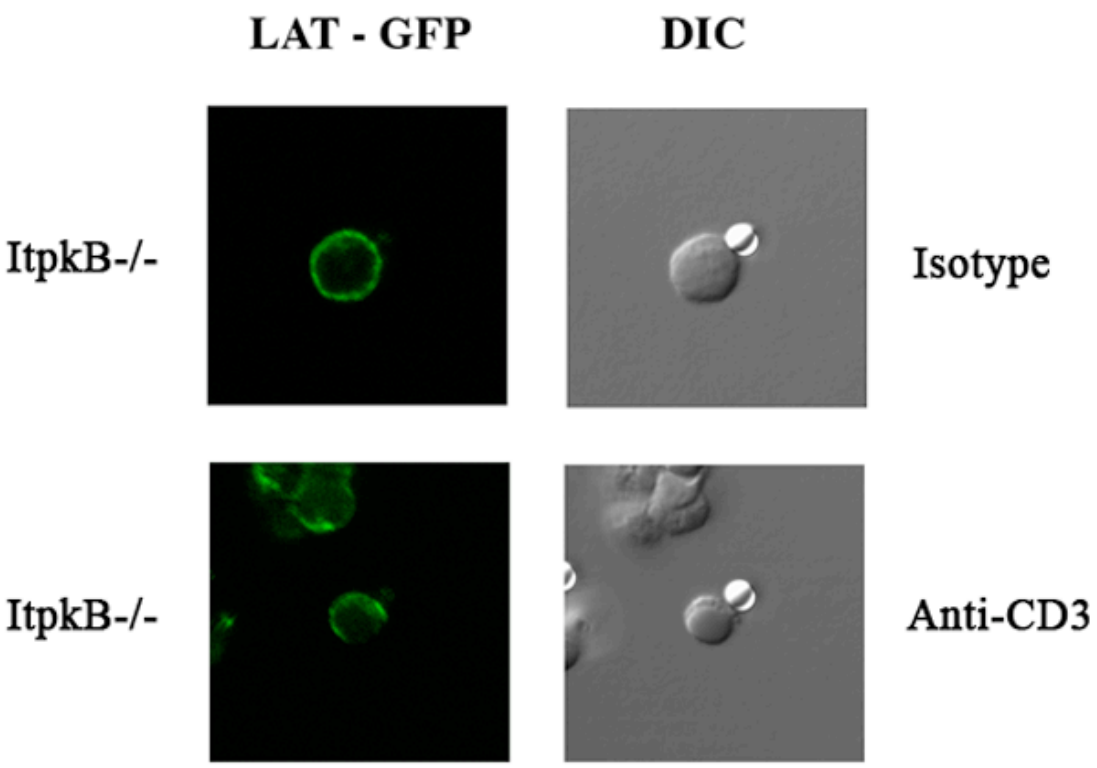

Figure 3.7. *The adaptor protein LAT inducibly localizes to the cell/bead interface upon stimulation irrespective of the presence of IP_4.*

ItpkB$^{-/-}$ DP thymocytes were nucelofected with LAT-GFP, mixed with anti-CD3ε coated microbeads or isotype control microbeads, and allowed to incubate at 37°C for 5 minutes.

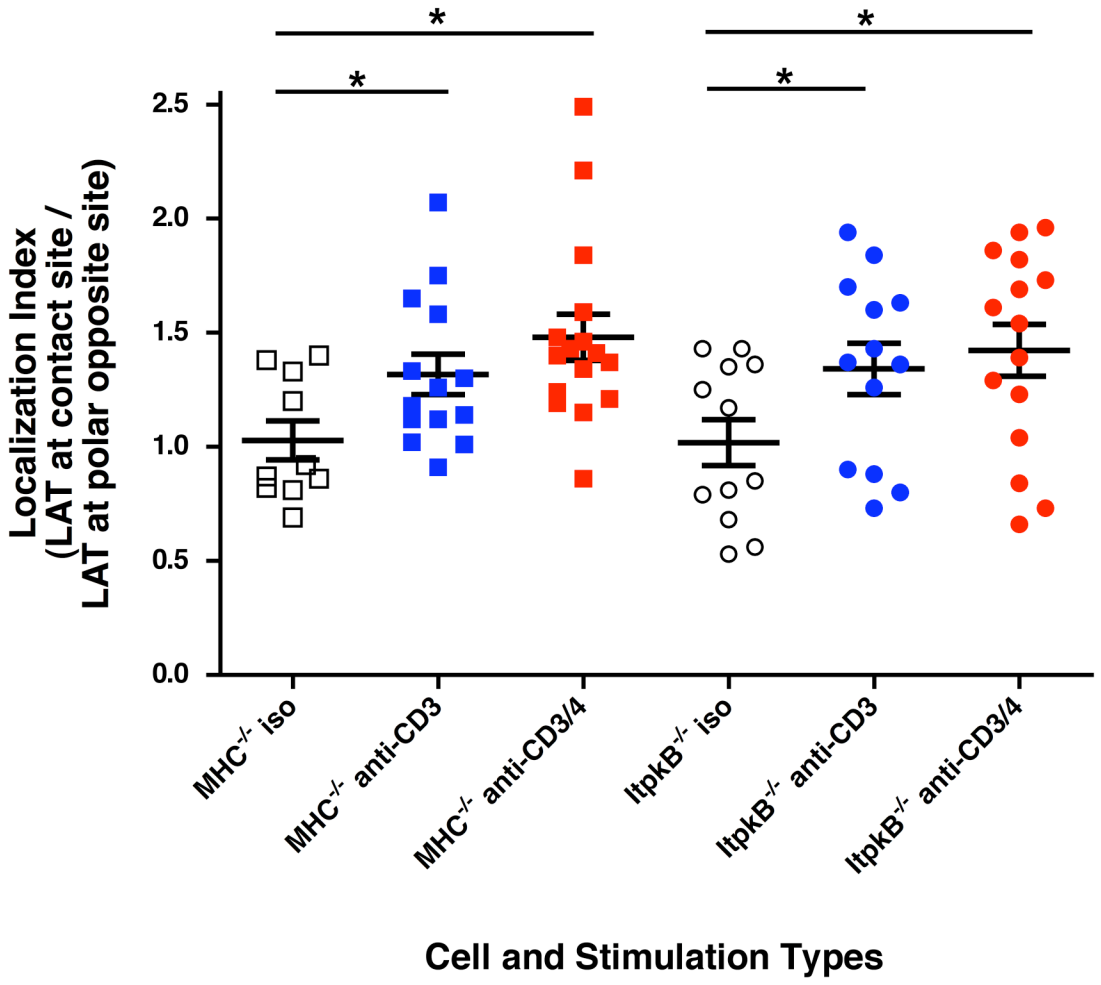

Figure 3.8. *LAT localizes to the cell/bead interface in the presence or absence of IP_4.*
Cells were treated as described in Figure 3.7, and analyzed for pixel intensity to determine the ratio of localization. Each symbol represents on cell/bead conjugate analyzed, while the lines in each group indicate the Mean ± SEM. The bars across with asterisks indicate a statistically significant change between compared groups (student t-test, $p < 0.05$).

In biochemical studies conducted by the Sauer lab, it was determined that IP_4 bestowed its effects through the PH domain of Itk. Consistent with data from Jurkat cells (11, 12), PH domain deletion abrogates Itk enrichment at the T cell/APC contact site (Figure 3.9). Localization of Itk to the contact site when its PH domain is absent was not affected by stimulation (Figure 3.9; anti-CD3, 0.93 ± 0.06; anti-CD3/4, 0.98 ± 0.07), nor the by the presence or absence or IP_4 (Figure 3.9; $ItpkB^{-/-}$; anti-CD3, 1.07 ± 0.08; anti-CD3/4, 0.94 ± 0.07). This suggests that ItpkB is required for PH domain dependent enrichment of Itk at sites of TCR engagement. This correlates with the reduction in Itk activation in CD3 or CD3/CD4 stimulated $ItpkB^{-/-}$ DP cells compared to controls (8). Further, Itk localization can be rescued with the addition of a Lck myristoylation sequence to the amino terminus of Itk, indicating that localization can be restored (Figure 3.10). The localization index of myr-ΔPH-Itk-GFP was restored in cells from both strains of mice when the cells engage stimulatory microbeads. The localization index within the $MHC^{-/-}$ DP thymocytes was 1.45 ± 0.06 when engaging anti-CD3 only beads and 1.50 ± 0.05 when engaging anti-CD3/4 beads. Likewise, the localization index with the $ItpkB^{-/-}$ DP thymocytes was 1.44 ± 0.04 when engaging anti-CD3 only beads and 1.46 ± 0.03 when engaging anti-CD3/4 beads. The observations indicate that ItpkB is required for optimal Itk activation through the Itk-PH domain.

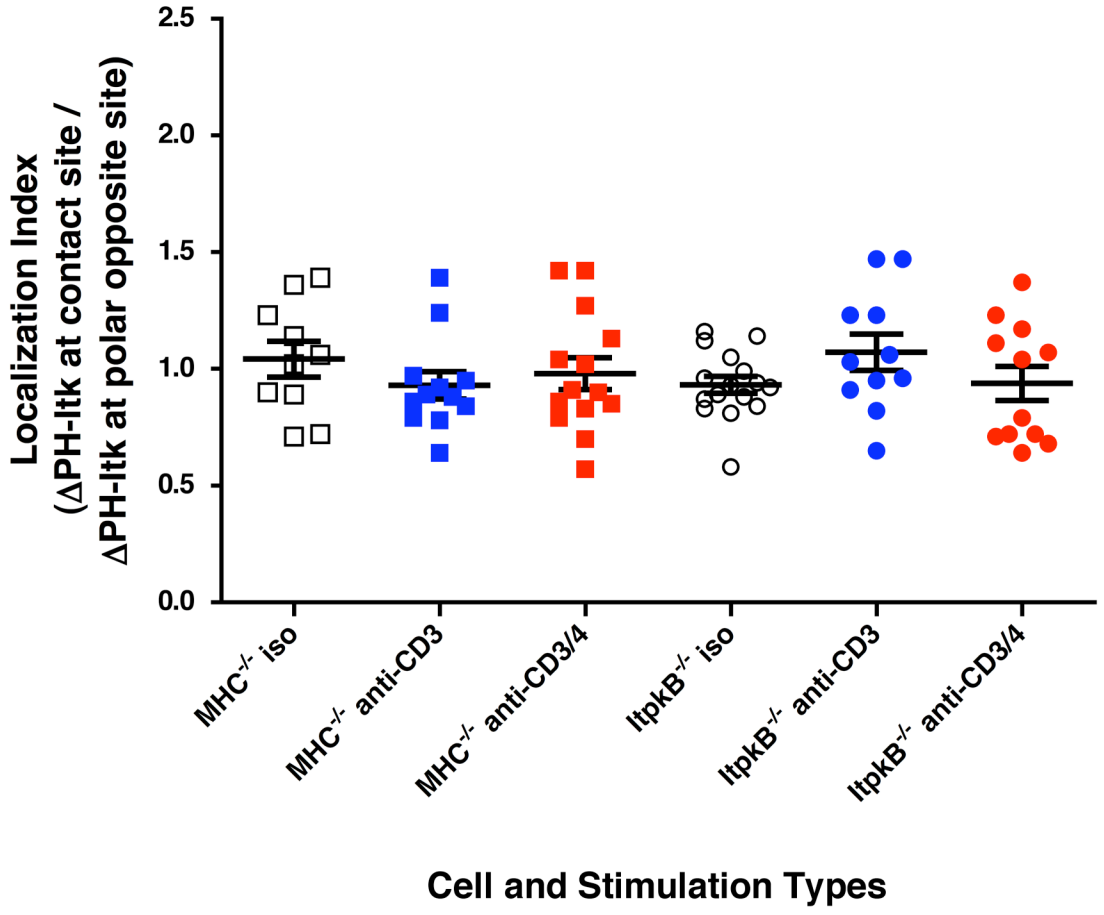

Figure 3.9. *Itk lacking the PH domain fail to localize to the inducible site of cell/bead interaction.*

MHC$^{-/-}$ DP or ItpkB$^{-/-}$ DP cells were nucleofected with ΔPH-Itk-GFP and mixed with non-stimulating or stimulating antibody coated microbeads. Cell conjugates were allowed to incubate for 5 minutes at 37°C. Each symbol represents one cell conjugate analyzed for Itk localization, with the ratio of localization determined by analyzing the pixel intensity of the site of interaction to an equal area at a site opposite. Lines within each group represent the Mean ± SEM.

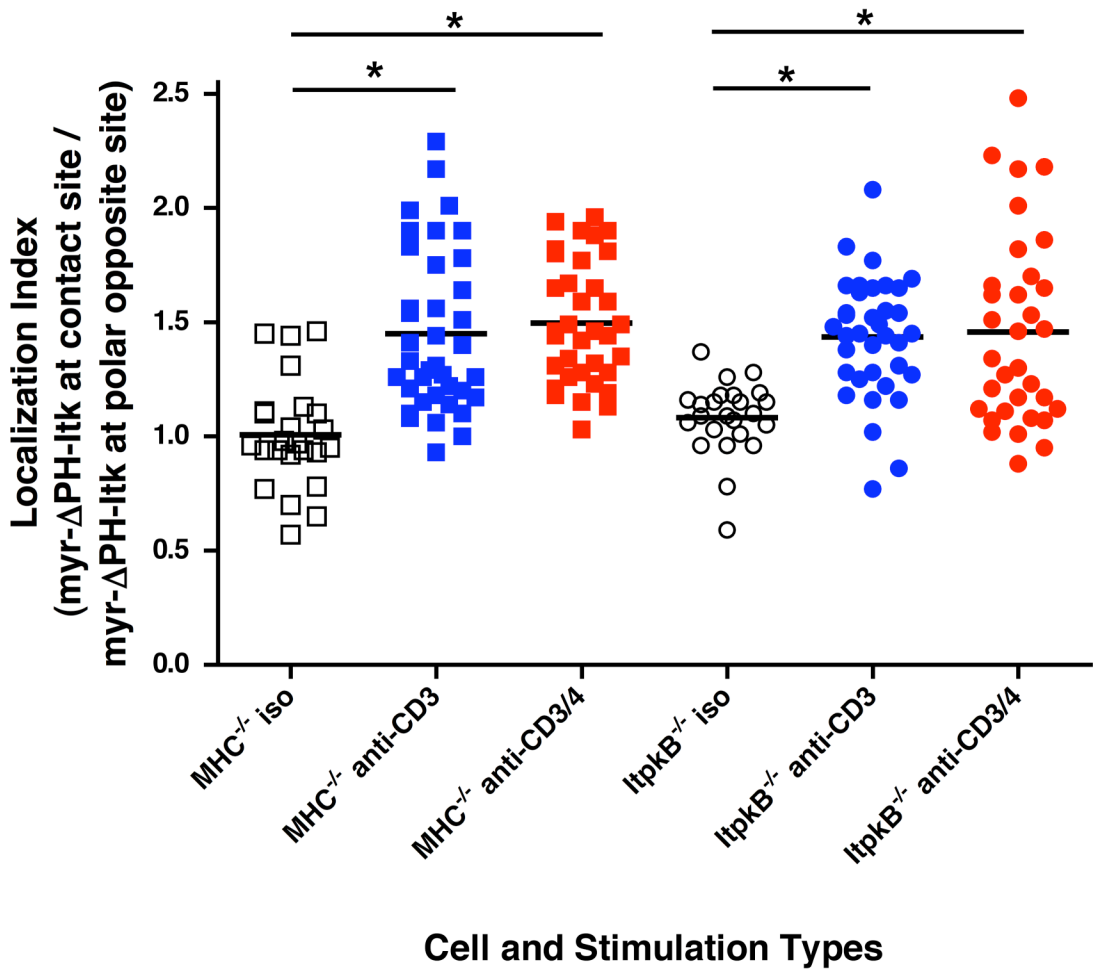

Figure 3.10. *Reconstituted ΔPH-Itk localization with the addition of a membrane myristoylation signal.*

Cells were treated as in Figure 3.9, with the exception of a Lck myristoylation signal genetically fused to the amino-terminus of ΔPH-Itk-GFP construct was transfected into each cell type. The bars across with asterisks indicate a statistically significant change between compared groups (student t-test, $p < 0.05$).

3.5 Discussion

TCR engagement results in the assembly of a complicated molecular signaling machinery originating at the sites of TCR stimulation (13-15). This machinery then relays signals into the interior of the cell, which in turn directs the cellular response, such as survival and maturation in positive selection, or cell death in negative selection. In peripheral T cells, Itk is assembled into signaling complexes containing PLCγ1 through interactions with SLP-76 and LAT (16-20). Since Itk uses a PH domain to localize to PI_3K-generated membrane PIP_3, it was hypothesized that reduced PIP_3 binding would result in impaired Itk recruitment in $ItpkB^{-/-}$ cells. To test this idea, the Sauer group conducted experiments that showed Itk association with PLCγ1 was strongly reduced in $ItpkB^{-/-}$ cells. In contrast, co-precipitation of LAT and PLCγ1 was largely ItpkB-independent (8). These results indicate a possible impairment of Itk recruitment into membrane signaling complexes in $ItpkB^{-/-}$ DP thymocytes. This was proven true by analyzing the localization of GFP-tagged Itk to the site of T cell engagement with a surrogate APC, or an antibody coated microbead, to determine that indeed, Itk localization is impaired in the absence of IP_4. Interestingly, and in support of the biochemical co-precipitation data between LAT and PLCγ1, LAT localization to the contact site remained intact in the absence of IP_4. Itk localization to the membrane site of interaction was dependent on its PH domain, while its localization could be reconstituted with the addition of a membrane localization signal.

Taken together, these data demonstrate that IP_4 binds to Itk in a PH domain dependent manner. They suggest that IP_4 production by ItpkB augments PIP_3 binding,

and is required for Itk recruitment to sites of TCR engagement and its activation. Membrane recruitment via the PH domain is required for Itk function (11, 12). However, constitutive membrane targeting and raft recruitment of myristoylated PH domain-deficient Itk did not allow for its phosphorylation by Lck (12). This suggests that the Itk PH domain has additional functions besides recruitment, possibly in modulating Itk conformation through intra- or intermolecular protein interactions involved in Itk regulation (21, 22). Thus, the first point of IP_4 action on Itk could occur in the cytosol, altering Itk conformation to enable its activation. If the assumption is made that free IP_4 is present in the cytosol, it could bind to the Itk PH domain to alter the conformation of this domain into one that is of high affinity for either PIP_3 or IP_4, depending on which is of a higher concentration at the site of localization. Additionally, Itk acts as a scaffold for multiple proteins, including Vav1, SLP-76, and WASp among others (23-26). Deletion of the PH domain disrupts this adaptor function, as Itk can no longer localize to the plasma membrane (12, 27). Thus, IP_4-regulation of the PH domain may control Itk at several levels, possibly including kinase-independent functions. In any case, these results suggest that one critical role of IP_4 is to allow proper Itk recruitment and activation.

Impaired Itk activation provides a mechanistic explanation for the defects in PLCγ1 activation, DAG production and MEK/Erk activation in ItpkB$^{-/-}$ DP cells (6, 28-32). Since the DAG analog PMA can rescue the block seen in ItpkB$^{-/-}$ thymocyte maturation, impaired DAG production due to reduced PLCγ1 activation, likely because of impaired Itk activation, is a major component of the defects causing the maturational block in ItpkB$^{-/-}$ mice. The coupled equimolar production of IP_3 and DAG by PLCγ1 has made it difficult to distinguish their individual contributions *in vivo*. ItpkB's unique role

to consume IP_3 while promoting PLCγ1 activity through regulation of Itk reveals the specific importance of DAG for thymocyte selection, underscored by the ability of PMA to rescue Erk activation, CD69 upregulation and in particular mature T cell development in the absence of ItpkB. Impaired positive selection after perturbation of the Ras/Erk pathway is consistent with a function for ItpkB upstream of DAG and RasGRP1 (33). However, the positive selection defect in RasGRP1$^{-/-}$ mice (3) is less severe than the block in ItpkB$^{-/-}$ mice, which cannot be rescued by transgenic expression of strongly selecting TCRs (34). Thus, other DAG effectors or other RasGRP isoforms may contribute to TCR signals required for positive selection.

Defective Itk function alone cannot explain all defects in ItpkB$^{-/-}$ thymocytes, because positive selection is less severely blocked in Itk$^{-/-}$ mice (35, 36). The more severe block in ItpkB$^{-/-}$ mice could possibly involve dysregulation of other IP_4-binding proteins, including GAP1^{M} and GAP1^{IP4BP} (37, 38). Promotion of PIP_3 binding through ItpkB/IP_4 may serve as a general mechanism required for membrane recruitment of PIP_3-binding proteins. Another level of regulation is provided by the inducible nature of IP_4 production (39). Different IP_4 levels might promote or inhibit activity, with higher levels possibly competing away PIP_3. Higher order inositol-polyphosphates have recently been ascribed unanticipated functions as regulators of chromatin remodeling complexes (40), or as nonenzymatic protein phosphorylating agents (41). These results suggest a novel function for IP_4 as an important *in vivo* regulator of proteins containing PIP_3-binding PH domains, exemplified by the Tec kinase Itk. PIP_3-binding proteins exist in all eukaryotes from yeast to mammals. Thus, the ability of IP_4 to serve as a physiologically relevant

ligand for PIP_3 binding PH domains is likely to have global implications as a novel biological regulatory mechanism.

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4 Managing Itk Function with a Cell-Permeable Peptide

4.1 Abstract

The interleukin-2 inducible T cell kinase (Itk) is critical for lymphocyte development and activation. Upon T cell receptor (TCR) engagement, Itk becomes transphosphorylated on tyrosine 511, an event dependent on the adaptor protein SLP-76. *In vitro* studies with Itk GST-SH3 fusion constructs have suggested that Itk and SLP-76 associate upon TCR engagement. However, the biological significance of this association on Itk function and cytokine production has not been demonstrated in live cells. To address this, utilization of a polyarginine-modified peptide (henceforth called R9-QQP) that represents a portion of the SLP-76 polyproline-rich region was generated as a competitive inhibitor aimed to disrupt the inducible interaction between Itk and SLP-76 in live cells. R9-QQP is readily taken up by cells as demonstrated by flow cytometric analysis and confocal microscopy of cells incubated with FITC-conjugated peptide. R9-QQP inhibits TCR-induced association of Itk and SLP-76, transphosphorylation of Itk on tyrosine 511, and expression of T_H2 cytokines in a dose-dependent manner. The inhibition mediated by R9-QQP is specific in comparison to the non-inhibitory effects by control peptides which represent either the polyarginine vehicle alone (R9) or a scrambled sequence of the polyarginine tagged cargo peptide (R9-SCR). Further evidence of specificity is the lack of inhibition by R9-QQP in the association between other SLP-76 binding proteins. The effects of R9-QQP on Itk activation and cytokine production are also demonstrated upon intraperitoneal administration of the peptide *in vivo*. In view of previous observations implicating Itk as a regulator of T_H2 cytokines,

the data presented here underscores the significance of Itk as a target for pharmacologic intervention.

4.2 Introduction

The Tec family of kinases plays a crucial role in lymphocyte development and activation through antigen receptors (1, 2). The Interleukin-2 inducible T cell kinase (Itk), a member of the Tec family, helps to regulate positive and negative selection during thymocyte development and affects the generation of T_H2 responses (3, 4). Phosphorylation of Itk on its tyrosine 511 by the Src family kinase Lck occurs early upon T cell receptor (TCR) engagement and is critical for the enzymatic activation of Itk (5, 6). Activated Itk targets and phosphorylates PLCγ1 on tyrosines 775 and 783, which is critical for activation of its phospholipase activity (7-9). In this fashion, Itk regulates the generation of secondary messengers and subsequently both intracellular and capacitative Ca^{2+} mobilization (10).

Recent studies discovered that Itk regulates the TCR-mediated reorganization of the actin cytoskeleton at the T cell-APC contact site (11, 12). Interestingly, and in contrast to the other properties of Itk discussed above, this function is independent of enzymatic activity and appears to be due to a putative adaptor role of Itk (11, 13). Additional evidence shows that regulation of actin polymerization by Itk cannot be compensated by other members of the Tec family (12, 13). Therefore, regulation of TCR-induced actin reorganization in T lymphocytes seems to be a unique property of Itk not shared by other members of the Tec kinase family.

The structural organization of Itk utilizes modular domains that are critical for its activation. Upon TCR engagement, Itk co-localizes with the T cell receptor in a process dependent on the Pleckstrin Homology (PH) domain of Itk (14, 15). It has recently been

demonstrated by Huang et al. that IP_4 is critical in promoting the interaction of Itk-PH domain with PIP_3 at the plasma membrane, thus indicating an additional role for the PH domain in Itk activation (16). Recruitment of Itk to the plasma membrane and subsequent activation also requires interaction with adaptor proteins such as LAT and SLP-76 (17, 18). The SH2 domain of Itk is critical for its inducible interaction with the LAT signaling complex, whereas the SH2 and the SH3 domains are required for interaction with SLP-76 in a co-operative fashion. Original *in vitro* studies using GST-fusion proteins revealed that the SH3 domain of Itk interacts with a proline rich region (PRR) of SLP-76, and that this interaction is pivotal for Itk activation. However, this has not been tested using full-length molecules in live cells.

As explained in the following background sections, Itk's ability to regulate T_H2-dependent responses renders it an attractive target for pharmacological intervention in diseases such as lung allergies. Interestingly, studies show that $Itk^{-/-}$ mice display no lung inflammation in an experimental model of bronchial asthma. *In vitro* studies have suggested that interference of Itk binding with its signaling partners, particularly with SLP-76, would result in the inhibition of Itk activation. However, a direct demonstration of such an effect has not been reported in live cells. In these studies, cell-penetrating cationic peptides (an emerging technology explained later) were generated that represent a portion of the proline-rich region of SLP-76 to which the Itk-SH3 domain binds. This was done to potentially manipulate the activation of Itk in both Jurkat cell culture T cells and primary T cells. Incubation of cells with these peptides *in vitro* causes the disruption of the inducible association between Itk and SLP-76, has significant inhibitory effects on the transphosphorylation of Itk tyrosine 511, and consequent diminished production of

T_H2 cytokines (both of which were evaluated using multi-parameter flow cytometry, again, explained later). Interestingly, *in vivo* delivery of the peptides by intraperitoneal administration in mice has similar effects to those seen with *ex vivo* cell treatment. Potentially, the data presented here may have implications on the potential pharmacological effects in disease situations through the manipulation of Itk activity.

4.2.1 T Helper Cell Lineage Commitment

The discovery that naïve $CD4^+$ T lymphocytes could differentiate into two distinct subsets was made over 20 years ago (19, 20). At this time, T helper cells were segregated into two categories, depending on their cytokine secretion and for their immune response. T helper 1 (T_H1) cells were defined by their ability to produce primarily IFNγ, IL-2, and TNFα, cytokines which mediate delayed type hypersensitivity and macrophage activation. T helper 2 (T_H2) cells express signature cytokines IL-4, IL-5, and IL-13, which help induce B cells to produce antibodies and for responses to allergies. In turn, cytokine secretion by antigen presenting cells (APC) controls this differentiation of T helper precursor (T_Hp) into T_H1 or T_H2 cells. For instance, IL-12 produced by activated dendritic cells is necessary for the differentiation of T_H1 cells, as shown by the deficient development of T_H1 cells from IL-12 defective mice (21, 22). Further, IL-4 is the primary cytokine expressed by APCs that differentiate T_Hp into T_H2 cells. Mice deficient for IL-4 or for the IL-4 receptor fail to develop T_H2 cells in response to stimulation (23-25).

The cytokine IL-2 is expressed exclusively in T cells and is the first produced during T helper cell differentiation. It is the primary cytokine produced by the naïve T_Hp cell, it causes the autocrine proliferative response necessary for clonal expansion. Differentiated T_H1 effector cells produce IL-2, albeit at levels lower than that of T_Hp cells, while T_H2 cells do not (26). IFNγ is expressed primarily in T_H1 $CD4^+$ effector cells, $CD8^+$ T cells, and natural killer cells. IFNγ is a potent activator of anti-viral response and as such is also antiproliferative. T_H1 lineage commitment and IFNγ gene transcription is controlled by a transcription factor named T-box expressed in T cells (T-bet) (27, 28). T-bet is expressed only in T_H1 cells and in NK cells. T-bet expression serves three purposes; it induces production of the T_H1 cytokine IFNγ, it transcriptionally activates the T_H1 differentiation pathway, and it simultaneously shuts off the production of T_H2 differentiated cytokines. T-bet expression also causes a decrease in IL-2 production in T_H1 cells when compared to T_Hp cells. Therefore, T-bet is a master regulator of T_H1 lineage commitment, activating T_H1 developmental programs while shutting off T_H2 differentiating pathways.

IL-4 expression is limited to a multitude of cells, including $CD4^+$ T_H2 cells, $CD8^+$ T cells, NK T cells, γδ T cells, mast cells, basophils, and eosinophils (29). The T cell specific zinc finger transcription factor GATA sequence binding protein 3 (GATA-3) is the primary activator of T_H2 specific differentiation and cytokine production. This protein is critical not only for T_H2 development, but for T cells in general, as thymocytes from GATA-3 deficient mice do not develop beyond the double-negative stage and therefore do not develop T cells (30, 31). In contrast to T-bet, GATA-3 inhibits T_H1 development and T_H1 cytokine production (32), making it a master regulator of T_H2

lineage commitment. Activation of GATA-3 is mediated through STAT6, leading to T_H2 differentiation and IL-4 production (33, 34). IL-4 was originally thought to be the paradigmatic cytokine involved in asthma pathogenesis, particularly through its regulation of the synthesis of the reaginic antibody IgE. IL-$4^{-/-}$ mice displayed no development of allergic symptoms when challenged with antigen (35, 36). Further, antibody neutralization of IL-4 prior to allergen sensitization prevents the development of allergic inflammation (37). An additional interesting finding from this work was that antibody neutralization administered after priming, but prior to allergen stimulation, was not effective at preventing allergic inflammation. Such studies suggest that IL-4 is essential for the development and expansion of antigen-specific T_H2 cells, but it is not sufficient for the effector phase of the allergic response.

Although T_H2 cytokines contribute to the immune response to environmental antigens, recent evidence has shown a singular role for IL-13 in the mediation of allergic diathesis. The cytokine IL-13 regulates IgE production, eosinophilic inflammation, airway-smooth muscle hyperplasia, mucous production, and the recruitment of leukocytes into airway spaces. In animal studies, IL-13 was shown to be both necessary and sufficient to induce features of allergic asthma independent of any other T_H2 cytokines (38, 39). Furthermore, IL-13 is overexpressed in the lungs of human asthmatics and genetic polymorphisms in the IL-13 gene may contribute to the susceptibility to asthma (40).

One of the key features of T_H1/T_H2 development is the silencing of the gene encoding the signature cytokine of the opposing differentiated state, as is the case with T-bet and GATA-3. One cytokine neither of these cell types produce, however, is IL-17.

In fact, fully differentiated T_H1 and T_H2 silence the IL-17 gene and inhibit the differentiation of a new subset of T helper cells, T_H17 (41). These newly characterized T helper cells seem to be important in the pathogenesis of autoimmune disorders (42, 43). Transcriptionally, T_H17 cells need the mobilization of Ca^{2+} to activate NFAT, as the proximal promoter of the IL17A gene contains two NFAT binding sites and needs active NFAT to produce IL-17 cytokine (44). A transcription factor preferentially expressed in T_H17 cells is the retinoic acid-related receptor gamma in T cells (RORγt), and its presence is also required for IL-17 production (45). RORγt$^{-/-}$ mice have reduced numbers of T_H17 differentiated cells, while transgenically overexpressed RORγt produces an excess of T_H17 cells. Experimentally, the overexpression of RORγt leads to an increase in susceptibility to experimental autoimmune encephalomyelitis (EAE), while the RORγt-deficient mice are less susceptible to this disease.

In the context of the following studies, it was sought to determine whether the cell-permeable peptide generated to disrupt the interaction between Itk and SLP-76 led to a downstream effect on cytokine expression and T helper cell differentiation. Given the involvement of Itk in T_H2 cytokine production, the peptide would be expected to affect cytokine production as it does Itk activity. Since the role of Itk is not known in IL-17 production or in T_H17 development, but given Itk's involvement in Ca^{2+} flux leading to NFAT activation, IL-17 cytokine expression was also tested in the presence of the peptide.

4.2.2 Cell-penetrating peptides

The hydrophobic nature of the lipid membrane bilayer makes it impermeable to most hydrophilic molecules creating an effective shield from the outside environment. This same barrier creates a major obstacle for introducing man-made therapeutic agents to act within the cell. Delivery of chemicals, nucleic acids, or proteins into cells is feasible, with caveats. Viral vector delivery is very effective, but suffers from capacity issues, protein production variability, and more alarmingly, possible *in vivo* adverse events due to genetic recombination and severe immunogenicity. Non-viral delivery, such as with lipids, are cost-effective and non-immunogenic, but are limiting with their ability to transfect only non-dividing cells, have low transfection efficiency levels compared to viral delivery, and suffer from toxicity issues. Recently, cationic and amphipathic peptides, called cell-penetrating peptides (CPPs), have become a useful tool for delivery of agents into a cell. These CPP mediated delivery have experimentally been successful at delivering a wide variety of entities, including proteins, peptides, nucleic acids (DNA vectors, siRNA), and even liposomes in both *in vitro* and *in vivo* settings. In a commonly accepted landmark paper lauding the usefulness of CPPs, intraperitoneal injection of a fusion protein consisting of the HIV transmembrane sequence Tat (a.a. 47-57) and β-galactosidase, was able to distribute the active enzyme to nearly every organ in the injected mouse, including being able to cross the blood brain barrier (46). Despite a still yet unknown (or debatable) mechanism for cellular entry, CPPs are beginning to become accepted as delivery vectors for biologically active cargos.

The discovery of cell-penetrating peptides was made through the observation that the Tat protein of HIV was independently able to translocate the plasma membrane and

that the portion of the Tat protein sufficient for this translocation was a positively charged sequence between amino acids 48 and 60 (47). It was later realized that this is an evolutionarily conserved phenomenon, as a 16 amino acid sequence (since named Penetratin) within the homeodomain of the antennapedia transcription factor in *Drosophila* is also able to translocate plasma membranes, and deliver the antennapedia protein into the nucleus (48). These naturally occurring peptide sequences have taken on an additional name, protein-transduction domains (PTDs) used here synonymously with CPP. These sequences of about 7 to 30 amino acids contain many positively charged amino acids, mostly arginines and/or lysines. Consequently, synthetic peptides consisting of a stretch of positively charged amino acids have been used successfully for cellular transduction, and an optimal range of positively charged amino acid sequence has been determined (49, 50).

One of the disadvantages of cell-penetrating peptides is their non-specific delivery to a cell regardless of whether the molecular target is available or not. Therefore, the challenge of CPP delivery is to either deliver the peptide in a tissue specific manner, so as to limit the activity of the peptide towards a target in a tissue where the activity is unwanted, or to design the peptide to target a specific protein or protein-protein interaction unique to certain cells or tissues. Tissue specific delivery can be accomplished by creating an anionic peptide sequence that non-covalently binds the cationic PTD, linked by a tissue-specific protease cleavage site (51). When the peptide enters the cell within the tissue where the active protease is present, the peptide be cleaved, releasing the biologically active peptide to act upon its target protein or mechanism. Alternatively, designing a CPP to target specific protein-protein interactions

has been shown to work effectively. For instance, a CPP designed to affect the interaction between NFAT and its activating phosphatase calcineurin was able to immunosuppress the graft versus host reaction in mismatched islet allografts in mice (52). In the following studies, the latter approach was employed to target the very specific protein-protein interaction of Itk and SLP-76 that occurs only in T cells and not in any other type of cell. Although this specifically designed CPP may get into any type of cell in the body, it has no available target outside of T cells, which whould limit any cross-indications, or side effects in other tissues.

4.2.3 Multi-parameter Flow Cytometry

Flow cytometry is a powerful tool for quantitating vast numbers of cells from a sample and determining relative populations of cells within that sample. The characterization is often done by staining the cell surface with antibodies specific for differentiated markers unique or characteristic of the cell types. However, this method does not provide much information about the functional response of the cells to stimuli. Furthermore, cells that can appear to be homogenous using surface characterization have been found to be heterogeneous when evaluated on their response to stimuli. Flow cytometry, therefore, has grown from merely a quantitative measurement to one that can measure qualitative responses and determine cellular function.

Several methods have been developed to measure cytokine expression. Among these are enzyme-linked immunosorbent assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR), enzyme-linked immunospot (ELISPOT),

immunocytochemistry, and intracellular cytokine staining (ICCS). ELISA measures the amount of secreted cytokine protein, RT-PCR measures the amount of induced mRNA (as does the RNase protection assay), ELISPOT estimates the frequency of cytokine producing cells, immunocytochemistry determines the localization of cytokine producing cells in tissues, while ICCS measures not only the frequency of cytokine producing cells, but the level of cytokine expressed by each cell. Intracellular cytokine detection by flow cytometry has emerged as the premier technique for studying cytokine production at the single-cell level. Multiparameter flow cytometry permits simultaneous detection of two or more cytokines within a single cell allowing for direct T_H1 versus T_H2 determination. Compared to ELISA, ICCS can be done using multicolor staining to determine exclusive expression or mutual co-expression of different cytokines within individual cells. This allows for the characterization of T cell subsets based on cytokine production rather than just cell surface markers. This capability, which when combined with the high throughput inherent in the instrumentation, gives intracellular cytokine staining an enormous advantage over existing single-cell techniques such as ELISPOT, limiting dilution analysis, and T cell cloning (the original method used to characterize lymphocytes with polarized cytokine profiles (20)). However, because of the greater level of non-specific binding inherent in fixed, permeabilized cells, greater care must be taken in designing specificity controls (53). Further, chemical inhibitors, such as Brefeldin A and Monensin, must be used to prevent cytokine secretion and allow the expressed cytokines to build up within the cell so that the antibody against the cytokine can bind and improve the signal to noise ratio. Thus, performing flow cytometric analysis on intracellularly stained cells allows for individual characterization of large

numbers of cells simultaneously in order to help elucidate the cellular characteristics among the heterogeneity of the cellular population.

Currently, the only technique that can simultaneously assess the phenotype, the magnitude, and functionality of a T cell response is flow cytometry. Throughout the T lymphocyte focused literature, the magnitude of a response is often cited as the paramount characteristic for the T cell mediated immune response. Recently, however, the quality of the T cell response can now be based upon the combination of a multitude of parameters determining the functionality of the immune response (54). Cellular functions, such as the ability to clonally expand, cause proliferation in other cells, organize immune responses by the secretion of chemokines, and carry out effector functions either by direct cytotoxic killing or by the secretion of cytokines are all multi-dimensional factors that determine how effective the immune response. Through this characterizing approach, cells with a greater degree of multifunctionality correlate to a less progressive disease state, or to one of effective protection from disease.

The analysis of cellular signaling pathways has been limited to biochemical techniques using cell populations that have been homogenized, often through lysis. Primary cell populations, usually heterogeneous or cells of a rare quantity, have been difficult to study biochemically as often they give little interpretable results. Further, cell populations that appear to be homogenous by cell surface staining or by Western blotting may not be when evaluating intracellular signaling on a single-cell level. The quantitative nature of multi-parameter flow cytometry allows the researcher to analyze a variety of signaling components simultaneously within a single cell. There are further advantages to multi-parameter flow cytometry and specifically phospho-epitope flow

cytometry which include shorter experimental time (half a day compared to 1-2 days using Western blotting), simultaneous analysis of multiple epitopes within the same cell, a large and dynamic quantitative range for data analysis (log scale), immediate quantitation through software algorithms (including population statistics and peak area characterization), and the ability to analyze complex or rare cell populations. However, one disadvantage to phospho-epitope flow cytometry includes the inability to analyze data with respect to the localization of the epitope within the cell. This remains the purvey of microscopy or of fractionation of the cell population sample prior to Western blotting. This disadvantage is rapidly dissolving with the advent of quantitative laser-scanning cytometry (55). Another disadvantage to phospho-epitope flow cytometry is the inability to detect low-abundance signaling proteins. Western blotting uses enzymatic amplification (e.g., HRP), which improves its signal-to-noise ratio. Also, since the cells have been lysed, the protein epitopes have already been denatured, so blotting does not have any issues with antigen accessibility or cellular autofluorescence.

Finally, the use of multi-parameter flow cytometry, particularly its simultaneous measurement of many cellular events, is extremely useful in the screening of protein inhibitors and their efficacy within the cell. Drug screening is often done using cell-free *in vitro* chemical libraries that obfuscate results when translated into cell-based systems. With multi-parameter flow cytometry, one can identify hyperactive pathways in a disease state and identify inhibitors for it, as well as observe the normally active pathways in normal cells that should be unaffected by the inhibitors. Further, this analysis could often be performed from non-manipulated biological specimens (e.g., blood samples), negating the use of tissue biopsies since the nature of flow cytometry could discriminate between

healthy and diseased cells without all the interference inherent in biological matrices, while being able to monitor the pharmacodynamic profile of the signaling pathways affected by the inhibitor.

In studies shown hereafter, a twelve amino acid peptide representing the binding site of Itk-SH3 domain on the PRR of SLP-76 was generated and rendered cell-permeable by the addition of nine arginines to its amino-proximal end. This cationic peptide is named R9-QQP after the nine arginines and the first three amino acids of binding sequence. The studies show that the peptide is taken up by T cells, disrupts the association between SLP-76 and Itk, affects the specific transphosphorylation of Itk on tyrosine 511, and consequently affects the production of T_H2 cytokines. This provides the first evidence of live cell consequences for the disruption of the specific interaction between the SH3 domain of Itk and the PRR of SLP-76. Further, it demonstrates that the addition of cell-permeable sequences to proteins has the useful potential to not only parse signal transduction pathways *in vivo*, but can have therapeutic potential as well. Finally, since Itk plays a significant role in the production of T_H2 cytokines, the data presented here provides a means to manipulate Itk pharmacologically in disease states.

4.3 Materials and Methods

4.3.1 Cells, Mice, Antibodies, and other reagents

Jurkat T cells (E6.1) were obtained from American Type Culture Collection; $SLP76^{-/-}$ (J14) and $LAT^{-/-}$ (JCaM2.5) Jurkat T cells were a kind gift from Dr. A. Weiss (University of California at San Francisco, San Francisco, CA). Cells were cultured in RPMI 1640 culture medium containing 5% FBS in a 37°C humidified 5% CO_2 chamber and stimulated according to each individual figure. Itk-GFP constructs were constructed and used according to previously used protocols (15). Anti-Itk (clone 2F12) and anti-Gads antibodies were purchased from Millipore (Temecula, CA). Anti-SLP-76 antibody was purchased from Cell Signaling Technology (Danvers, MA). Anti-Lck (clone 2102) and anti-PLCγ1 (clone 1249) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing phospho-epitopes, anti-Itk pY511 (clone 24a), anti-PLCγ1 pY783 (clone 27) were purchased from BD Biosciences (San Jose, CA), while anti-Lck pY394 (also anti-Src pY416) was purchased from Cell Signaling Technology (Danvers, MA). Antibodies recognizing mouse cytokines anti-IL-4 (clone 11B11), anti-IFNγ (clone XMG1.2), and anti-IL-2 (clone JES6-5H4) were purchased from BioLegend (San Diego, CA) while anti-IL-13 (clone eBio13A) and anti-IL-17A (clone eBio17B7) were purchased from eBioscience (San Diego, CA). Normal rabbit IgG and rabbit anti-mouse IgG were purchased from Jackson Immunoresearch (West Grove, PA). Protein G sepharose was obtained from Sigma-Aldrich (St. Louis, MO). $Itk^{-/-}$ mice were provided by Dr. D. Littman (New York University School of Medicine,

New York, NY) through Dr. T. Kawakami (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Male C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were handled in accordance with the guidelines of the IACUC at San Diego State University, and were experimented upon between the ages of 6-12 weeks. All graphing and statistics were done using GraphPad Prism (GraphPad Software, San Diego, CA).

4.3.2 Cell permeable synthetic peptides and treatment

The peptides RRRRRRRRRQQPPVPPQRPMA (R9-QQP), RRRRRRRRRPQMPAPQRPQPV (R9-SCR), and RRRRRRRRR (R9) were synthesized on an Applied Biosystems 433A peptide synthesizer on Fmoc amide resin 4-(2',4'-dimethoxyphenyl-F-moc-aminomethyl)-phenoxy (Novabiochem) using standard Fmoc protocols (56). FITC was linked to the N-terminus of the peptide through an aminocaproic spacer. Peptides were purified on a C18 preparative reverse phase-HPLC column (Vydac) to 95% purity. The mass was confirmed by matrix-assisted laser desorption mass spectrometry (MALDI) on a Micromass TOF spec 2E (Waters).

Twenty million Jurkat cells or 70 million murine splenocytes per ml of medium (RPMI 1640 without serum), were incubated with various amounts of the indicated peptide for 30 minutes at 37°C in a humidified atmosphere of 5% CO_2-air. Cells were washed and stimulated in the various protocols described below. There was no cellular toxicity with any of the peptides and concentrations tested.

4.3.3 Flow cytometry

Phosphorylation events measured by flow cytometry were conducted in a manner similar to a published work (57). Stimulated cell reactions were stopped with 2% paraformaldehyde and allowed to fix. Cells were then permeabilized with 100% methanol, and afterwards were blocked with 30 μg/mL species-specific normal serum in PBS. Cells were stained with phospho-specific antibodies and then analyzed by flow cytometry. Intracellular cytokine staining involved stimulating isolated splenocytes with both anti-CD3ε and anti-CD28 antibodies overnight and then restimulating for 6 hours in the presence of Brefeldin A. Cells were first stained with 5 μg/mL Alexa Fluor 647-conjugated anti-mouse CD4 antibody (clone RM4-5), fixed with 4% paraformaldehyde, and then permeabilized with 100% methanol. Cells were then blocked with 30 μg/mL species-specific normal serum in a saponin containing PBS buffer (0.1% saponin, 0.5% BSA, in PBS), stained with an anti-cytokine antibody, and then analyzed by flow cytometry on $CD4^+$ cells. Isotype controls were used for each dye to set the cytometry gates. The collection was completed on a FACSAria Flow Cytometer (BD Biosciences, San Jose, CA), and analysis was done using either FACSDiva software (BD Biosciences, San Jose, CA) or with FlowJo software (Tree Star, Inc., Ashland, OR).

4.3.4 Immunoprecipitations and Immunoblotting

Lysates were precleared with 10 μg/mL anti-Rabbit IgG for two hours, followed by 1 hour of incubation with protein G conjugated sepharose beads. Precleared lysates were incubated overnight with 5 μg/mL anti-SLP-76 antibodies and incubated with

protein G sepharose beads for one hour. Immunocomplexes were washed three times with 1X lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris (pH 7.3), 0.4 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 μM sodium o-vanadate, and 1 μM PMSF) and denatured in 1X Laemmli's buffer by boiling for five minutes. Samples were run on a 4-12% gradient SDS-PAGE and then transferred to PVDF membrane. The membrane was first probed for co-immunoprecipitating proteins (e.g. anti-Itk, then anti-Gads, etc.), stripped (2% SDS, 100 mM β-mercaptoethanol, 62 mM Tris, pH 6.7), re-probed between each blotting, and finally probed for anti-SLP-76. All blotting antibodies were used at 1 μg/mL. All blots were scanned by densitometry to measure ratios of co-immunoprecipitating proteins and results were cumulated using the Mean ± SEM, with statistics using the student t-test.

4.3.5 Analysis of Itk Localization

Itk-GFP localization was performed similar to that of a previous work (16). Briefly, transfected Jurkat cells were incubated with polystyrene beads (6 μm diameter, Polysciences Inc., Warrington, PA) precoated with anti-CD3ε (OKT3) or with isotype control antibodies (UPC10). The cell-bead mixture were centrifuged for five minutes at 50*g* to ensure contact, and then incubated at 37°C for 2 minutes. The cells were then fixed on slides using 4% paraformaldehyde and visualized using a 100X objective on a laser scanning confocal microscope (Leica TCS SP2, Leica, Heidelberg, Germany). A region of interest was drawn at the cell/bead membrane interface and its pixel intensity for that area was measured, while an equal area region of interest pixel intensity was

measured at a membrane site not engaging a bead. The localization index was calculated as the ratio between the pixel intensity at the contact site and the pixel intensity of an equal area at the control site.

4.4 Results

4.4.1 SLP-76 is critical for the TCR-induced activation

The adaptor proteins LAT and SLP-76 associate with Itk in a TCR-inducible signaling complex. However, since this observation was determined using GST-fusion proteins in vitro, it was sought to determine whether this interaction occurred within intact cells. In Figure 4.1, this observation is confirmed in Jurkat (clone E6.1) T cells stimulated with anti-CD3ε antibodies for up to 10 minutes demonstrating that SLP-76 association with Itk is both time-dependent and transient. The SLP-76/Itk association increases within 1 minute of stimulation and becomes resolved after 5 minutes (Figure 4.1). Furthermore, the presence of SLP-76 is critical for the transphosphorylation of Itk, as comparative studies in Jurkat cells containing (E6.1) and deficient in SLP-76 (J14) show (Figure 4.2). Since transphosphorylation of Itk is critical for its activation, these data suggest that SLP-76 plays an important role in the activation of Itk.

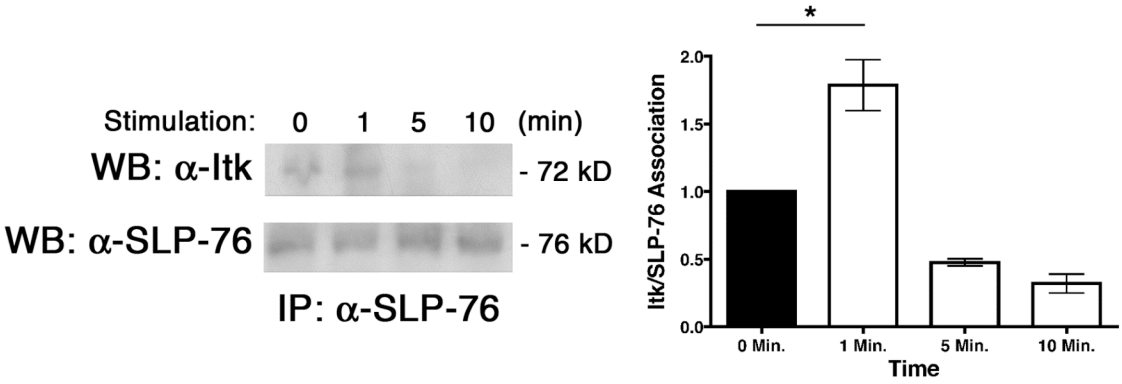

Figure 4.1. *Itk Inducibly Associates with SLP-76 upon Stimulation.*

Jurkat T cells were stimulated with anti-CD3ε antibodies for up to 10 minutes, lysed, immunoprecipitated with anti-SLP-76 antibodies and then run on SDS-PAGE. Western blotting was conducted with anti-Itk antibodies, and then re-probed with anti-SLP-76 antibodies for loading. The graph represents the Mean ± SEM for the association of Itk with SLP-76 over time cumulated from three experiments. The bar with asterisk above represents a statistically significant ($p < 0.05$) increase in association.

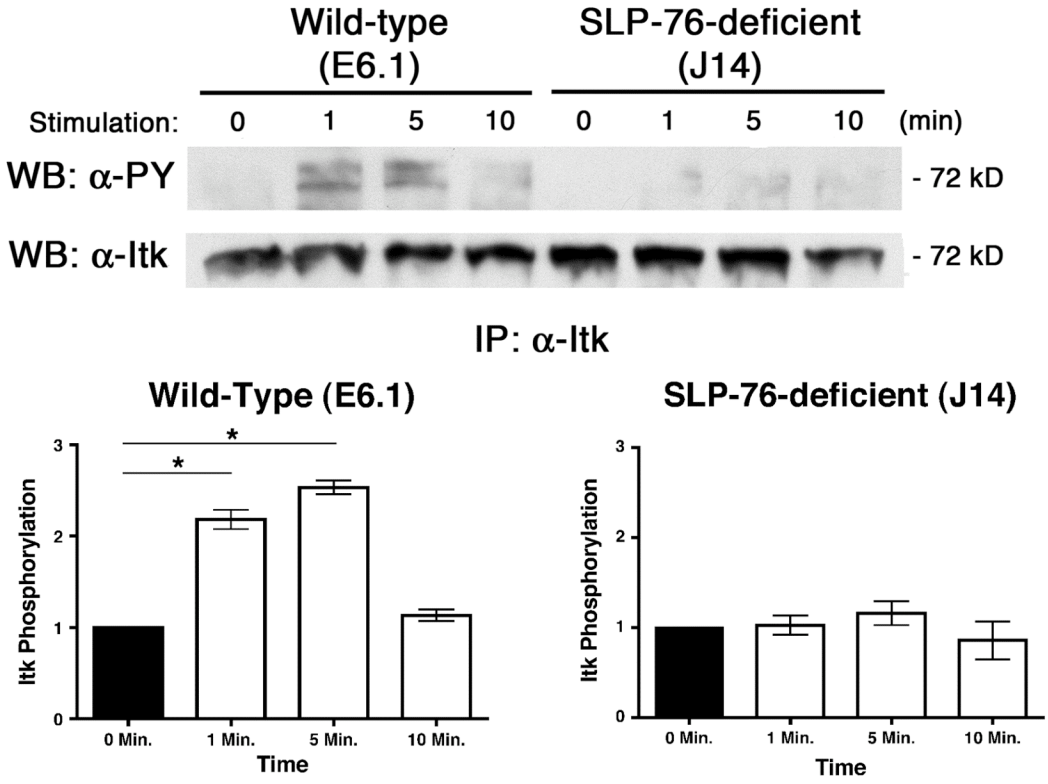

Figure 4.2. *SLP-76 is Necessary for the Activation of Itk.*

Jurkat T cells, either wild-type (E6.1) or SLP-76-deficient (J14) were stimulated with anti-CD3ε antibodies as indicated for the above time-points, lysed, and immunoprecipitated with anti-Itk antibodies. Samples were resolved on SDS-PAGE and Western blotted with anti-Phospho-Tyrosine antibodies and then re-probed with anti-Itk antibodies for loading controls. Densitometric analyses of the Western blot bands of the Itk phospho-tyrosine signals were performed and values were normalized to loading control bands. The left panel graph represents the tyrosine phosphorylation of Itk over time in wild-type Jurkat T cells while the right panel graph represents the tyrosine phosphorylation of Itk over time in SLP-76-deficient Jurkat T cells. Each graph represents three cumulative experiments displaying the Mean ± SEM.

4.4.2 SLP-76 is required for the recruitment of Itk to the T cell APC engagement site

It has been previously demonstrated that upon TCR engagement Itk undergoes intracellular relocalization from a diffuse cytoplasmic pattern to a distinct aggregation at the T cell-APC interface (15). LAT is another adaptor protein whose presence is required for Itk activation. It has previously been reported that Itk and LAT participate in a signaling complex upon TCR engagement and that in the absence of LAT, Itk does not become transphosphorylated and enzymatically activated (17, 58). Since SLP-76 and LAT appear to be important for the transphosphorylation/activation of Itk, the question is raised whether they play any role in its recruitment to the T cell-APC interface. To address this, a surrogate APC system where polystyrene beads coated with stimulatory anti-CD3ε or isotype control antibodies were incubated with Jurkat cells and the cytoplasmic redistribution of Itk in the cells was assessed using confocal microscopy (Figure 4.3).

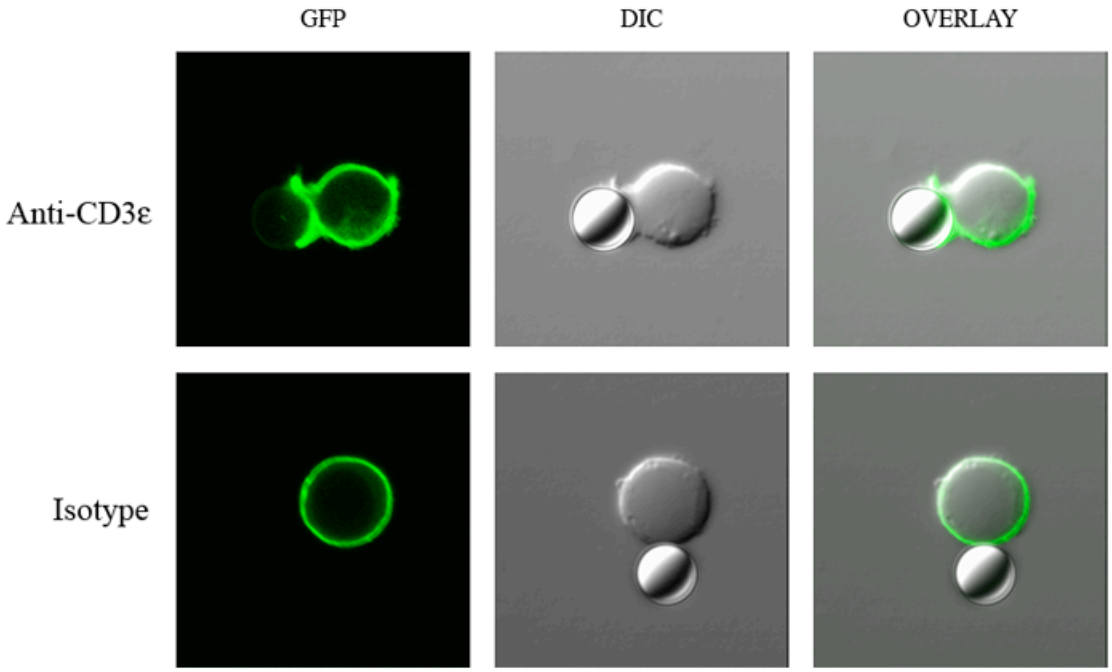

Figure 4.3. *Itk localizes to the T cell surrogate antigen presenting cell contact site.* WT-Itk-GFP was transfected into Jurkat T cells, mixed with polystyrene beads coated with anti-CD3ε antibodies (top panels) or isotype control antibodies (bottom panels) and visualized by confocal microscopy. GFP pixel intensity was evaluated at the T cell-bead contact site and at a membrane site of equal area opposite the interaction site, the ratio of which was calculated to establish a localization index.

Figure 4.4 demonstrates that Jurkat T cells transfected with WT-Itk-GFP engaging beads coated with stimulatory antibody (Figure 4.4, left side, filled circles) causes a significant increase in the localization index compared to isotype control beads (Figure 4.4, left side, unfilled circles). Importantly, Jurkat cells deficient in SLP-76 expression (J14) did not display such an increase (Figure 4.4, middle, compare filled to unfilled squares). Surprisingly, the absence of LAT had no effect on Itk recruitment

(Figure 4.4, right side, compare filled to unfilled diamonds). These data indicate that Itk recruitment to the TCR-induced signalosome is dependent on SLP-76.

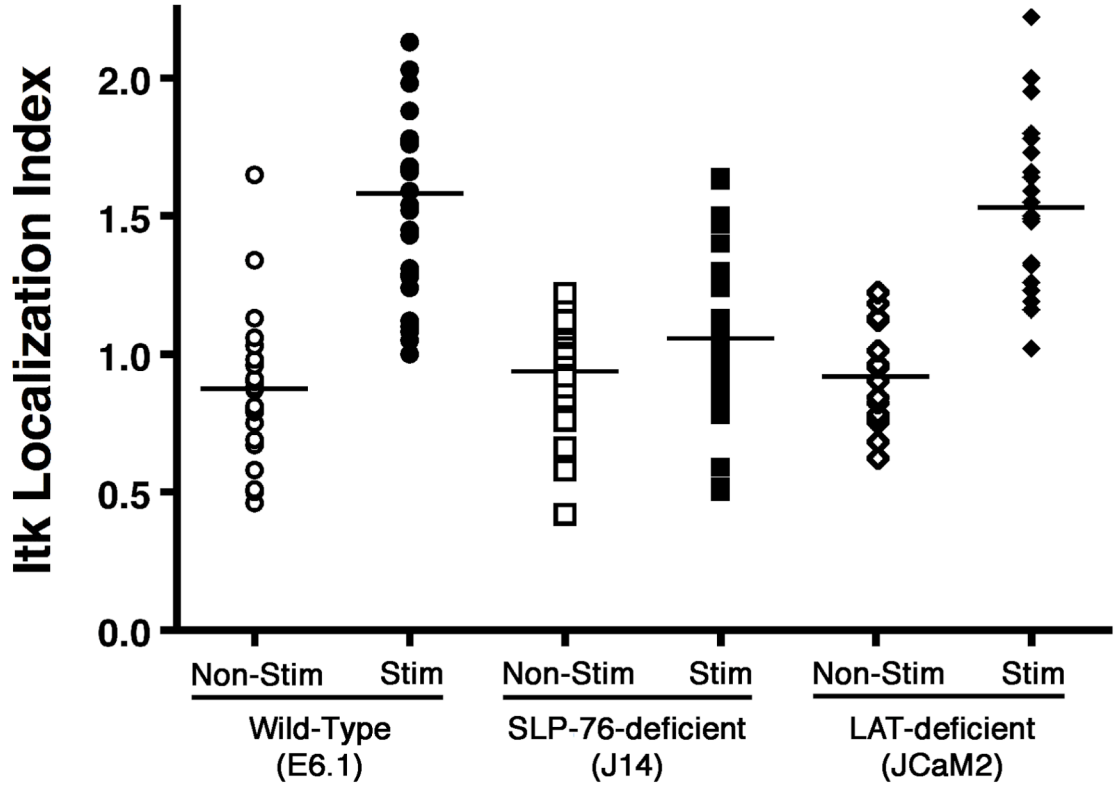

Figure 4.4. *Itk recruitment to the T cell/APC interface is dependent upon the presence of SLP-76, but not LAT.*

Jurkat T cells, either wild-type (E6.1), SLP-76-deficient (J14), or LAT-deficient (JCaM2), were transfected with Itk-GFP and stimulated through engagement with either non-stimulating (unfilled) or stimulating (filled) antibody coated polystyrene beads. Itk localization was imaged through 100X objective confocal microscopy and analyzed by ImageJ. The localization index is defined as the amount of Itk localization (as measured by mean pixel intensity) at the T cell/APC contact interface divided by the identical area directly opposite from that contact site. Each plot represents one cell conjugate measured cumulated over three experiments.

4.4.3 Internalization of Arginine-modified peptides

The above data validate that SLP-76 is critical for both the recruitment of Itk to the T cell-APC interface and for its activation. *In vitro* studies have demonstrated that the association of Itk with SLP-76 is regulated by the Itk SH2 and SH3 domains in a cooperative fashion (18). In particular, the SH3 domain of Itk interacts with amino acids 184-208 of the PR region of SLP-76 and synthetic peptides representing this region can inhibit the interaction *in vitro* (59). These data, along with the studies discussed above, support the assertion that the interaction between Itk and SLP-76 is important for the activation of Itk. However, the consequences disrupting this interaction have not been demonstrated with intact proteins in live cells and thus, its biological significance is only suggestive. To further pursue the biological significance of the interaction between Itk and SLP-76, a peptide representing amino acids 184-195 of the SLP-76 proline rich region (PRR) was synthesized, and rendered cationic by the addition of nine arginine residues to its N-proximal end. This peptide is referred to as R9-QQP (relating to the 9 tandom arginine tag and the first three residues of the SLP-76 PRR). To demonstrate that R9-QQP can penetrate the cell membrane, a fluorescein tag was conjugated to the peptide and was then tested for its intracellular translocation. Jurkat cells incubated with various concentrations of labeled peptide were analyzed by flow cytometry and by confocal microscopy. The data in Figure 4.5A clearly demonstrate a peptide dose-dependent increase in fluorescence intensity. Confocal microscopic analysis of the peptide-treated cells indicated that intracellular translocation of the peptides and fluorescence were not due to extracellular attachment to the membrane (Figure 4.5B).

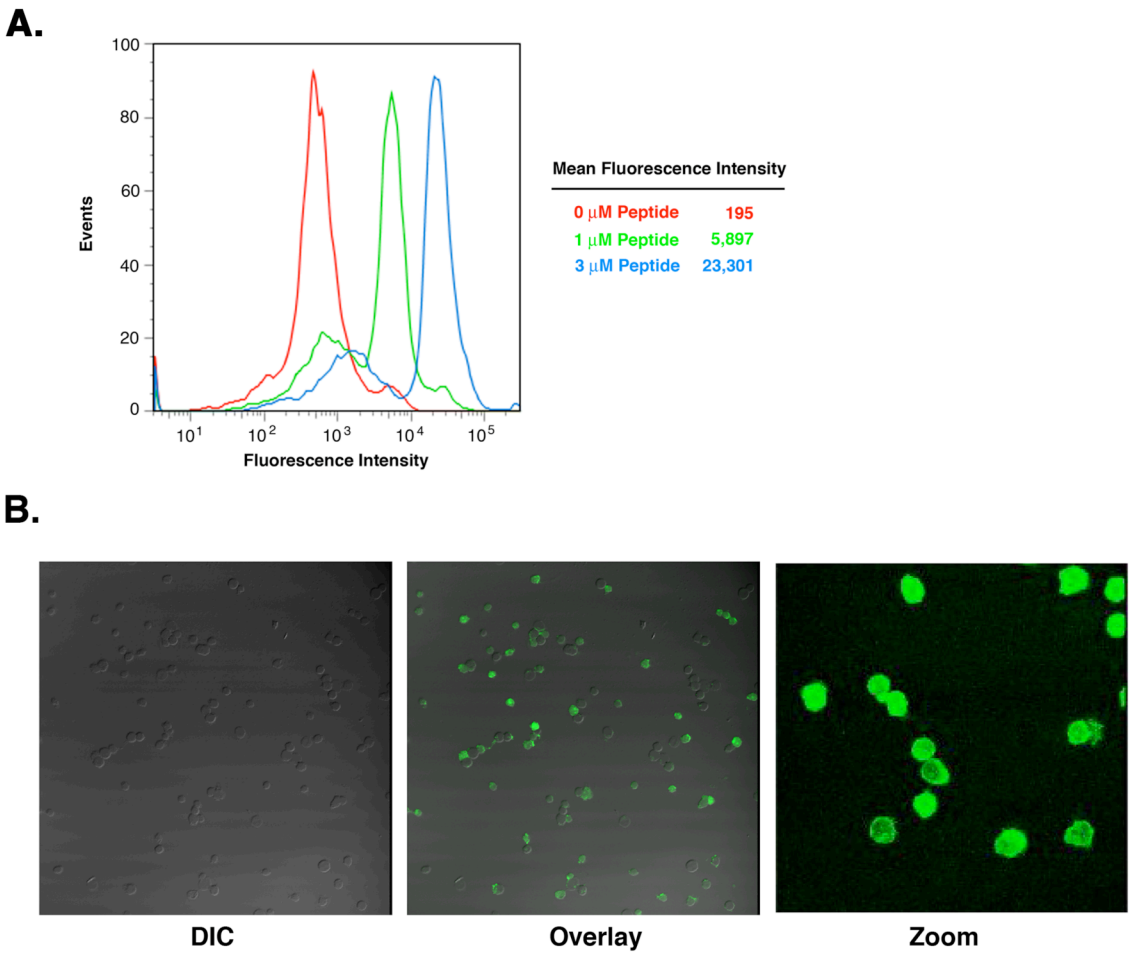

Figure 4.5. *Cell-penetrating peptides can enter live T cells.*
A.) FITC-conjugated R9-QQP peptides at various concentrations were cultured with Jurkat T cells for 30 minutes at 37°C and then analyzed by flow cytometry. Each colored peak represents the amount of peptide cultured, with the mean fluorescence intensity for each condition shown. B.) Confocal images of cells treated with 1 μM of FITC-conjugated R9-QQP. Left panel shows DIC image, middle panel shows entry of FITC-peptide in cells, and right panel shows 8X digital zoom of peptide presence within individual cells.

4.4.4 Disruption of Itk and SLP-76 association in live cells

To assess the *in vivo* effects of R9-QQP peptide entry, Jurkat T cells were incubated with various concentrations of R9-QQP or control peptides (R9-SCR or R9), and stimulated through their antigen receptors to evaluate the inducible association

between Itk and SLP-76. As exhibited in Figure 4.6, the inducible association between Itk and SLP-76 was disrupted upon addition with R9-QQP peptide in a dose-dependent manner. Quantification of the Western blot bands indicates that fifty percent inhibition of Itk and SLP-76 association was affected by approximately 0.8 μM of R9-QQP peptide treatment (Figure 4.6B). This disruption was specific for this particular R9-QQP peptide as a similarly cationic scrambled peptide (R9-SCR) or a simple poly-arginine peptide (R9) did not cause significant inhibition at the highest concentration tested (Figure 4.6). Furthermore, the inhibition was specific for the interaction between Itk and SLP-76, as the interaction between Gads and SLP-76 was not affected by the incubation with R9-QQP (Figure 4.6A). *In vitro* studies have previously shown that the SH3 domain of Gads also interacts with the PRR region of SLP-76 in an area that is distinct of Itk binding (18, 60), however the R9-QQP peptide had little effect on their interaction. Therefore, the disruption of the inducible interaction between Itk and SLP-76 by the R9-QQP peptide is specific.

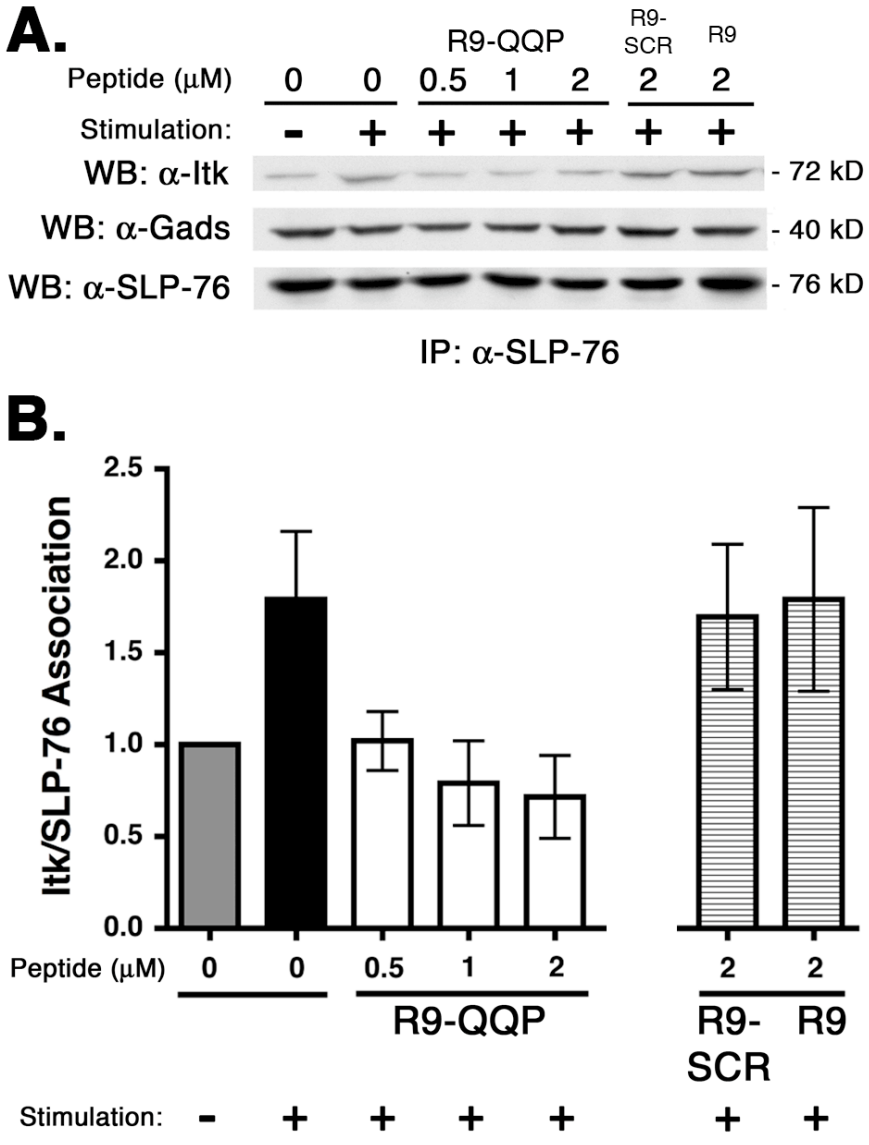

Figure 4.6. *The cell-penetrating peptide R9-QQP can specifically disrupt the association between Itk and SLP-76.*

A.) Jurkat T cells were incubated with various amounts of R9-QQP peptide or control peptides (R9-SCR or R9), stimulated with anti-CD3ε cross-linked antibodies for 1 minute, lysed, immunoprecipitated with anti-SLP-76 antibodies and the immune complexes were resolved by SDS-PAGE. The membrane was probed with anti-Itk antibodies (top panel), and then re-probed with anti-Gads antibodies (middle panel) and anti-SLP-76 antibodies (bottom panel). B.) The graph represents the densitometric analyses of the Western blot bands of the Itk/SLP-76 association in the presence of various concentrations of peptide. Densitometric analyses values were normalized to loading control bands. The graph is the cumulative mean from five individual experiments ± SEM.

4.4.5 Inhibition of Itk transphosphorylation by cell penetrating peptides

As mentioned above, SLP-76 is critical in this process of transphosphorylation of Itk on tyrosine 511 by the Src kinase Lck (Figure 4.2 and (61)). However, this effect on transphosphorylation has been demonstrated only in genetically altered systems, such as in SLP-76 Jurkat mutant cell lines or in knockout mouse null backgrounds, or with *in vitro* assays using purified proteins, and thus the biological significance of the association between Itk and SLP-76 in live cells has only been inferred. Since R9-QQP was found to inhibit the interaction between Itk and SLP-76 in live cells, it was sought to determine its effects on Itk transphosphorylation. A phosphoflow cytometric assay was employed using specific anti-phospho-Y511-Itk antibodies in order to assess the status of Itk transphosphorylation. To ensure the specificity of this assay, Jurkat cells or splenic lymphocytes from either wild-type or $Itk^{-/-}$ mice were stimulated with anti-CD3ε antibodies and analyzed by flow cytometry. The data in Figure 4.7 demonstrate that the anti-phospho-Y511-Itk antibodies measure specific phosphorylation, as the percentage of positive cells increased in the case of stimulated Jurkat (12-15% positive cells) and normal splenocytes (7-9% positive cells), but not with splenocytes from $Itk^{-/-}$ animals (<1% positive cells). By evaluating the fold increase in stimulated versus non-stimulated conditions, specific Itk phosphorylation increased nearly 3-fold in both Jurkat cells and in normal splenocytes, while there was no increase in Itk-deficient cells, indicating non-specific background staining by the antibody (Figure 4.7B).

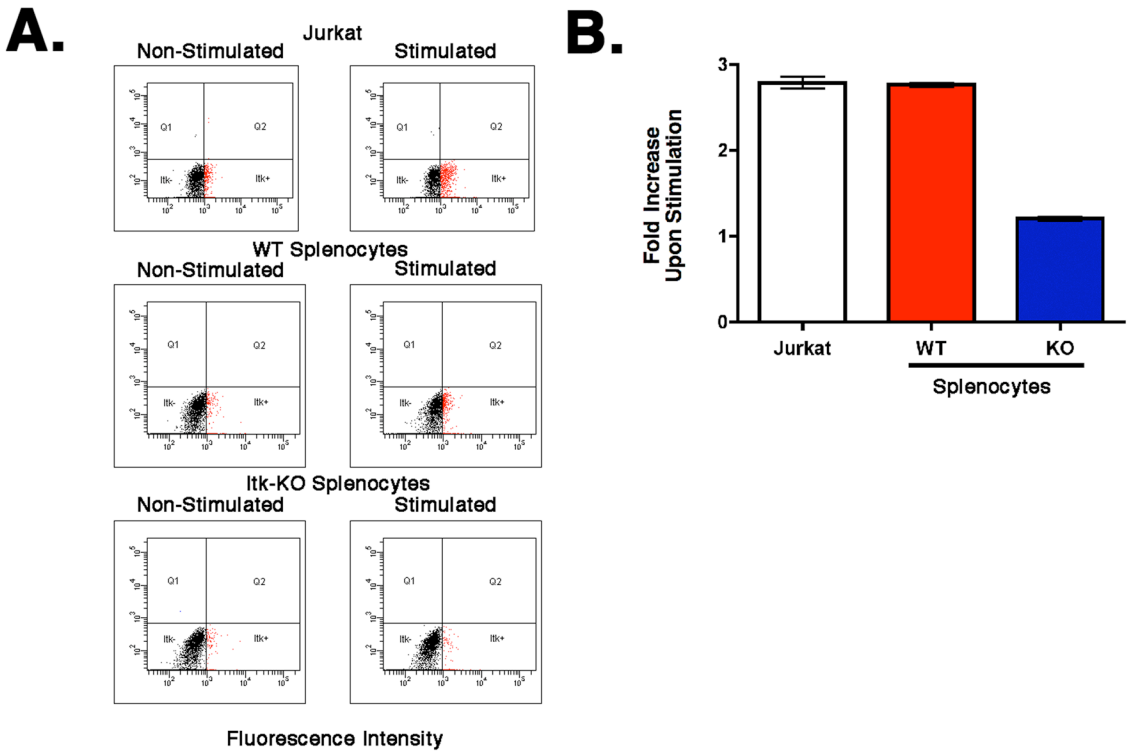

Figure 4.7. *The specific tyrosine phosphorylation of Itk can be measured by use of PhosphoFlow.*

A.) Jurkat T cells and mouse splenocytes (wild-type and Itk$^{-/-}$) were stimulated with anti-CD3ε cross-linked antibodies (stimulated) or isotype control cross-linked antibodies (non-stimulated), and then analyzed by PhosphoFlow using phospho-specific antibodies for tyrosine 511 of Itk. B.) This graph represents percent positive Itk tyrosine 511 phosphorylated cells in each condition cumulated over three experiments. The results show the mean fold increase upon stimulation ± SEM.

Utilizing this phospho-flow assay, the effects of R9-QQP peptide treatment on Itk-Y511 phosphorylation were assessed. Briefly, Jurkat cells or isolated splenocytes from normal mice were treated *ex vivo* with various R9-QQP concentrations and then stimulated with anti-CD3ε cross-linked antibodies. As evidenced in Figure 4.8, R9-QQP induced a peptide dose-dependent inhibition of Itk-Y511 phosphorylation both in Jurkat cells (Figure 4.8A) and in *ex vivo* treated splenocytes (Figure 4.8B). In three replicate experiments using Jurkat T cells, the average amount of peptide required to cause 50% inhibition of Itk tyrosine 511 phosphorylation was approximately 0.8 μM (Figure 4.8A). The R9-QQP mediated inhibition was specific, as treatment with the control R9 peptide did not cause significant inhibition at the highest concentrations tested (Figure 4.8A). However, there was a 30% inhibition of specific Itk tyrosine 511 phosphorylation using R9-SCR, although this most likely reflects non-specific effects as equivalent concentrations of R9-QQP caused 95% inhibition (Figure 4.8B). Further, inhibition with R9-SCR was not reproducible in all experiments, as treatment with this peptide failed to produce similar effects in mouse splenocytes (Figure 4.8B). In comparison, the effects of R9-QQP incubation on Itk phosphorylation was tested in mouse splenocytes *ex vivo* and were found to be reproducible (Figure 4.8B). R9-QQP incubation of splenocytes led to the inhibition of TCR-induced Itk tyrosine 511 phosphorylation in a dose-dependent fashion (50% inhibition occurs at approximately 0.85 μM peptide treatment). The effects of this R9-QQP incubation in primary mouse splenocytes were specific as indicated by the lack of significant inhibition by the control peptides, R9-QQP and R9 (Figure 4.8B).

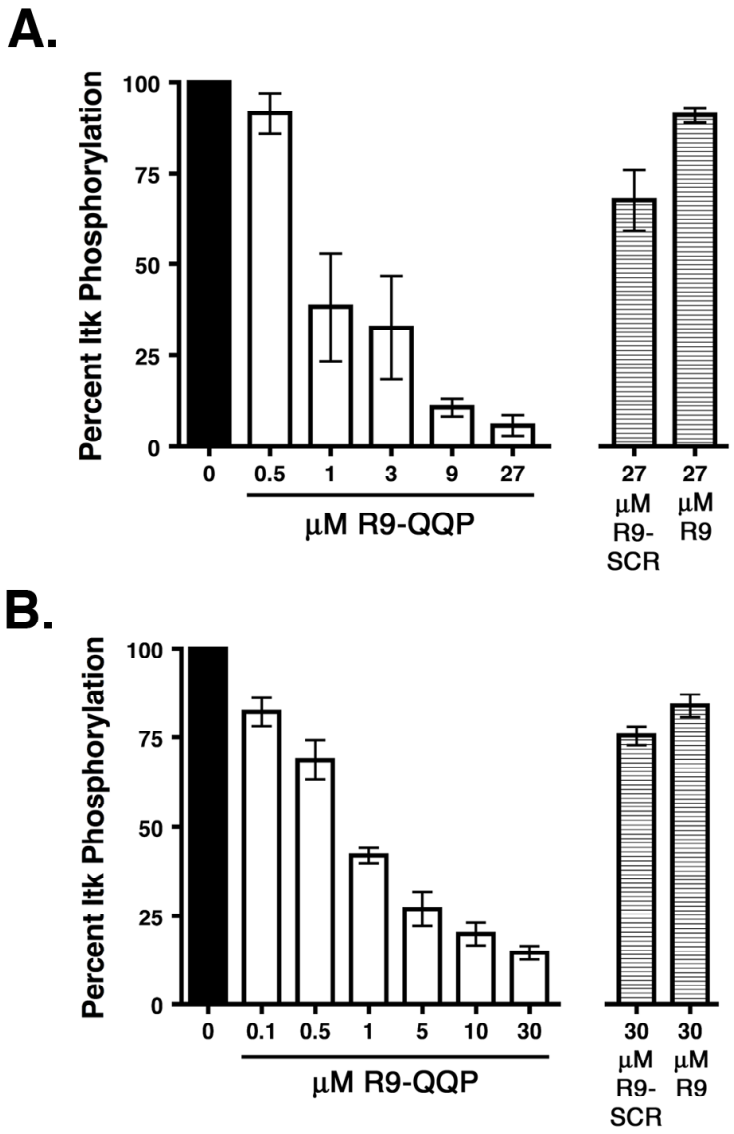

Figure 4.8. *The cell-penetrating peptide R9-QQP can inhibit the specific tyrosine phosphorylation required for Itk activation.*

A.) Jurkat T cells were incubated with various concentrations R9-QQP peptide or control peptides, stimulated with anti-CD3ε cross-linked antibodies, and then analyzed by PhosphoFlow using phospho-specific antibodies for tyrosine 511 of Itk. The graph represents percent positive Itk tyrosine 511 phosphorylated cells in each condition. B.) Mouse splenocytes were incubated *ex vivo* with various concentrations R9-QQP peptide or control peptides, stimulated with anti-CD3ε cross-linked antibodies, and then analyzed by PhosphoFlow using phospho-specific antibodies for tyrosine 511 of Itk. This graph represents percent positive Itk tyrosine 511 phosphorylated cells in each condition. Each graph shows the cumulative mean from three experiments ± SEM.

4.4.6 Selective inhibition of T_H2 cytokines by R9-QQP

It has been demonstrated that Itk controls the secretion of T_H2 cytokines (4, 62, 63). To further assess the biological significance of R9-QQP inhibition, the effects of R9-QQP treatment on TCR-mediated cytokine production were determined. In initial studies, mouse splenocytes were stimulated with anti-CD3ε plus anti-CD28 antibodies and production of both T_H1 and T_H2 signature cytokines was measured by intracellular flow cytometry. Cells were treated with peptides either 30 minutes before stimulation, 8 hours after stimulation, or both. Interestingly, the T_H2 signature cytokine IL-4 was significantly inhibited by the peptide treatment, but IFNγ that is characteristic for T_H1 cytokines was not significantly affected when the cells were treated with the peptide prior to stimulation (Figure 4.9). Treatment of cells with peptide 8 hours after stimulation reversed IL-4 cytokine production, resulting in a small increase, indicating that once cytokine production had been stimulated through the Itk pathway, it could no longer be inhibited. This is confirmed with data of cells treated with peptide 30 minutes before and 8 hours after stimulation displayed the same IL-4 cytokine production inhibition as was seen with a 30 minute pre-treatment alone. Furthermore, the inhibition was specific as control R9 or R9-SCR peptides had no significant effect.

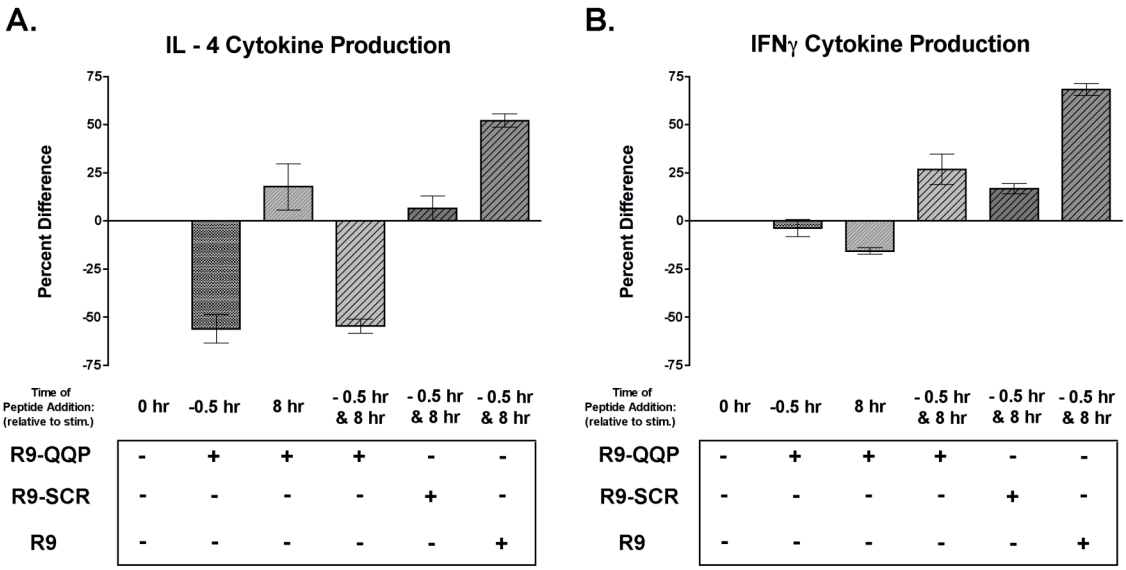

Figure 4.9. *Cell-penetrating peptide R9-QQP inhibits the TCR-induced expression of T_H2 cytokines.*

Wild-type (C57Bl/6) mouse splenocytes were treated with 5 μM peptide *ex vivo* for either 30 minutes prior to stimulation, 8 hours after stimulation, or for 30 minutes prior to and 8 hours after stimulation. Cells were allowed to incubate under stimulation conditions for 24 hours and were then analyzed for specific cytokine expression using intracellular cytokine staining. $CD4^+$ cells were stained and gated by flow cytometry and then specific intracellular cytokines were measured as shown in A.) IL-4 and B.) IFNγ. Data is presented as Percent Difference as calculated from stimulated cells not treated with peptide. Data is presented as the Mean ± SEM cumulated from two experiments.

Lymphocytes from Itk-deficient mice fail to express T_H2 cytokine genes (4, 63). In view of this evidence and the data described above, studies on the effects of R9-QQP on cytokine expression were expanded to determine the dose-dependent response to peptide treatment and to test for other affected cytokines. Murine splenocytes treated with R9-QQP, or control peptides, were stimulated with anti-CD3ε plus anti-CD28 antibodies and the intracellular expression of IL-4, IL-13, IL-2, and IFN-γ cytokines were assessed by intracellular cytokine staining and flow cytometry. Figure 4.10A shows that treatment with R9-QQP causes a dose-dependent inhibition of IL-4 with 50% inhibition

seen with 5 μM peptide incubation. R9-QQP also caused significant inhibition of IL-13 as treatment with 1 μM of peptide resulted in 50 percent inhibition of IL-13 as compared to no-peptide control (Figure 4.10A). The effect on both IL-4 and IL-13 is specific, as neither of the two control peptides causes significant inhibition of cytokine expression (Figure 4.10A). The values of the R9-QQP treated groups for IL-4 and IL-13 expression were statistically significantly different from 'no peptide' controls ($p < 0.05$). Treatment with R9-QQP has a marginal, though significant, effect on IL-2 expression (Figure 4.10B, $p = 0.014$), consistent with a moderate reduction in IL-2 expression in Itk-deficient mice. Interestingly, R9-QQP has no significant inhibitory effect on the expression of the signature T_H1 cytokine, IFNγ (Figure 4.10C, $p > 0.05$). Thus, the inhibitory effect of R9-QQP appears to be primarily selective for T_H2 cytokine expression.

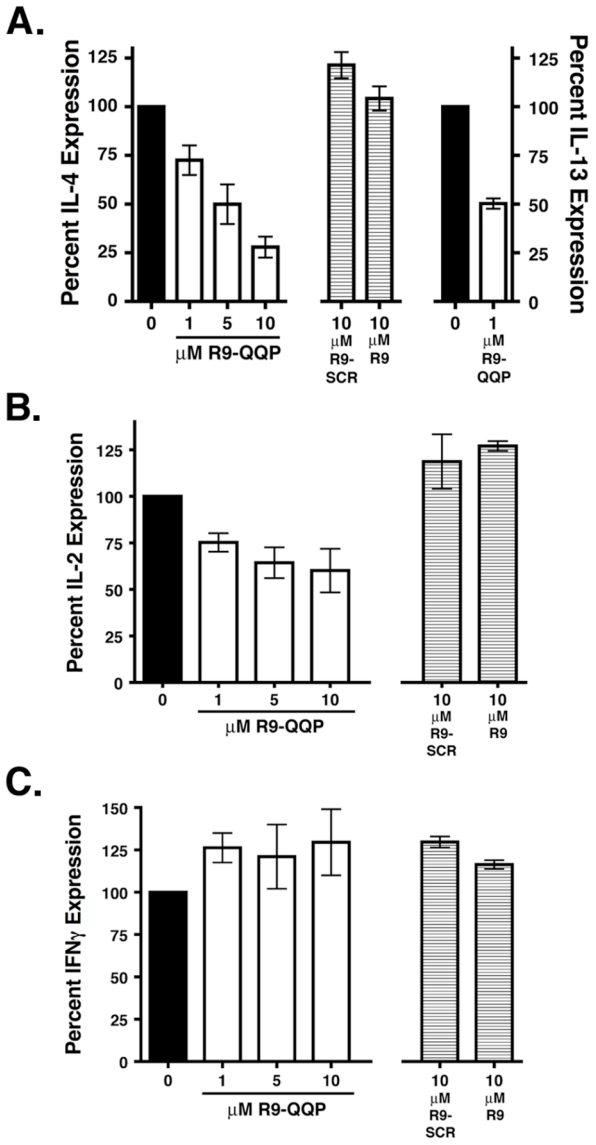

Figure 4.10. *Ex vivo treatment of splenocytes has specific effects on T_H2 cytokine production.*

Murine splenocytes pre-treated with various amounts of R9-QQP, control peptides or no peptide, as indicated, were stimulated with anti-CD3ε and anti-CD28 antibodies as described in Materials and Methods section. Cells were stained with anti-CD4 antibodies and the $CD4^+$ cells were analyzed for IL-4 and IL-13 (panel A), IL-2 (panel B), and IFNγ (panel C) expression using intracellular cytokine staining and flow cytometry as described in the Materials and Methods section. The data represent averages of three replicate experiments (±SEM) presented as percent of cells positive for each indicated cytokine. The results are normalized to 100% of cytokine expression in cells not treated with peptide that ranged from 6-20% of the cells displaying signal for the various cytokines tested.

4.4.7 In vivo administration of cationic peptides specifically inhibits Itk/SLP-76 association

The above findings strongly support the possibility that R9-QQP may be useful as an inhibitor of T_H2 cytokine production *in vivo*. Even though assessing the clinical utility of cell penetrating peptides is beyond the scope of this investigation, it was sought to determine whether R9-QQP could be effective when administered in *in vivo* mouse model systems. To this end, wild-type C57Bl/6 mice were injected intraperitoneally with various concentrations of R9-QQP 24 hours and 30 minutes before the removal of spleens. Isolated splenocytes were stimulated with anti-CD3ε and anti-CD28 cross-linked antibodies for 1 minute at 37°C prior to lysis. Lysates were immunoprecipitated with anti-SLP-76 antibodies, resolved on 4-12% gradient SDS-PAGE, and Western blotted for the indicated co-immunoprecipitating proteins. The data displayed in Figure 4.11 demonstrate that intraperitoneal administration of R9-QQP disrupts the activation-induced association between Itk and SLP-76. This inhibition is specific as other signal transducers reported to associate with the PRR region of SLP-76; Lck, PLCγ1 and Gads, are not affected (Figure 4.11).

In another series of experiments, wild-type C57Bl/6 mice were injected intraperitoneally with only 20 mg of either R9-QQP or control peptide per kg of mouse weight 24 hours and 30 minutes prior to excision of spleens. Samples were then processed as in the above experiment. Similar to the effects seen previously, R9-QQP inhibits the activation-induced association between Itk and SLP-76 in a peptide-specific manner, as neither the R9-SCR nor the R9 control peptides had significant effects on this interaction (Figure 4.12). Reproducibly, the inhibitory effects of R9-QQP on Itk/SLP-76

interaction was specific (Figure 4.13) since only in the presence of this peptide was this association affected and not in any other association with SLP-76 (Figure 4.14). The specific 50% inhibitory concentration for Itk association with SLP-76 using *in vivo* administration was about 10 mg/kg. No other proteins associating with SLP-76 displayed a significant reduction.

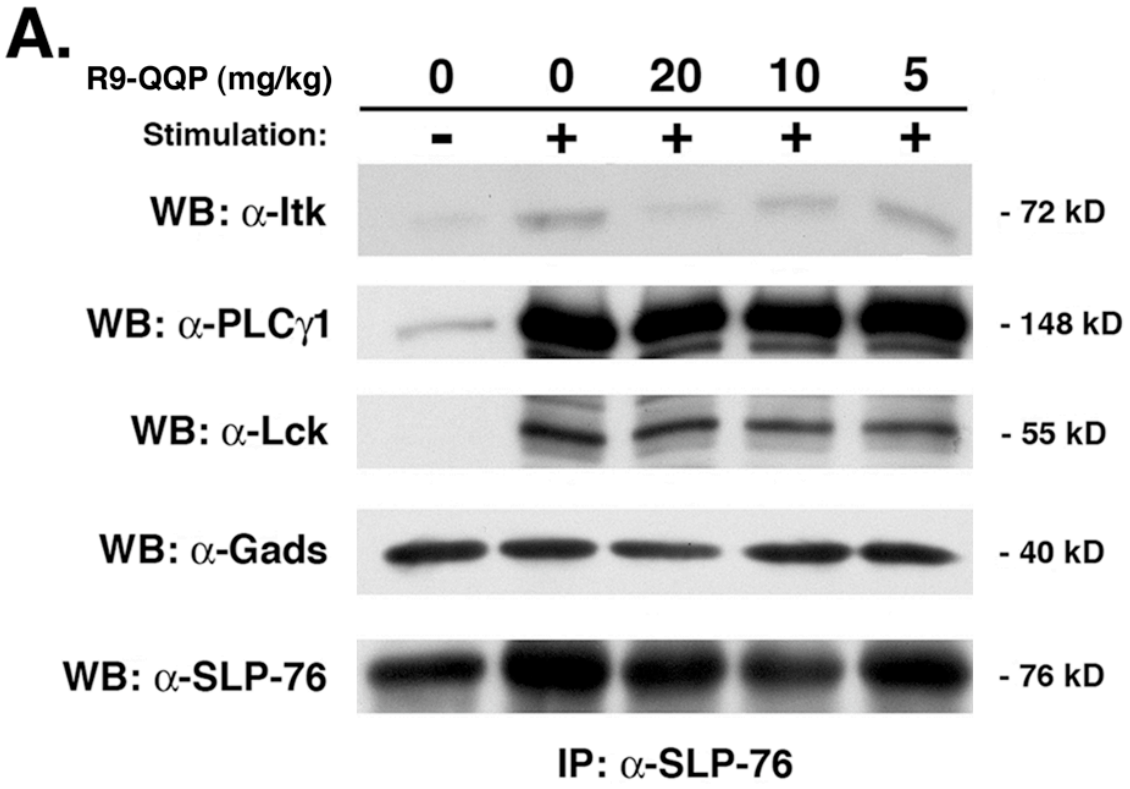

Figure 4.11. *Delivery of cell-penetrating peptide R9-QQP in vivo specifically inhibits the inducible association between Itk and SLP-76 in a dose-dependent manner.*

Wild-type (C57Bl/6) mice were injected intraperitoneally with different concentrations of R9-QQP peptide in PBS at 24 hours prior to harvest and at 30 minutes prior to harvest. Non-treated mice were similarly injected with equal volume of PBS vehicle control. The spleens were then excised from the mouse, and stimulated with anti-CD3ε and anti-CD28 cross-linked antibodies for 1 minute at 37°C. An aliquot of cells were then lysed, immunoprecipitated with anti-SLP-76 antibodies, and resolved on 4-12% gradient SDS-PAGE. The membrane was Western blotted with anti-Itk, anti-SLP-76, anti-Lck, anti-PLCγ1, and anti-Gads antibodies in subsequent re-probing.

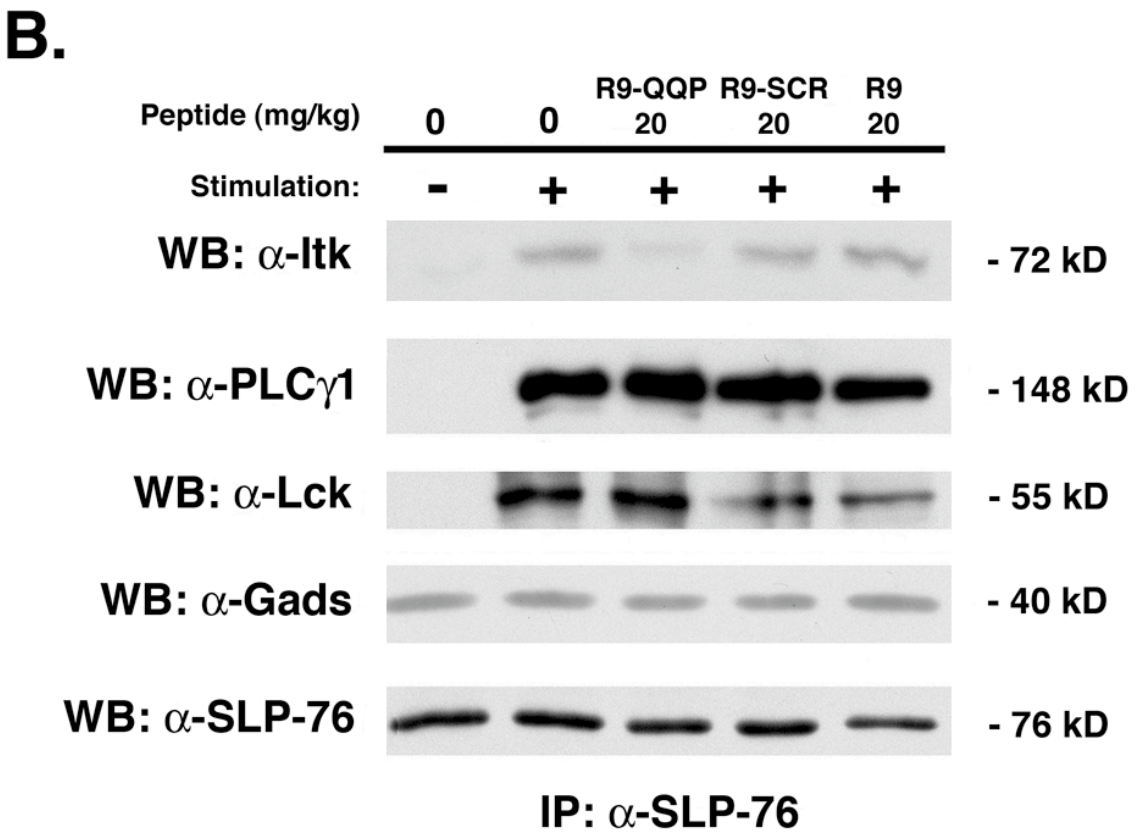

Figure 4.12. *In vivo administration of R9-QQP and control peptides effects on SLP-76 protein-protein interactions.*

Wild-type (C57Bl/6) mice were injected intraperitoneally with 20 mg/kg of R9-QQP peptide or control peptides in PBS at 24 hours and at 30 minutes prior to harvest. Non-treated mice were injected with an equal volume of PBS vehicle control. The murine spleens were excised, and splenocytes were stimulated with anti-CD3ε and anti-CD28 cross-linked antibodies for 1 minute at 37°C (+) or non-stimulated on ice (-). An aliquot of cells were then lysed, immunoprecipitated with anti-SLP-76 antibodies, and resolved on 4-12% SDS-PAGE. The membrane was Western blotted with anti-Itk, anti-SLP-76, anti-Lck, anti-PLCγ1, and anti-Gads antibodies in subsequent re-probing.

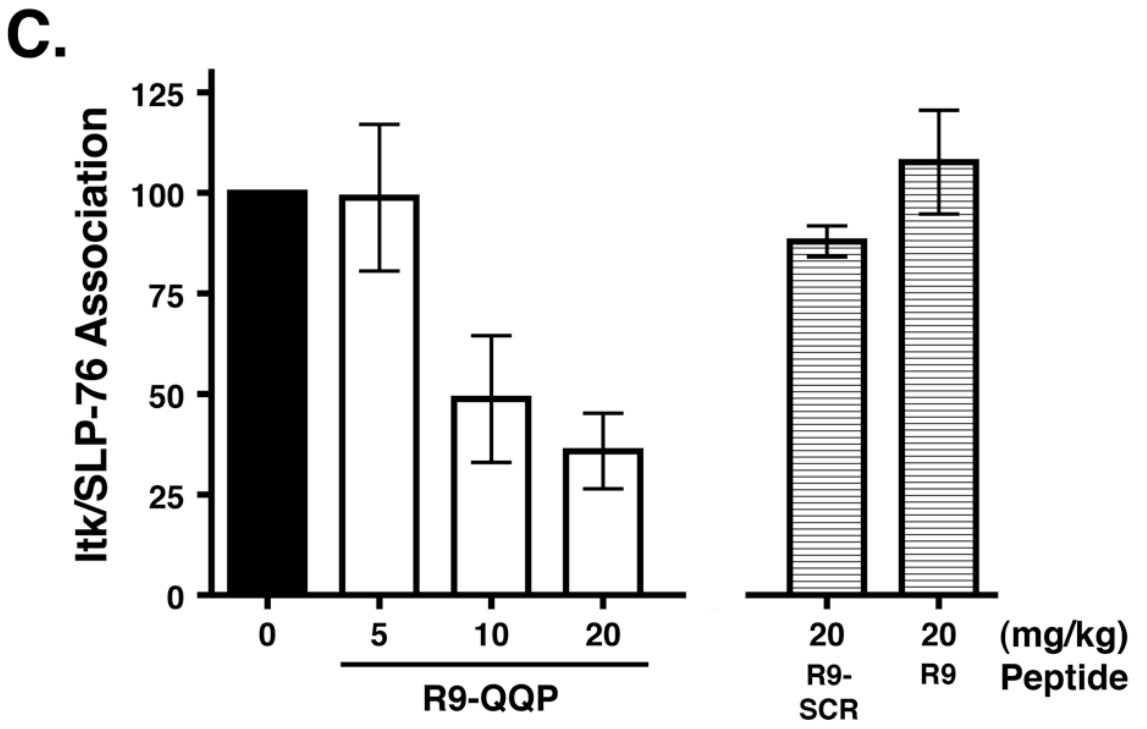

Figure 4.13. *Delivery of cell-penetrating peptide R9-QQP in vivo specifically inhibits the inducible association between Itk and SLP-76.*

Wild-type (C57Bl/6) mice were injected intraperitoneally with various amounts of R9-QQP or control peptides in PBS per kg weight at 24 hours and at 30 minutes prior to spleen harvest. Non-treated mice were injected with equal volume of PBS vehicle control. Splenocytes were stimulated with anti-CD3ε and anti-CD28 cross-linked antibodies for 1 minute at 37°C, while non-stimulated samples were kept on ice. Samples were immunoprecipitated with anti-SLP-76 antibodies, resolved on 4-12% SDS-PAGE, Western blotted with anti-Itk antibodies and re-probed with anti-SLP-76 antibodies for loading controls. Densitometric analyses of the Western blot bands were conducted on the Itk/SLP-76 association in the presence of various concentrations of peptide. Densitometric analyses values were normalized to loading control bands (SLP-76). The results are normalized to 100% of Itk/SLP-76 association in cells not treated with peptide. The graph represents the dose-response curve of Itk associated with SLP-76 and the effects of the R9-QQP peptide and the control peptides on that interaction. The bars represent the Mean ± SEM cumulated from three independent experiments.

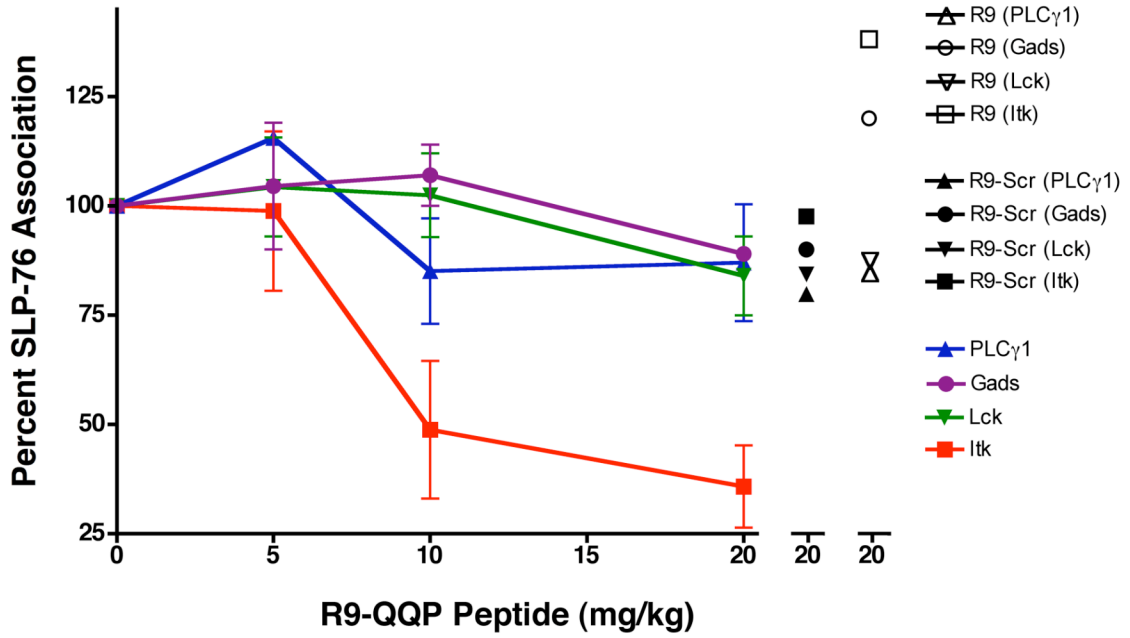

Figure 4.14. *In vivo delivery of peptide R9-QQP specifically inhibits Itk/SLP-76 association and not that of SLP-76 with other associating proteins.*

Wild-type (C57Bl/6) mice were injected intraperitoneally with various amounts of R9-QQP or control peptides in PBS per kg weight at 24 hours and at 30 minutes prior to spleen harvest. Non-treated mice were injected with equal volume of PBS vehicle control. Splenocytes were stimulated with anti-CD3ε and anti-CD28 cross-linked antibodies for 1 minute at 37°C, while non-stimulated samples were kept on ice. Samples were immunoprecipitated with anti-SLP-76 antibodies, resolved on 4-12% SDS-PAGE, Western blotted with antibodies for co-immunoprecipitating proteins and re-probed with anti-SLP-76 antibodies for loading controls. Densitometric analyses of the Western blot bands were conducted on the association of the various proteins with SLP-76 in the presence of various concentrations of peptide. Densitometric analyses values were normalized to loading control bands (SLP-76). The results are normalized to 100% of SLP-76 association with the indicated protein in cells not treated with peptide. Shown is a line graph representing dose-response curve of SLP-76 associating molecules with the effects of each dose of peptide on that specific protein-protein interaction cumulated from three independent experiments. The error bars represent the ±SEM.

4.4.8 In vivo injection of R9-QQP affects the specific tyrosine phosphorylation of Itk

The *in vivo* administration of peptide was also used to analyze the specific effect on tyrosine phosphorylation. In these experiments, an aliquot of cells gleaned from the

co-immunoprecipitation assay were used to test for specific phosphorylation using phospho-flow. Two other proteins, Lck and PLCγ1, were also tested for their specific phosphorylation. Since Lck phosphorylates Itk and binds to SLP-76, it was tested to determine whether the R9-QQP peptide had an effect on its specific phosphorylation on tyrosine 394, the activating Lck autophosphorylation site (64-66). If the peptide affects Lck phosphorylation, it could then be surmised that it is Lck affecting Itk activity. The other protein tested for its phosphorylation was PLCγ1, the lipase downstream of Itk that also binds SLP-76. If the peptide affects Itk activity, then PLCγ1 tyrosine 783 phosphorylation would also be reduced. Admittedly, if the peptide affects PLCγ1 phosphorylation more so than that of Itk, the peptide might preferentially be acting on PLCγ1, which would be the reason for its reduction in activity. To this end, the *in vivo* administered treated cells were stimulated with anti-CD3ε and anti-CD28 cross-linked antibodies and tested for specific tyrosine phosphorylation using phospho-flow. As seen in Figure 4.15, R9-QQP peptide treatment had a dose-dependent effect on the tyrosine phosphorylation for each of these proteins at varying degrees. The most significant effect of R9-QQP was on specific Itk tyrosine phosphorylation (Figure 4.15, red filled squares), with the most severe and sustained dose-dependent decrease in phosphorylation as compared to the other proteins. PLCγ1 had a similar, though not completely overlapping effect on its specific tyrosine phosphorylation (Figure 4.15, green filled upward pointing triangles) as compared to Itk phosphorylation. PLCγ1 displayed statistically similar reduction in phosphorylation as that of Itk at 5 mg/kg of peptide treatment, but slightly less comparative reduction seen in phosphorylation at higher doses. Lck had a very slight reduction in its specific tyrosine phosphorylation (Figure 4.15, blue filled downward

pointing triangles) at lower administered doses of R9-QQP peptide, with greater reduction observed at the highest dose administered. The control peptides, in agreement with previous studies, displayed little statistical effects on the phosphorylation of any of these proteins even when administered at the highest concentration (Figure 4.15, black filled symbols for R9-SCR and open symbols for R9).

To further address the question whether the peptide was preferentially acting upon Itk and not on PLCγ1, R9-QQP peptide was administered to excised splenocytes from wild-type (C57Bl/6) or $Itk^{-/-}$ mice. If the peptide affects PLCγ1 association with SLP-76 irrespective of the presence of Itk, then the effects seen on PLCγ1 tyrosine phosphorylation is due to the peptide and not the upstream effect on Itk activity. Splenocytes from each strain of mice were treated *ex vivo* with R9-QQP peptide at 1 or 10 μM, or without peptide. As seen in Figure 4.16, treatment of splenocytes from wild-type (C57Bl/6) mice had an immediate effect on Itk association with SLP-76 at 1 μM, but 10 times more peptide was needed to disrupt the association between SLP-76 and PLCγ1. Interestingly, in the absence of Itk, even with the high concentration of R9-QQP peptide, the association between SLP-76 and PLCγ1 remained intact. This indicates that the effects seen on PLCγ1 phosphorylation is indirectly due to the specific effects the R9-QQP peptide has on Itk activity upstream of PLCγ1.

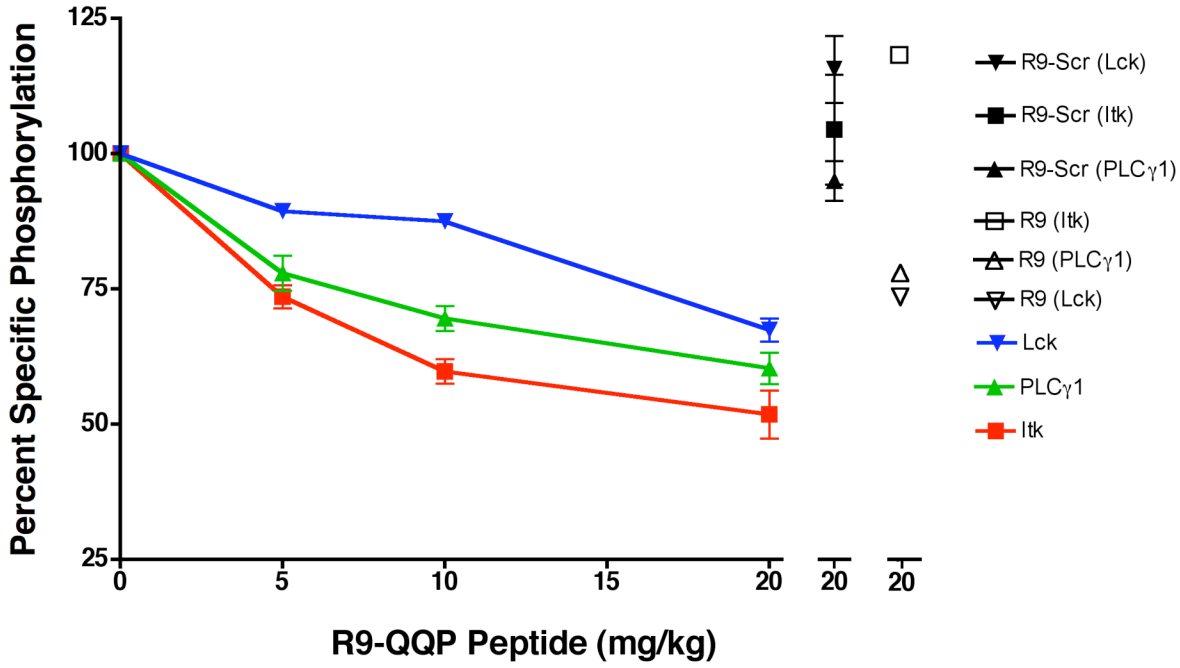

Figure 4.15. *In vivo delivery of peptide R9-QQP inhibits the specific tyrosine phosphorylation of Itk.*

Mice were treated with peptide and stimulated as described in Figure 4.11 and Figure 4.12. The cells were then analyzed for specific tyrosine phosphorylation using phospho-flow similar to the methods used in Figure 4.7. This line graph represents the dose-response curve of specific tyrosine phosphorylation of Itk (pY511, squares), PLCγ1 (pY783, upward pointing triangles), and Lck (pY394, downward pointing triangles) in the presence of various concentrations of peptide cumulated from three independent experiments. Each point is the mean for that concentration, with the error bars representing the ±SEM.

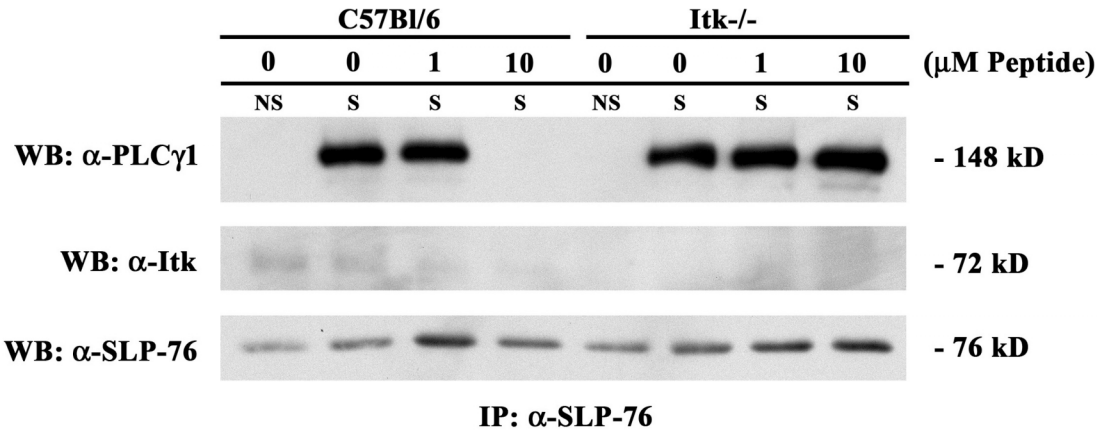

Figure 4.16. *PLCγ1 is unaffected by the peptide in the absence of Itk.*
Excised splenocytes from wild-type (C57Bl/6) or Itk$^{-/-}$ mice were treated ex vivo with R9-QQP peptide similar to previous experiments at the concentrations indicated. Cells were stimulated for 1 minute at 37°C with anti-CD3ε antibodies (S), or were left unstimulated on ice (NS). Cells were lysed, pre-cleared with rabbit IgG, immunoprecipitated with anti-SLP-76 antibodies, immunocomplexes were run on SDS-PAGE, transferred to PVDF, and Western blotted with anti-Itk and anti-PLCγ1 antibodies.

4.4.9 In vivo delivery of the R9-QQP peptide dramatically affects CD4$^+$ cytokine expression

The *in vivo* regulation of Itk association with SLP-76 and the consequent regulation of Itk activity means little if downstream processes, particularly that of T cell output or cytokine expression, is not affected. As such, the effects of *in vivo* administration of the peptide were tested for expression of two T_H1 cytokines (IFNγ and IL-2), two T_H2 cytokines (IL-4 and IL-13), and a cytokine from another subset of T helper cells, the T_H17 cells (IL-17). An aliquot of cells was culled from the *in vivo* administration co-immunoprecipitation assay and stimulated as in the *ex vivo* cytokine expression assay in Figure 4.10. As seen in Figure 4.17, T_H2 cytokine expression was

dramatically affected by the *in vivo* administration of R9-QQP peptide (IL-4, filled red squares; IL-13, red filled upward pointing triangles). T_H1 cytokine expression yielded heterogeneous results. IFNγ expression was roughly unaffected by peptide treatment, and similar to the *ex vivo* peptide treatment studies, displayed a slight increase in expression (Figure 4.17, green filled diamonds). By contrast, IL-2 displayed a slight reduction in expression at all *in vivo* dosage levels, again, similar to the small reduction seen in the *ex vivo* treatment (Figure 4.17, green filled downward pointing triangles). T_H17 cytokine expression, as measured through the expression of IL-17, was unaffected by R9-QQP treatment (Figure 4.17, blue filled circles). As seen in previous experiments, the effects on cytokine expression seen here were due to the presence of the R9-QQP peptide, and were not observed with treatment from either of the control peptides (Figure 4.17, black filled for R9-SCR and open symbols for R9). These results conclude that the R9-QQP peptide is a specific inhibitor of Itk function *in vivo* leading to the specific interruption of T_H2 cytokine production.

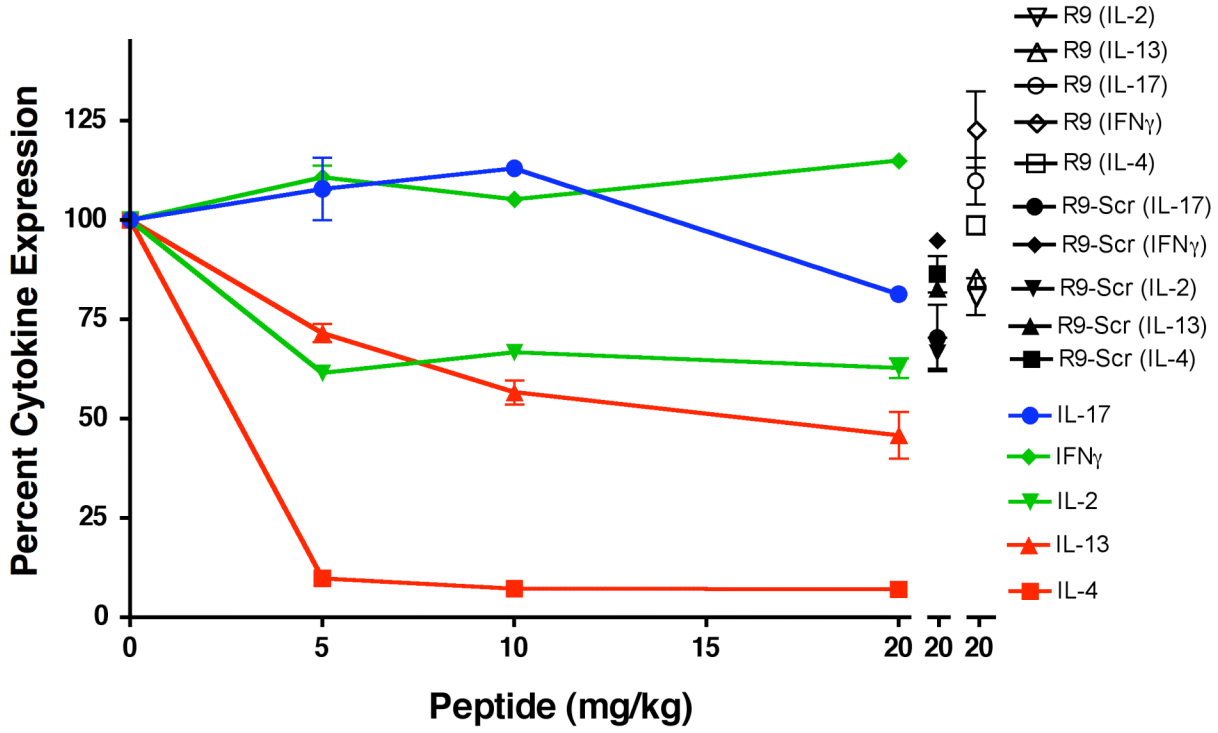

Figure 4.17. *In vivo administration of the R9-QQP peptide dramatically affects CD4$^+$ cytokine expression.*

Cells were treated as in Figure 4.11 and Figure 4.12, then stimulated with anti-CD3ε and anti-CD28 antibodies to activate the cells and promote cytokine production. The cells were incubated for 24 hours, re-stimulated with anti-CD3ε and anti-CD28 antibodies in the presence of Brefeldin A, and after an additional 6 hours of stimulation, the cells were stained for intracellular cytokine production as described in the Materials and Methods.

4.5 Discussion

Previous investigations have demonstrated that mice deficient in Itk expression display defects in T cell development, TCR-mediated activation, and T_H2 cytokine production (3, 4, 63, 67-73). The activation of Itk requires its membrane targeting of the T cell at the site of antigen presenting cell (APC) interaction. This recruitment is regulated by membrane phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and inositol 1,3,4,5-tetraphosphate (IP_4) that interact with the Pleckstrin Homology (PH) domain of Itk (15, 16). Upon its recruitment to the membrane, Itk interacts with various other signal transducers that putatively alter its conformation and render it enzymatically active. Of particular interest is the interaction of Itk with two adaptor proteins, the linker of activated T cells (LAT) and the Src homology 2 domain-containing leukocyte protein of 76 kilodaltons (SLP-76) (17, 18, 58, 61, 74). In the present investigation, cell permeable peptides were utilized as competitive inhibitors to provide direct evidence that disruption of the specific interaction between the Src Homology 3 (SH3) domain of Itk and the Proline Rich Region (PRR) of SLP-76 in live cells. This specific protein-protein disruption impedes the inducible activation of Itk and selectively inhibits the expression of T_H2 cytokines thus providing direct evidence for the biological significance of this interaction.

The recruitment of Itk to the TCR signaling complex positions Itk as a target of transphosphorylation on tyrosine 511 by Lck, which is critical for the activation of Itk (5, 6). Previous experiments have demonstrated that Itk associates with LAT in a TCR-inducible manner and its activation requires the presence of this adapter protein (17, 58).

Similar findings have been reported with SLP-76. Interestingly, in these studies SLP-76 appears to be important for the recruitment of Itk to the T cell-APC contact, while LAT does not (Figure 4.4). One possible interpretation of these results could be that LAT may be required for the conformational change of Itk to render it active (75), but Itk proximity to LAT might require the presence of SLP-76. Alternatively, the SH3 domain of Itk may be initially important for binding to SLP-76, while the SH2 domain (most likely through binding tyrosine 145 of SLP-76) is of critical importance for Itk activation. This is supported by recent studies from the Koretzky lab demonstrating that the interaction of the N-terminal tyrosine 145 of SLP-76 with the SH2 domain of Itk is critical for the optimal activation of the kinase (76). Furthermore, the Yablonski lab has demonstrated that only when Itk associates with SLP-76 does it become enzymatically active (61). Figure 4.1 and Figure 4.2 in these studies agree with this conclusion.

The use of GST-fusion proteins of Itk domains (not full-length protein) to probe for molecular interactions with other T cell proteins was useful for the initial observation that Itk and SLP-76 associate (18). It was also useful for the screening of peptide libraries to identify sequences that could interfere with this interaction *in vitro*, as the specific peptide sequence of the SLP-76 PRR (a.a. 184-195) was identified to inhibit the SH3 domain of Itk binding to SLP-76 (18). However, the biological significance of this interaction had never been determined. To address the significance of the interaction between Itk and SLP-76, synthetic competitive peptides representing the interactive binding sites were rendered cell-permeable by the addition of a nine arginine tag (referred to as R9-QQP).

The use of a cell permeable sequence to the peptide interaction site allows for transport of non-permeable cargo into the intracellular environment. Addition of cell permeable peptide sequence to the SLP-76 PRR that interacts with Itk SH3 domain enables the peptide to enter T cells (Figure 4.5). While the method of uptake for cell-permeable peptides may be a matter of controversy, they are either taken up through an ATP-independent endocytic pathway or through a direct translocation through the plasma membrane, depending on the cell type and/or the cell-permeable peptide sequence and the cargo its carrying (77-87). For instance, cargos rich in prolines, such as the one used in the above studies, have a greater propensity of efficient cellular penetration (88).

The intracellular presence of R9-QQP in both Jurkat T cells and mouse splenocytes enabled this peptide to have profound effects on the inducible association between Itk and SLP-76 (Figure 4.6 and Figure 4.11-Figure 4.15). This disruption led to the specific inhibition of Itk transphosphorylation on tyrosine 511 (Figure 4.8 and Figure 4.15). These effects of R9-QQP on Itk are specific, as two control peptides, the scrambled cargo sequence with the R9 tag (R9-SCR) and the R9 tag alone (R9), displayed no significant effects on Itk and SLP-76 association nor did they affect Itk transphosphorylation. This was not due to a lack of cellular entry, as FITC-conjugated R9 was as efficient entering Jurkat T cells as FITC-conjugated R9-QQP (data not shown).

The disruption by R9-QQP peptide treatment on Itk/SLP-76 association was specific, as this peptide had little specific effect on the association of SLP-76 with any other protein that associates with it. The adaptor protein, Gads, which is acknowledged to link the SLP-76 signalosome to the LAT signalosome, was unaffected by R9-QQP despite binding to the SLP-76 PRR, albeit at a site distinct from that of Itk SH3 domain

(89). The binding site of Gads SH3 domain encompasses amino acids 224-244 within the PRR of SLP-76 (89), whereas the SH3 domain of Itk binds within amino acids 184-208 of the PRR of SLP-76 (18). The inducible association between SLP-76 and that of the molecule activated by Itk, the lipase PLCγ1, was similarly unaffected by the presence of R9-QQP. This is despite the observation that the SH3 domain of PLCγ1 binds an overlapping sequence to that of Itk within the PRR of SLP-76 (a.a. 157-223) (90, 91). Since PLCγ1 is a target of Itk, the inhibition of Itk phosphorylation would theoretically lead to the inhibition of PLCγ1 phosphorylation. However, given the overlapping nature of these two molecules binding to SLP-76, the effects of R9-QQP could be due either to the disruption of Itk binding to SLP-76 leading to its inactivation, or to the direct inhibition of the PLCγ1/SLP-76 interaction. R9-QQP inhibited the inducible interaction between PLCγ1 and SLP-76 at approximately ten times higher concentrations than that required for the inhibition of Itk/SLP-76 interaction (Figure 4.16). Further, PLCγ1 association with SLP-76 was no longer affected by the R9-QQP peptide when Itk was not present (Figure 4.16). Therefore, PLCγ1 binds to SLP-76 directly, or at least does not require Itk to do so, and its dissociation from SLP-76 probably has more to do with the activity of Itk than through the direct dissociation of PLCγ1/SLP-76 by R9-QQP. Lastly, the inducible association of SLP-76 with the tyrosine kinase responsible for Itk activation, Lck, was uninhibited by the presence of the R9-QQP peptide, regardless of the concentration. This despite the observation that the SH3 domain of Lck associates with the SLP-76 signalosome through SLP-76's PRR, at a completely overlapping sequence of amino acids 185-194 (92, 93). It is difficult to reconcile these results, other than to continue studies to determine whether Itk or Lck preferentially bind this site on SLP-76,

in the absence of one or the other kinase. Given the above effects of the R9-QQP peptide on Itk and the lack of effect on Lck, it would seem Itk binds SLP-76 preferentially.

The data presented in these studies provide the first demonstration of the biological significance of the inducible interaction between Itk and SLP-76, as determined by the effects of the disruption of this interaction has on cytokine production (Figure 4.9, Figure 4.10 and Figure 4.17). R9-QQP had a profound selective inhibition on T_H2 cytokine production, particularly that of IL-4 and IL-13. By contrast, R9-QQP had little inhibitory effect on the production of T_H1 cytokine IFNγ. There is a small, though significant, effect on IL-2 production in the presence of R9-QQP. The R9-QQP effects on these cytokines is consistent with observations from studies using $Itk^{-/-}$ mice (4, 63, 73).

The truly intriguing aspects of these data includes the intraperitoneal injections displaying similar R9-QQP peptide effects as seen in cells treated *in vitro* or *ex vivo*. *In vivo* treatment with peptide reproduced the disruption of inducible Itk/SLP-76 interaction (Figure 4.11 and Figure 4.14), the inhibition of the inducible transphosphorylation of Itk on tyrosine 511 (Figure 4.15), and the selective reduction of T_H2 cytokine production (Figure 4.17). One difference in the results was between the cytokine expression of IL-4 and IL-13 when the cells were treated *ex vivo* versus *in vivo* (compare Figure 4.10 to Figure 4.17). The difference may be due to the treatment protocols, as the *in vivo* protocol involved treating with peptide twice as opposed to a single treatment in the *ex vivo* protocol. The pharmacokinetics of peptide effects as well as peptide clearance have yet to be determined, though the *in vivo* protocol was based on published work using a cell-permeable peptide that inhibits NFAT function *in vivo* (52). Further, it is difficult to

determine the amount of effective peptide concentration *in vivo*, making a direct comparison between the two types of treatment complicated. Irrespective of the delivery method, R9-QQP has a specific effect on T_H2 cytokine production.

The use of cell permeable peptides has increasingly been used to act as competitive inhibitors of signaling pathways. In experiments done be Eibert, et al., cell-penetrating peptides were generated to inhibit the interaction between cofilin and the actin cytoskeleton (94). Cofilin induces filamentous actin to depolymerize, which promotes cytoskeletal remodeling. T cells incubated with the cofilin inhibitory cell-penetrating peptide could no longer form immunological synapses and functionally could no longer proliferate upon stimulation. In studies by Rothbard, et al., nine arginine resides were conjugated to cyclosporin A (CsA) to deliver this protein through the skin of both mice and men (95). Unmodified CsA was unable to transverse the epithelial barrier. Entry of R9-CsA into the dermal T lymphocytes inhibited cutaneous inflammation in a mouse model of contact dermatitis. In another study by Noguchi, et al., a cell-penetrating peptide was designed to competitively inhibit the interaction between calcineurin and NFAT (52). Incubation of Jurkat cells with this peptide resulted in the inhibition of the inducible translocation of NFAT into the nucleus, thereby reducing its transcriptional activity and the production of IL-2. Noguchi, et al., proceeded to inject the peptide intraperitoneally and were able to significantly prolong the survival of mismatched pancreatic islet allografts in mice. These studies demonstrate the power of cell-penetrating peptides to dissect and affect cell signaling pathways *in vitro* and *in vivo*.

Itk-deficient mice are defective in the expression of T_H2 cytokines, whose expression is imperative in the response to allergies. An allergic response is

characterized by the differentiation into the T_H2 effector class of cells in which the transcription factor GATA-3 dictates the activation of a gene cluster that leads to the secretion of the cytokines IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF (96). These cytokines mediate the allergic response through the class switching of B cells to IgE synthesis (IL-4 and IL-13), mast cell recruitment (IL-4, IL-9, and IL-13), the development and recruitment of eosinophils (IL-3, IL-5, and GM-CSF), basophils (IL-3 and IL-4), and airway remodeling (IL-13). Conversely, for autoimmune responses, the T_H1 phenotype controlled by T-bet primarily exudes the cytokine IFNγ. The imbalance between these responses, by T_H1 and T_H2, creates a chronic asthmatic condition.

Once allergic sensitization has occurred, encounters with allergens thereafter will cause an immediate and late immune response. The immediate response involves the activation of allergen-specific T_H2 effector cells that dictate B cell IgE synthesis. IgE cross-linking to high-affinity IgE receptor (FcεRI) on mast cells causes these cells to degranulate, releasing histamine and leukotrienes that cause mucus production, vascular permeability, smooth-muscle contraction, and the wheal and flare reaction in skin. Immediate hypersensitivity symptoms include edema and itching, and in some severe cases, anaphylactic shock. This initial response occurs in less than 30 minutes. Chemokine secretion by resident mast cells recruits other effector cells for the late immune response. The late response consists of the arrival of the effector cell eosinophils and basophils, within a time frame of 6-72 hours. Eosinophils are granulocytes recruited by IL-5 that normally secrete toxic proteins that help to eliminate a helminth infection, but are unwanted in an asthmatic response (97-99). Basophils are similar to mast cells in that they produce histamines in response to activation, but they

also secrete toxic granules like eosinophils do which contributes to the allergic response (100). Clinical manifestations of this late allergic reaction include edema, excessive warmth, redness of the area, and for some, pain.

Current treatments for asthma involve using corticosteroids and agonists for the β-adrenonergic receptor. Corticosteroids inhibit the T_H2 response by preventing the expression of cytokines, chemokines, and adhesion molecules regulated by NFκB (101). This is a temporary relief in asthmatic symptoms, as corticosteroid treatment has no long-term benefits in the amelioration of the disease (102). β-adrenonergic receptor agonists come in two flavors, short-acting and long-acting. The short-acting β-adrenonergic receptor agonists rapidly relieve bronchoconstriction in an asthma attack by mediating smooth muscle relaxation. It does this by binding the β-adrenonergic receptor, activating $G\alpha_s$ to produce the secondary messenger cAMP, which activates the kinase PKA. This kinase phosphorylates myosin light-chain kinase causing Ca^{2+}-dependent K+ channels to open and relieve the smooth-muscle constriction. The long-acting agonists do this in a time-released manner, but are not effective alone because they inhibit other inflammatory responses. Therefore, long-acting β-adrenonergic receptor agonists are often combined with corticosteroids for a short and long-term benefit.

Another treatment for some allergies and bronchial asthma is the use of allergen-specific immunotherapy (SIT). SIT induces tolerance to the antigen through repeated exposure, which decreases the recruitment of mast cells, basophils, and eosinophils to the mucosa. SIT increases the production of blocking antibodies IgG_1, IgG_4, and IgA, while decreasing the production of IgE. SIT also prevents the expansion of T_H2 effector cells and the cytokines they produce. Finally, SIT increases the production of IL-10, a

cytokine that suppresses the allergic response through the induction of regulatory T cells. Induced tolerance does have some side effects, however, as anaphylaxis has occurred in some patients after treatment (103).

Blocking IgE binding to FcεRI has proven to be an effective modality in moderating allergic responses. The FcεRI is highly expressed on mast cells and when bound and activated by IgE causes degranulation of the mast cell leading to an allergic reaction (104). Mast cells secrete histamine, prostaglandins, and leukotrienes, which leads to bronchoconstriction, mucous secretion and mucosal edema, all features of an asthmatic response. Blocking IgE binding to FcεRI inhibits allergen-induced inflammatory responses in both mice and humans. The anti-IgE antibody, Omalizumab (Xolair), has been shown to be useful in the treatment of allergic asthma (105-107). The only drawback to its use is that it takes up to 16 weeks of doses to establish a clinical effect. Mast cell inhibitors and histamine blockers are also effective at mediating asthma. Preventing mast cell degranulation was first used as a treatment for asthma in 1968 with the use of sodium cromoglicate and later with nedocromil sodium in 1984, both of which inhibit chloride flux in mast cells thereby increasing the threshold for activation (108). FcεRIs and chloride channels also reside on other cells outside of mast cells, so although these therapies are effective at limiting mast cell degranulation and the allergic response, they often have unanticipated side effects.

Immunotherapies concentrated on the biological effects of cytokines have become a popular means of regulating an allergic or asthmatic response. In particular, T_H2 cytokines and their cognate receptors have become key targets in the quest to benefit asthmatics. IL-4 is crucial for maintaining the T_H2 phenotype, so it has become an

appealing drug candidate. Soluble IL-4R (altrakincept) proved to be effective through clinical Phase II trials, but failed to be effective in Phase III trials due to bioavailability issues (109, 110). Antibodies against IL-4 have worked as vaccines in mice to limit IL-4 titer and reduce lung inflammation, but these studies have not yet progressed into human studies (111, 112). Since IL-13 is invaluable in many airway functions, it too has become a key druggable target. Soluble IL-13Rα2, which binds to IL-13 but not to similar IL-4, has been able to reduce airway hyper-responsiveness and mucus production in mice (38). Antibodies against IL-13 inhibited eosinophil and neutrophil movement into the lungs of monkeys (113) and prevented inflammation in the lungs of mice (114). These IL-13 blocking antibodies have since moved into clinical trials and are now in Phase II.

The connection between the T_H2 defects seen in $Itk^{-/-}$ mice and asthma was made in the August lab. Using a murine model, they were the first to demonstrate the involvement of Itk in the development of lung allergy symptoms (115). Mice lacking Itk display significantly reduced lung inflammation, recruitment of eosinophils, production of mucous, as well as reduced airway hyper-responsiveness upon allergen challenge (115, 116). Furthermore, the kinase activity of Itk is required for the recruitment of T cells in the lung, production of T_H2 cytokines, and induction of the inflammatory response in allergic asthma (117). In view of these data, Itk is an attractive target for the development of inhibitors as potential anti-allergy therapeutics. In related recent studies by McCusker et al., a STAT6 cell-penetrating peptide was used to selectively inhibit T_H2 cytokine expression similar to these experiments with R9-QQP (118). Furthermore, this STAT6 cell-penetrating peptide could diminish the symptoms of bronchial asthma when delivered intranasally in an experimental mouse model. It would thus be prudent to

determine whether R9-QQP could produce similar effects and ameliorate asthmatic symptoms by inhibiting Itk activity.

In summary, these experiments demonstrate the biological significance of the specific interaction between the Itk SH3 domain and the SLP-76 PRR in live cells. Previously, the importance of this interaction between these two signaling partners had only been assessed from *in vitro* studies. These findings may pave the way for the development of novel lung allergy therapies that target early signaling events (i.e. disruption of Itk/SLP-76 interaction) in the production of inflammatory mediators. Such drugs in combination with other peptide inhibitors that interfere with later signaling events, such as the one reported by McCusker and colleagues (118), could provide an effective treatment of allergic diseases. Such an approach is distinct from that of current anti-allergy therapies which target the already produced effectors that cause the disease symptoms (e.g. corticosteroids, β-adrenoreceptor agonists, anti-histamines and leukotriene inhibitors). Finally, the proposed use of cell-permeable peptides to disrupt a specific signaling event may provide the impetus for extending the use of this technology in dissecting additional immunological signaling pathways in live cells and expand the ability for rational drug design.

Portions of Chapter 4 have been submitted as: Grasis, J.A., K. Herman, and C. D. Tsoukas. (Submitted) Inhibition of Itk Activation and Cytokine Production by Cell Permeable Peptides. *J. Immunol*.

4.6 References

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